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ORIGINAL ARTICLE

Analysis of gene expression profiles in response to *Sclerotinia sclerotiorum* in *Brassica napus*

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Abstract *Sclerotinia sclerotiorum* is a necrotrophic plant pathogen which causes serious disease in agronomically important crop species. The molecular basis of plant defense to this pathogen is poorly understood. We investigated gene expression changes associated with *S. sclerotiorum* infection in a partially resistant and a susceptible genotype of oilseed *Brassica napus* using a whole genome microarray from *Arabidopsis*. A total of 686 and 1,547 genes were found to be differentially expressed after infection in the resistant and susceptible genotypes, respectively. The number of differentially expressed genes increased over infection time with the majority being up-regulated in both genotypes. The putative functions of the differentially expressed genes included pathogenesis-related (PR) proteins, proteins involved in the oxidative burst, protein kinase, molecule transporters, cell maintenance and development, abiotic stress, as well as proteins with unknown functions. The gene regulation patterns indicated that a large part of the defense response exhibited as a temporal and quantitative difference between the two genotypes. Genes associated with jasmonic acid (JA) and ethylene signal transduction pathways were induced, but no salicylic acid (SA) responsive genes were identified. Candidate defense genes were identified by integration of the early response genes in the partially resistant line with previously mapped quantitative trait loci (QTL). Expression levels of these genes were verified by Northern blot analyses. These results indicate that genes encoding vari-

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ous proteins involved in diverse roles, particularly WRKY transcription factors and plant cell wall related proteins may play an important role in the defense response to *S. sclerotiorum* disease.

Keywords *Brassica* · Gene expression profile · Microarray · *Sclerotinia* · *Arabidopsis*

Abbreviations

ET	Ethylene
hpi	Hours post-inoculation
JA	Jasmonic acid
RT-PCR	Reverse-transcriptase polymerase chain reaction
SA	Salicylic acid

Introduction

Plants have evolved multiple and inducible mechanisms to defend themselves from biotic stress. While some of these mechanisms are common to all types of assaults, such as tissue reinforcement and the production of anti-microbial metabolites, others are tailored to combating specific types of pathogen interactions (McDowell and Dangl 2000). The most well studied mechanism is directed toward non-necrotrophic pathogens and is mediated by the specific recognition of pathogen avirulence (Avr) protein by a corresponding receptor encoded by a plant resistance (*R*) gene. This leads to an immediate local defense response, known as the hypersensitive response (HR), which is followed by down-stream signaling that activates defense response genes, subsequently resulting in a persistent, broad-spectrum resistance termed systemic acquired resistance (SAR; Rathjen and Moffett 2003). A different form of systemic resistance, referred to as induced systemic resistance (ISR), is mediated by interactions with non-pathogenic organisms such as *Pseudomonas fluorescens* (Pieterse et al. 1998; Feys and Parker 2000). *Sclerotinia sclerotiorum* is a necrotrophic fungal pathogen, which causes severe disease in most agronomical important crops, such as oilseed (*Brassica napus*), soybean, and sunflower. The defense responses mounted against this pathogen appear to be multi-factorial and remain poorly understood.

Three distinct, yet interconnected signaling pathways, mediated by salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are involved in the plant disease response (Dong 1998; Feys and Parker 2000; McDowell and Dangl 2000; Martinez et al. 2001). These signaling pathways are differentially regulated and act individually, synergistically, or antagonistically depending on the pathogen involved (Glazebrook 2005; Kachroo and Kachroo 2007). The coordinate regulation of defense responses has been revealed using microarray analysis and examining *Arabidopsis* mutants in specific signaling pathways (Maleck et al. 2000; Cheong et al. 2002). Although JA/ET-dependent pathways were reported to be involved in the plant response against necrotrophic pathogens, the gene expression changes and the corresponding signal transduction pathways associated with *S. sclerotiorum* infection have not been reported.

Microarray analysis is a powerful technology that allows the simultaneous assessment of the expression of thousands of genes, even entire genomes (e.g., dissection of signal transduction pathways involved in plant defense). The transcriptome of *Arabidopsis* has been examined during SAR using a variety of microarray technologies (Maleck et al. 2000; Schenk et al. 2000). The quantitative nature of gene expression in *Arabidopsis* during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae* has revealed a common set of responsive genes (Tao et al. 2003). Marathe et al. (2004) reported that the "resistome" of *Arabidopsis* in response to *Cucumber Mosaic Virus* infection involved 80 genes commonly induced by both viral and bacterial pathogens.

The advances that have been made in *Arabidopsis* genomics have great potential to be used in closely related (phylogenetically) plant species. *Brassica* and *Arabidopsis* belong to the same family Brassicaceae and the levels of genome synteny regions between *Arabidopsis* and *Brassica* species have been reported (Cavell et al. 1998; Parkin et al. 2005). Girke et al. (2000) used an *Arabidopsis* cDNA microarray to investigate gene expression in the seeds of *B. napus* for the purpose of assessing cross-species use of microarray technology. Similarly, Horvath et al. (2003) reported that an *Arabidopsis* cDNA microarray may also be used in a more distantly related plant species such as leafy spurge, poplar, and wild oat. In these cases, the arrays comprised only a portion of the *Arabidopsis* genome. Also, cDNA microarray may not be able to distinguish among members of multigene families, related genes, and differentially spliced genes. Another limitation of using cDNA microarrays is that arrayed cDNA clones may not match their respective EST sequences found in databases due to errors in sample tracking.

Synthetic oligonucleotides (oligos) offer an attractive alternative to cDNA amplicons due to the lack of amplification and the errors associated with sample tracking. The uniform length and concentration of oligos provide more precise estimation of gene expression levels (Lee et al. 2004). Oligos can be synthesized to match any known or predicted gene sequence. Specifically, 70-mer oligos for all ~26,000 predicted genes within the *Arabidopsis* genome have been developed (http://www.operon.com/arrays/omad.php). Lee et al. (2004) compared the *Arabidopsis*

oligo-arrays with a cDNA array, and found that oligos have potentially more sensitivity for detecting changes in gene expression. More importantly, they found that oligo arrays designed from *Arabidopsis* gene sequences are effective for use in *Brassica* species (*B. oleracea*).

Quantitative trait loci (QTL) for resistance to *S. sclerotiorum* were previously mapped in a segregating population of *B. napus* derived from the partially resistant parent, RV289 (Zhao et al. 2006). In this study, we used an oligonucleotide microarray representing 26,000 genes from *Arabidopsis* for the purpose of investigating changes in gene expression in the partially resistant line RV289 and a highly susceptible line Stellar. The objectives of our study were to examine gene expression changes of *B. napus* at different time points after infection, to identify commonly and specifically expressed genes in the two genotypes and to classify signal transduction pathways during the early stage of infection. Certain genes that were up-regulated early and specifically expressed in the partially resistant line co-localized with the map positions of previously identified QTL. Our results provide a better understanding of the complex interactions between plants and necrotrophic pathogens, such as *S. sclerotiorum*, and the identification of candidate genes for further investigation.

Materials and methods

Plant materials

Two *B. napus* genotypes were used, the partially resistant line RV289 and the highly susceptible cultivar Stellar. RV289 is a single plant selected from a Chinese winter line Hua dbl-2, provided by Dr. J. Meng (Huazhong Agricultural University, China). A doubled haploid line derived from Stellar, a Canadian spring cultivar, was provided by Dr. R. Scarth (University of Manitoba, Winnipeg, Canada). These two lines have been used to build molecular linkage maps (Ferreira et al. 1994; Udall et al. 2005) and to locate QTLs for resistance to *S. sclerotiorum* (Zhao et al. 2006).

Plant growth, inoculation, and tissue harvest

Seeds were planted in 8-cm pots containing Scott's Metromix 336p soil (Scott's-Sierra Horticultural Products Co., Marysville, OH, USA) in flats of 24 plants and arranged as a randomized complete block design. The plants were grown in a controlled growth chamber under 450 μmol m^{-2} s^{-1} light intensity with 16-h photoperiod provided by fluorescent and incandescent lamps. The temperature was maintained at a constant 21°C. Plants were watered automatically with 1/4 Hoagland's solution daily for 11 min and relative humidity was maintained at 60%.

Inoculation was performed on 3-week-old seedlings as previously reported (Zhao et al. 2004). Briefly, petioles of the third fully expanded leaf were severed 2.5 cm from the stem, and the open-end of the petiole was infected with *S. sclerotiorum* mycelium. The inoculated petiole and a 1-cm section of stem adjacent to the inoculation point were harvested using a razor blade at 12, 24, and 48 h post-inoculation (hpi) with mock-inoculated plants as a control. Eight individual plants were pooled for one RNA sample. Another RNA sample was collected with separated sets of growing plants and inoculated with separated cultured colonies as a second biological replicate. Harvested tissues were immediately placed in liquid nitrogen and stored at –80°C.

RNA preparation, probe labeling, slide hybridization, and scanning

Total RNA extraction, mRNA purification and probe preparation were conducted according to Wang et al. (2005). Labeled probes were hybridized to slides at 58°C for ~16 h in the dark. After hybridization, the slides were washed four times at room temperature; once with 2 × SSC, 0.2% SDS for 4 min, followed by two washes in 0.2 × SSC for 2 min each and a final wash in 0.05 × SSC for 4 min. The slides were rinsed in 0.05 × SSC several times and dried by centrifugation at 850 rpm for 5 min at room temperature. A Genepix 400B scanner (Axon Instruments Inc., Union City, CA, USA) was used to scan and quantify the intensity of each feature using GenePix Pro5.0 software.

Arabidopsis 70-mer oligo microarray and hybridization design

The 26,090 oligonucleotides (see Wang et al. 2005 for oligo design) were spotted on SuperAmine slides (TeleChem International Inc., Sunnyvale, CA, USA) using an OmniGrid Accent microarrayer (Genemachines, San Carlos, CA, USA). Each slide contained 27,648 features (26,090 oligo-features plus controls). The printed slides were UV cross-linked, baked, and post-treated according to Wang et al. (2005). Gene names and GenBank accession numbers of the 26,090 oligos and their corresponding cDNA sequences can be found at http://www.operon.com/arrays/omad.php. Duplicated dye swaps were used in our experiments to compare gene expression changes. Each dye-swap consisted of two slide hybridizations: (1) two samples labeled with Cy3-dCTP and Cy5-dCTP, respectively, and (2) the same two samples reversely labeled with Cy3-dCTP and Cy5-dCTP. Further details of the experimental design and analysis methods can be found in Craig et al. (2003) and Wang et al. (2005).

Data analysis

The background-corrected intensity values were used as data for the statistical analysis. A robust local regression (the lowess function in the R package; Cleveland 1979) was used to normalize the background corrected data. Analysis of variance (ANOVA) models were employed to detect differentially expressed genes using the normalized data. Two different analyses were conducted to detect significant genes. The first analysis assumes a common variance for all genes (Craig et al. 2003; Jiang 2004), while the second analysis is based on a per-gene-variance assumption (Jiang 2004).

The ANOVA model under the common variance assumption is

$$\log(y_{ijkgr}) = \mu + A_i + D_j + T_k + G_g + AG_{ig} + DG_{jg} + TG_{kg} + \varepsilon_{ijkr},$$

where $\log(y_{ijkgr})$ is the background corrected and normalized natural logarithm of the intensity of the r^{th} replicate of gene g on array i, with dye j and treatment/condition k; μ is the average natural logarithm of gene intensity over all the genes, arrays and dyes. A, D, T, and G are the array, dye, treatment and gene main effects, respectively, and ε_{ijkr} is normally distributed with mean zero. Differential expression is tested using a null hypothesis that reflects the average treatment. Specifically, $H_0 : T_k + TG_{kg} = T_{k'} + TG_{k'g}$ is tested for each gene g.

When the variance of each unique gene is acknowledged, a per-gene-variance ANOVA model is employed:

$$\log(y_{ijkgr}) = \mu_g + A_{ig} + D_{jg} + T_{kg} + \varepsilon_{ijkgr}.$$

μ, A, D, and T are the gene specific average natural logarithm of the intensity, array, dye, and treatment effects, respectively. Since the subscript g in every term is suppressed, then we have the ANOVA model for a single gene g:

$$\log(y_{ijkr}) = \mu + A_i + D_j + T_k + \varepsilon_{ijkr}.$$

Our justification for using both the common gene and individual gene variance assumption is as follows: if all genes have the same variation across replicates, the common-variance approach is more powerful than the per-gene-variance approach as it has more degrees of freedom for testing. However, the assumption of a common variance across all genes does not hold, i.e., the variation is different across genes. Therefore, the per-gene-variance assumption and approach is more plausible than the common-variance assumption. Unfortunately, the degrees of freedom in the test for a per-gene-variance approach is smaller since the number of biological replicates is limited. As a compromise between a false common variance assumption and a test based on a small number of biological replicates per gene, both approaches are employed in independent analyses to detect the statistically significant differentially expressed genes.

Because we are testing more than 26,000 features for differential expression, it is necessary to adjust the Type-I error rate. False Discovery Rate (FDR; Benjamini and Hochberg 1995) was used for multiple comparison corrections. The significance level α was chosen as 0.05.

RT-PCR and Northern-blot analysis

Total RNA (12 μg), the same as that used in the microarray experiment, was fractionated in a 1.2% formaldehyde agarose gel, and then transferred to a Hybond-N^+ membrane. Probes were labeled with ^{32}P-dCTP using reverse-transcriptase polymerase chain reaction (RT-PCR) amplified cDNA fragments of *B. napus* with primers designed based on the conserved regions of *Arabidopsis* gene and had a product size of 200-500 nucleotides. Only a subset of the genes derived from the integration of differentially expressed genes in RV289 at 24 hpi and which were co-localized with positions of previously mapped QTL was selected for verification of the microarray. Primer pairs used in the RT-PCR were shown in Table 1. Nine probes derived from amplification of a single and distinct band from *B. napus* cDNA were used in the northern hybridization. The hybridization was performed at 60°C using Church buffer (0.5 M Na_3PO_4, 2.5% SDS). Blots were washed twice in 0.5 × SSC, 0.5% SDS at room temperature for 5 min and then twice in 0.2 × SSC, 0.2% SDS at 65°C each for 5 min. The blots were exposed to an X-ray film at –80°C for 1–3 days depending on the signal intensity.

Results

Gene expression profiles in response to *S. sclerotiorum*

We used an *Arabidopsis* 70-mer oligo-microarray representing 26,000 annotated genes for the purpose of investigating the transcriptional differences of genes in *B. napus* after *S. sclerotiorum* infection. Two ANOVA models, each reflecting different model assumptions, were employed to estimate and test for differential expression between genotypes at each of three time points. The expression ratio was determined by mRNA abundance in mycelium-inoculated versus mock-inoculated plants to eliminate the circadian clock and plant growth stage effects, since the samples were collected at different times of day. Using the

Table 1 RT-PCR primers used in the verification of gene expression for the 12 candidate genes

At locus	Forward primer	Reverse primer
At2g32190	5′ CAM AAC CAA TCT TCA GGA G	5′ CAA CAG CAA CAC ATG GCC GCA AG
At2g35980	5′ CCY GCC TTC TAC GGT CCA TC	5′ GGC TAG GTT ATA CCT TAA AAT G
At2g38470	5′ GTT CTA TCT TTC AAT CWC AGG	5′ GWC CTT CCA AAG ATC TCT CSA C
At3g46080	5′ CAT CCT TGT CCG ATA TGT GG	5′ AAW CTK AGN TCC AYA CAA GCC
At3g47380	5′ CCT CTT GCA AAR CCA CAA CAT AC	5′ TGA GCA TTR CTC ATG TGG AAC
At3g52400	5′ TGT RCT TCA CCG TCA CCG GCG	5′ CCT GRT GCT CAA CCA ACA CRG C
At3g52450	5′ CAT GAA AGA TCC GGT GAT AG	5′ ACG KCG AAG AGT GTG GTT TG
At5g05320	5′ GAA GCA TRG TGC TGG AAT CTT G	5′ GAG AGA GTA ACC ATC TTG TAG
At5g05730	5′ GGA ACC CTG ATC CTC AAC TAG TTC	5′ GTC TGA AGA TTC ACG TTA CCT G
At5g13080	5′ TGG AGG AAR TAY GGC CAA AAR GC	5′TCR TGS TSG AAG TTW TCG GWG G
At5g15890	5′ GAY G CSA AGT CCA ACT TCT TCA GCC	5′ GGT CCA GGC AGG CAC CAA TG
At5g62150	5′ GAC ATG AGT GCT TGG TGT TCT GC	5′ CAT GGA TAT GCG GGT TTC GCT C

results from both statistical models we report the statistically significant differentially expressed genes detected by FDR, and examine the intersection (Table 2) of these two analyses. The statistically significant differentially expressed genes across genotypes at the same time point, as well as those statistically significant genes across time within a genotype were compared using the resulting gene lists.

Sclerotinia sclerotiorum infection altered the expression of ~7% of the 26,090 genes (Table 2, Fig. 1). At the 12 hpi, we detected only one gene (At4g02380, which encodes a late embryogenesis abundant family protein) that showed a statistically significant differential expression between RV289 and Stellar. The changes of gene expression at this time point could be too low to be detected by the microarray experiment; therefore, only those genes differentially expressed at 24 and 48 h were reported. A total of 686 and 1,547 genes were differentially expressed in RV289 and Stellar, respectively. Most of the statistically significant genes exhibited a threefold to tenfold change (Fig. 2), although some showed changes in expression of over 100-fold. Complete lists of differentially expressed genes and their corresponding *P*-values at each time point

Table 2 Number of statistically significant differentially expressed genes detected by common variance and per-gene-variance at significance level of 0.05 using FDR correction at three different time points post-inoculation in RV289 and Stellar

RNA samples[a]	Common variance	Per-gene-variance	Both[c]
RV 12 hpi[b]	190	696	1
RV 24 hpi	589	4,124	316
RV 48 hpi	820	4,534	523
ST 12 hpi	160	54	1
ST 24 hpi	491	2,031	252
ST 48 hpi	1,924	9,538	1,494

[a] RV and ST indicate resistant genotype RV289 and susceptible genotype Stellar, respectively

[b] hpi denotes hours post-inoculation

[c] Number of genes detected by FDR under assumptions of both common variance and per-gene-variance

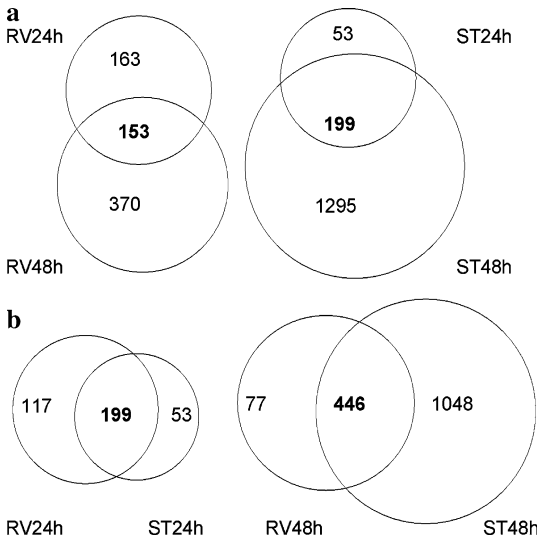

Fig. 1 a and b *Venn* diagrams showing the number of overlapping and specific genes which had significantly differential expression changes at 24 and 48 h post-inoculation in *Brassica napus*. RV indicates the partially resistant genotype RV289 and ST denotes the susceptible genotype Stellar. Results were based on the number of significantly differentially expressed genes detected by ANOVA from eight slides involving two biological and two technical replicates. Number of genes specifically regulated within each genotype and time point was generated from a direct comparison of resulting gene lists from each genotype and time point. **a** Comparisons within each genotype at two time points. **b** Comparisons between genotypes at the same time point. A complete list of genes from all 26k oligo arrays with the ratios and corresponding *P*-values from the data analysis are accessible from the supplementary tables

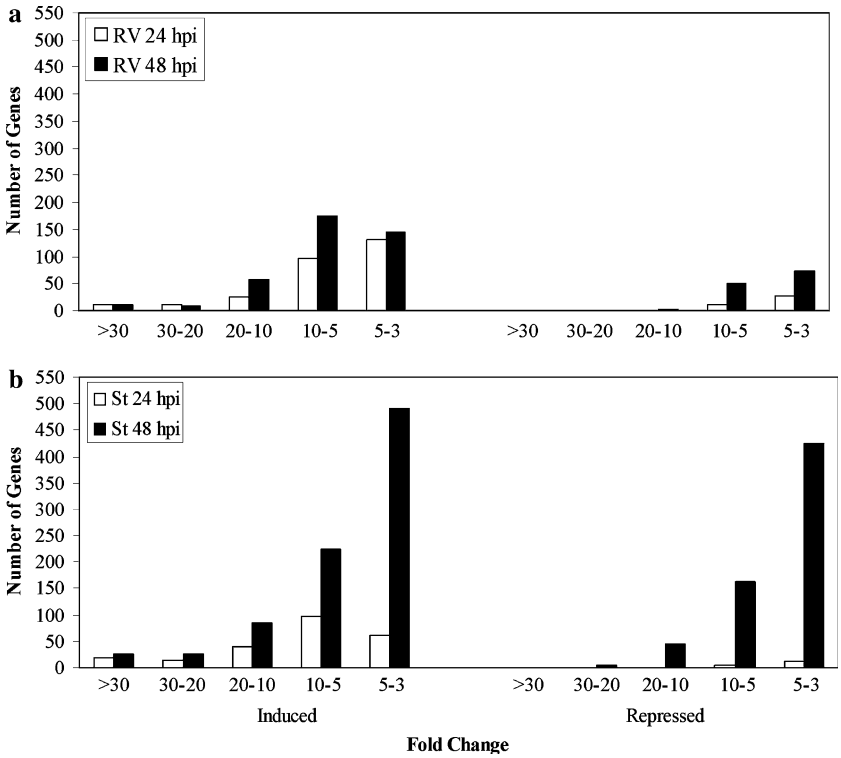

Fig. 2 a and b Number of significantly differentially expressed genes and the levels of expression at 24 and 48 h post-inoculation in RV289 (**a**) and Stellar (**b**). Number of genes differentially regulated within each genotype and time point was generated from ANOVA analysis of eight slides involving two biological and two technical replications. Levels of expression were determined by the mRNA transcript abundance in mycelium-inoculated plants versus mock-inoculated controls

within each genotype are available in the supplementary information (Supplementary Tables 1 to 4).

Co-regulation patterns of significantly differentially expressed genes

Within each genotype, the number of differentially expressed genes increased over time (Fig. 1). In RV289, the number of genes that were significantly differentially expressed increased from 163 to 370 over the 24 to 48 hpi, whereas in Stellar the number increased from 53 genes at 24 hpi to 1,295 statistically significant genes at 48 hpi. A similar number of genes were found at both time points within each genotype (Fig. 1a); however, the expression levels increased with infection time for most of the genes that over-lapped in both time points in the partially resistant genotype RV289; while in the susceptible genotype Stellar most of the overlapping genes decreased their expression levels over time.

Genes detected at the same time point (24–48 h) were compared between genotypes and grouped as being either genotype-specific or common (Fig. 1b). At 24 hpi, RV289 had more specifically expressed genes than Stellar, while at 48 hpi the opposite was observed. We also found that 199 and 446 genes overlapped between RV289 and Stellar at 24 and 48 hpi, respectively (Fig. 1b). The overlapping genes had similar patterns of expression, i.e., were either activated or repressed in both genotypes and no significant difference in expression levels was observed between the two genotypes in a two-sample *t*-test (188 of 199 at 24 hpi, and 426 of 446 at 48 hpi).

Functional classification of the significantly differentially expressed genes

The differentially expressed genes detected at 24 hpi in RV289 were categorized into 13 groups according to the putative function of each gene, which was inferred from metabolic processes known to be related to each gene in *Arabidopsis* (Fig. 3). These categories included pathogenesis-related (PR) proteins, proteins involved in defense-related pathways, the oxidative burst, hormonal responses, transport, abiotic stress, cell maintenance and development, cell wall modification, protein degradation, secondary metabolism, and signal transduction (Table 3). A complete list of the functional groups of genes is shown in supplementary Table 5.

As expected, most of the genes (278 of 316) were up-regulated; in particular those involved in the defense-related pathways, hormonal responses, and molecule transporters. Most of the down-regulated genes included those encoding proteinases, proteins involved in cell maintenance and development. This may indicate that immediately after pathogen infection, defense-related genes leading to the generation of antimicrobial compounds to block pathogen growth are activated and that nutrient

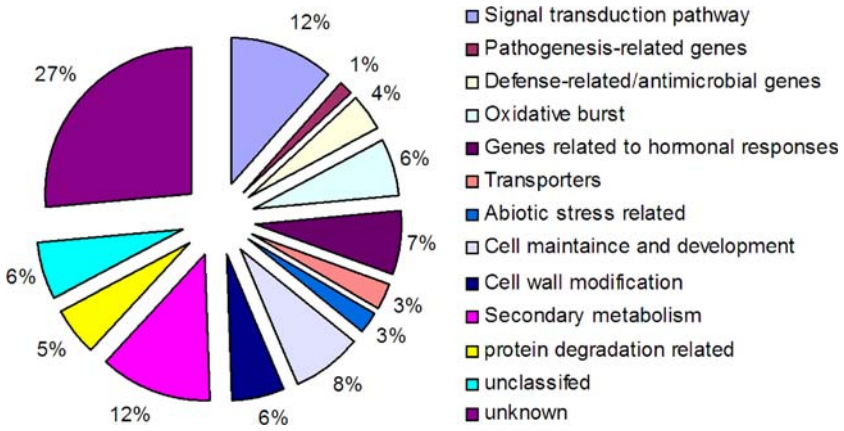

Fig. 3 Functional classification of genes which displayed significantly differential expression in RV289 at 24 h post-inoculation with *S. sclerotiorum*

transport to the infection site to meet this increased energy demand is accelerated. Interestingly, some genes with similar functions were up-regulated while others were down-regulated; one obvious case being the protease inhibitors. Down-regulation of protease inhibitors might indicate that these genes were negative regulators in response to *S. sclerotiorum*.

We identified groups of genes involved in signal recognition and transduction pathways that were differently expressed in response to *S. sclerotiorum* infection. The first group included genes responsive to JA, ET, gibberellin, and auxin. Most of these genes were up-regulated, except for one gene (At1g45015) which is putatively involved in lipid recognition in the JA pathway in *Arabidopsis* was down-regulated. The second group of genes included proteases, phosphatases, kinases, and calcium-binding proteins which were shown previously to be involved in defense responses (Cheong et al. 2002; Ali et al. 2003; Marathe et al. 2004). Proteases such as aspartyl protease and cysteine proteases have been shown to mediate plant-pathogen interactions (Marathe et al. 2004; Xia et al. 2004); however, most genes in the protease pathway detected in our study were down-regulated. The third group contained genes encoding transcription factors, such as the AP2, bZIP (HY5), zinc finger, basic helix-loop-helix, myb (MYB55), WRKY, NAC, and homeobox-leucine zipper family proteins. These transcription factors accounted for half of the signal transduction proteins detected in our study. We also found other groups of genes encoding transporters, including nuclear, protein, sugar, monosaccharide, amino acid, ABC, and magnesium transporters.

Integration of the significantly differentially expressed genes with mapped QTLs

Several QTL with the resistance alleles from RV289 were mapped to a *B. napus* linkage map (Zhao et al. 2006). This allowed us to determine whether genes uniquely induced in the partially resistant line RV289 were located in the QTL regions. We used a comparative mapping method to align QTL locations in *B. napus* to the collinear regions in *Arabidopsis* (Parkin et al. 2005). Sequences of the flanking RFLP markers in QTL regions spanning 10–15 cM on linkage groups N2/N12, N5, and N14 were used to query the *Arabidopsis* genome (http://www.arabidopsis.org/Blast) using BLAST (Altschul et al. 1990) and were found to be collinear with *Arabidopsis* chromosomes 3, 5, and 2 (Fig. 4). Twelve genes located in these regions and which also displayed significantly differential expression changes between RV289 and Stellar at 24 hpi were identified. Annotations of the 12 genes were shown in Table 4.

Verification of the microarray data and the identification of candidate genes

Microarray analysis revealed hundreds of genes, which were statistically significant in differential expression. In order to narrow down the number of genes in the verification experiment, we only used 12 genes derived from the integration of gene expression and previously mapped QTL in the following Northern blot analysis (Table 1). Homologous fragments of the 12 genes from *B. napus* were amplified by PCR using primers designed from conserved regions of *Arabidopsis*. Nine of the twelve primer pairs produced a single band and were used in the subsequent verification experiments (At3g46080 did not amplify; At5g15890 and At5g62150 had more than one band). Northern blot analysis showed that seven of the nine genes were up-regulated in the inoculated samples compared with the control and that transcript abundance increased with time (Fig. 5, only five genes are shown, and the other two genes, At2g32190 and At5g13080, were hybridized in different blots). Transcription of these five genes was present at higher levels in RV289 than in Stellar at 24 hpi. Transcripts at 12 hpi were undetectable using Northern blot analysis, confirming the results from the microarray data.

Table 3 A subset of functional genes in RV289 at 24 hpi from the microarray data

Class	Putative functions	Oligo ID	At locus	F.C.[a]	*P*-value[b]
Pathogenesis-related genes					
	Pathogenesis-related protein	A014787_01	At4g33720	3.46	2.28×10^{-3}
	Disease resistance protein (TIR-NBS-LRR class)	A015225_01	At4g36150	5.27	1.57×10^{-4}
	Disease resistance-responsive family protein	A021290_01	At4g11180	5.01	1.62×10^{-3}
Defense-related/anti-microbial genes					
	Hevein-like protein (HEL)	A011810_01	At3g04720	6.58	2.36×10^{-7}
	Peroxidase	A015933_01	At5g06730	6.87	0.00028641
	Germin-like protein	A010503_01	At3g10080	4.85	0.00060715
	S-locus lectin protein kinase family protein	A015496_01	At4g21390	7.81	0.0012154
	Isoflavone reductase, putative	A024039_01	At1g75300	6.45	5.22×10^{-9}
Oxidative burst					
	Cysteine proteinase	A024966_01	At4g11310	0.20	1.05×10^{-6}
	Glutathione S-transferase	A003708_01	At1g69920	9.04	1.34×10^{-6}
	Cytochrome P450 family protein	A007017_01	At2g29090	0.29	0.0001602
	Cytochrome P450 71A16	A018456_01	At5g42590	3.96	7.69×10^{-5}
Signal transduction pathway					
Phosphatase and kinase					
	Protein phosphatase 2C (PP2C)-related	A002290_01	At1g48040	31.47	8.49×10^{-7}
	Protein kinase-related	A004377_01	At1g53060	7.81	2.84×10^{-8}
	Protein kinase family protein	A011332_01	At3g25250	29.32	2.66×10^{-7}
Tanscription factor					
	AP2 domain-containing transcription factor	A019144_01	At5g67180	3.65	5.56×10^{-6}
	bZIP protein HY5 (HY5)	A020209_01	At5g11260	0.28	8.39×10^{-5}
	Zinc finger protein, putative	A014232_01	At4g09700	7.48	6.92×10^{-5}
	RING zinc finger protein-related	A023621_01	At2g31510	7.31	1.01×10^{-5}
	Basic helix-loop-helix (bHLH) family protein	A015111_01	At4g09180	22.67	7.71×10^{-6}
	CHP-rich zinc finger protein-related	A014397_01	At4g01340	0.18	7.18×10^{-4}
	myb family transcription factor (MYB55)	A013871_01	At4g01680	3.83	1.50×10^{-6}
	WRKY family transcription factor	A017767_01	At5g13080	3.81	1.01×10^{-6}
	WRKY family transcription factor	A019924_01	At1g64000	4.01	3.08×10^{-3}
	NAC family (no apical meristem)-related protein	A018005_01	At5g18270	4.16	1.08×10^{-6}
	WRKY family transcription factor	A006343_01	At2g38470	5.89	4.65×10^{-6}
	Homeobox-leucine zipper family protein	A019286_01	At5g52170	12.55	4.50×10^{-5}
Genes related to hormonal responses					
Jasmonic acid pathway					
	Lipase class 3 family protein	A001120_01	At1g56630	3.42	0.00029992
	Family II extracellular lipase	A016305_01	At5g42170	37.34	1.63×10^{-6}
Auxin related pathway					
	Auxin-responsive protein	A005177_01	At3g15540	8.73	4.89×10^{-5}
	Auxin-responsive GH3 family protein	A013467_01	At4g03400	18.04	4.82×10^{-6}
Ethylene pathway					
	Ethylene-responsive factor	A010537_01	At3g23230	4.82	0.00054292
	1-Aminocyclopropane-1-carboxylate oxidase	A020836_01	At1g05010	3.70	0.00057564
Transporters					
	Nuclear transport factor 2 (NTF2) family protein	A003012_01	At1g71480	3.69	3.47×10^{-5}
	Protein transport protein-related	A005508_01	At5g27220	5.40	5.05×10^{-5}
	Sugar transporter family protein	A007381_01	At2g35740	5.31	4.24×10^{-6}

Table 3 continued

Class	Putative functions	Oligo ID	At locus	F.C.[a]	*P*-value[b]
	Monosaccharide transporter	A002937_01	At1g34580	103.4	2.08×10^{-6}
	Amino acid transporter family protein	A014868_01	At4g35180	3.22	0.00013558
	ABC transporter family protein	A001205_01	At1g15520	19.79	3.95×10^{-7}
	Magnesium transporter CorA-like family protein	A015754_01	At5g09710	6.56	2.64×10^{-5}
Abiotic stress related					
	Universal stress protein (USP) family protein	A011007_01	At3g11930	3.58	0.00012799
	Early responsive to dehydration stress protein	A012143_01	At3g25760	5.34	1.19×10^{-5}
	Heat shock protein 70	A013416_01	At4g37910	11.34	8.39×10^{-8}
Cell maintaince and development					
	Pentatricopeptide (PPR) repeat-containing protein	A009905_01	At3g06430	3.72	0.0014675
	Ribosomal protein L34 family protein	A020835_01	At1g29070	0.23	6.74×10^{-6}
	O-methyltransferase family	A003292_01	At1g76790	3.81	3.76×10^{-5}
Cell wall modification					
	Invertase/pectin methylesterase inhibitor protein	A009600_01	At3g47380	3.82	0.00029387
	Proline-rich family protein	A010441_01	At3g09000	3.82	3.79×10^{-5}
	Syntaxin, putative (SYP122)	A012579_01	At3g52400	3.23	0.0032615
Secondary metabolism					
	Anther-specific protein agp1	A000755_01	At1g24520	3.52	0.00032882
	rcd1-like cell differentiation family protein	A023430_01	At2g32550	3.77	0.0010074
	MADS-box protein-related	A004676_01	At1g31640	9.84	1.70×10^{-7}
	Dormancy-associated protein-related	A004704_01	At1g28330	0.26	6.58×10^{-7}
Protein degradation related					
	F-box family protein contains F-box	A023793_01	At1g32430	0.17	0.0066487
	U-box domain-containing protein	A010325_01	At3g52450	3.65	0.00047019
	Protein–protein interaction regulator family	A020245_01	At1g15200	3.25	0.00013835
	Protease inhibitor	A021288_01	At4g12500	0.16	4.18×10^{-6}

[a] F.C. indicates fold change of gene expression in inoculated plants versus mock-inoculated controls

[b] *P*-value indicates probability of a gene showing significantly differentially expression between *S. sclerotiorum* infected samples compared with mock-infected samples at significance level of 0.05 using FDR correction

The mRNA abundance of the gene homologous of At2g38470 was examined by quantitative RT-PCR analysis in RV289 and Stellar at 24 hpi. The expression ratio was higher in RV289 than in Stellar after corrected PCR efficiency and normalized using the gene *Actin* (AF111812) in the treatments relative to the mock-inoculated controls (data not shown).

Discussion

We used *Arabidopsis* arrays to examine gene expression changes in *B. napus* in response to *S. sclerotiorum*. RV289 is a partially resistant line and had revealed a significantly reduced disease symptom compared with Stellar (Zhao et al. 2004). In this study, we observed temporal and quantitative differences in gene expression between two genotypes of *B. napus* in response to *S. sclerotiorum* infection. RV289 tended to induce the expression of genes associated with defense mechanisms earlier (24 hpi) than the susceptible genotype, with many of these same genes being expressed later (48 hpi) in the susceptible line Stellar. This suggests that the difference in the defense response of the two genotypes to *S. sclerotiorum* may due to temporal and quantitative differences in gene expression. This seems plausible since our previous screening data indicate that the defense response is multi-genic and the partially resistance phenotype was observed as restricting lesion progression rather than preventing infection.

Several plant hormones are important molecules in regulating plant defense response. SA is involved in plant *R* gene-mediated disease resistance to biotrophic pathogens, whereas JA and ET are implicated in the response to necrotrophs (McDowell and Dangl 2000). Auxin was also reported to be involved in the plant response to various stresses and is interconnected with these disease resistance

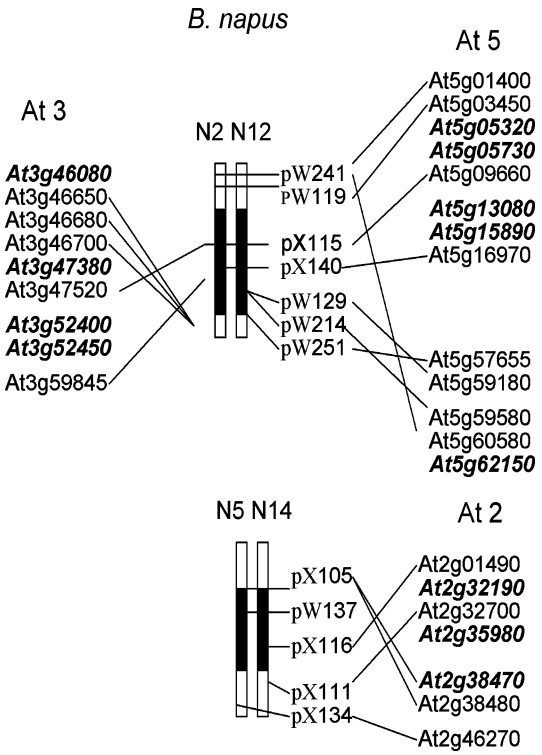

Fig. 4 Integration of the mapped QTL regions on linkage groups of N2, N12, N5, and N14 in *B. napus* and the collinear chromosome positions in *Arabidopsis* chromosomes 2 (At 2), 3 (At 3), and 5 (At 5). *Black* boxes indicate QTL intervals in corresponding linkage groups of *B. napus*. *Slash marks* on *vertical lines* indicate relative positions of RFLP marker loci flanking QTL regions. Alignments between RFLP marker loci and corresponding *Arabidopsis* genes are shown. Twelve candidate genes localized in these collinear regions are shown in *black* and *italics*

signaling pathways (Cheong et al. 2002; Dowd et al. 2004; Reymond et al. 2004). In our study, we detected few genes known to be specially regulated by SA but several that are specially regulated by JA and ET in the response to *S. sclerotiorum* infection. This indicates that response to *S. sclerotiorum* is SA-independent, and mainly JA and ET-dependent, although this hypothesis requires further examination. We also found that auxin-responsive genes were up-regulated after *S. sclerotiorum* infection, suggesting the concomitant activation of both auxin and JA/ET pathways in the response to *S. sclerotiorum* infection.

Transcription factors play important roles in gene regulation (Riechmann et al. 2000). AP2, WRKY and zinc finger proteins have been reported to be differentially regulated by various stresses (Chen et al. 2002). *Arabidopsis* transcription factor *AtWRKY33* (At2g38470) was recently reported to be required for resistance to necrotrophic fungal pathogens *Botrytis cinerea* and *Alternaria brassicicola* (Zheng et al. 2006). In this study, at least two WRKY family transcription factors homologous to At2g38470 and At5g13080 were identified. These two genes were co-localized with the positions of previously mapped QTL. The expression levels of these two genes were verified by Northern-blot analysis, and for At2g38470, also confirmed by quantitative RT-PCR. These two genes were also highly induced after *S. sclerotiorum* infection in another partially resistant *B. napus* line with a lineage similar to RV289 (D. H. Hegedus, Saskatoon Research Center, Canada, personal communication). WRKY transcription factors regulate down-stream gene expression via combining the W box of the promoter regions of the target genes. How the transcription factors identified in this study regulate the response to *S. sclerotiorum* infection remains to be elucidated by the identification and characterization of the target genes and its regulatory sequences.

Two types of transporters have been reported to function in the defense response, the monosaccharide transporter gene, *STP4* (Truernit et al. 1996; Fotopoulos et al. 2003), and the *Arabidopsis* ATP transporter, *AtPDR12* (Campbell et al. 2003; Jasinski et al. 2003). These reports suggested a model for increased demand for carbohydrate and energy caused by environmental stress. We identified several

Table 4 Annotation of 12 candidate genes identified from the integration of differentially expressed genes in RV289 at 24 hpi and mapped QTL

Oligo ID	At locus	Description
A021628_01	At2g32190	Expressed protein
A008079_01	At2g35980	Hairpin-induced family protein (YLS9)
A006343_01	At2g38470	WRKY family transcription factor
A021247_01	At3g46080	Zinc finger-related protein
A009600_01	At3g47380	Invertase/pectin methylesterase inhibitor family protein
A012579_01	At3g52400	Syntaxin, putative (SYP122)
A010325_01	At3g52450	U-box domain-containing protein
A016617_01	At5g05320	Monooxygenase
A016574_01	At5g05730	Anthranilate Synthase
A017767_01	At5g13080	WRKY family transcription factor
A017545_01	At5g15890	Expressed protein
A016171_01	At5g62150	Peptidoglycan-binding LysM domain-containing protein

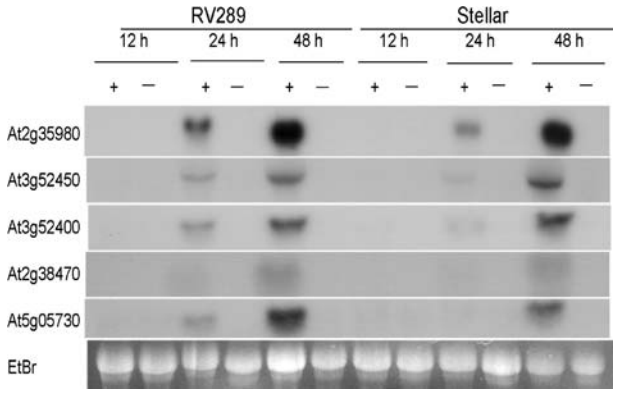

Fig. 5 Northern-blot analysis of selected genes revealed from the integration of microarray data and mapped QTL. Total RNA of 12 μg from RV289 and Stellar at 12, 24, and 48 hpi was separated on 1.2% formamide gel. Probes were cDNA fragments generated from PCR amplification of *B. napus* cDNA using primers designed from conserved regions of the target *Arabidopsis* genes revealed from microarray results. + mycelium-inoculated samples,—mock-inoculated controls. EtBr stained total RNA was shown as a loading control. Only RNA samples from one biological replicate was shown. The second biological replicate gave the same result

genes encoding putative transporters including a monosaccharide transporter, an ABC transporter, amino acid transporters and sugar transporters that were highly up-regulated in response to this infection. This is consistent with the previous observations on the defense functions of monosaccharide and ATP transporters, and may also suggest that besides monosaccharide transporter and ABC transporter, other types of transporters are demanded in the infection point.

Gene expression profile studies using microarray usually result in hundreds of genes showing up- or down-regulation. Verification of hundreds of genes is not practical. Thus, which and how many genes to choose has been a debate for researchers. In this study, we used a comparative mapping method to integrate the differentially expressed genes with the previously mapped QTL to narrow down the number of genes. Four genomic regions on N2, N12, N5, and N14 from *B. napus* containing resistance alleles from the partially resistant parent RV289 correspond to two regions in *Arabidopsis* due to a transposition of the N2 region containing the QTL onto N12 and homeology between N5 and N14 in the regions of the QTLs (Udall et al. 2005; Zhao et al. 2006). Conserved blocks between *B. napus* and *Arabidopsis* were found between N2/N12 and At3/At5, and N5/N14 and At2 by blast analysis. This result agrees with the detailed alignment reported by Parkin et al. (2005). Candidate genes were chosen based on two criteria: (1) location in these collinear regions of *Arabidopsis*, and (2) a significant expression change in RV289 at 24 hpi and a consistent expression change at 48 hpi. Twelve candidate genes were selected based on these criteria from the integration. Northern-blot analysis verified seven of the nine genes which were amplified as a single and distinct band from *B. napus*. A quantitative RT-PCR was also used to verify the expression level of these seven genes. In general, the quantitative PCR gave higher expression values than the microarray data. However, only three of the seven genes were verified, including a homolog of At2g38470. The main reason for this is that the PCR primers were designed based on *Arabidopsis* sequence and in *B. napus* genome, each homologous single gene was assumed to have at least six copies. Thus, the PCR amplification is not specific enough. Further work is needed to design and test primers for gene specificity. Cross-amplification should be considered in verification of homologous genes or members of a gene family. Products of these seven genes that were confirmed by Northern-blot analysis include hairpin-induced family protein (YLS9), WRKY and zinc finger proteins, plant cell wall related proteins, and ET-responsive proteins. Although functions of these genes need further characterization, our data provide new insights into the mechanism of defense response to *S. sclerotiorum* and suggest that this defense might involve various proteins and signaling pathways. Early induction of WRKY and zinc finger proteins and plant cell wall related proteins may play important roles in enhancing resistance to *S. sclerotiorum* disease.

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