Patterns of differential gene expression in *Brassica napus* cultivars infected with *Sclerotinia sclerotiorum*

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SUMMARY

The fungal pathogen *Sclerotinia sclerotiorum* infects a broad range of dicotyledonous plant species and causes stem rot in *Brassica napus*. To elucidate the mechanisms underlying the defence response, the patterns of gene expression in the partially resistant *B. napus* cultivar ZhongYou 821 (ZY821) and the susceptible cultivar Westar were studied using a *B. napus* oligonucleotide microarray. Although maximum differential gene expression was observed at 48 h post-inoculation (hpi) in both cultivars, increased transcript levels were detected in cv. ZY821 at the earlier stages of infection (6–12 hpi) for many genes, including those encoding defence-associated proteins, such as chitinases, glucanases, osmotins and lectins, as well as genes encoding transcription factors belonging to the zinc finger, WRKY, APETALA2 (AP2) and MYB classes. In both cultivars, genes encoding enzymes involved in jasmonic acid, ethylene and auxin synthesis were induced, as were those for gibberellin degradation. In addition, changes in the expression of genes encoding enzymes involved in carbohydrate and energy metabolism appeared to be directed towards shuttling carbon reserves to the tricarboxylic acid cycle and generating reactive oxygen species. Transcripts from genes encoding enzymes involved in glucosinolate and phenylpropanoid biosynthesis were highly elevated in both cultivars, suggesting that secondary metabolites are also components of the response to *S. sclerotiorum* in *B. napus*.

INTRODUCTION

Sclerotinia sclerotiorum is a necrotrophic plant pathogen that infects important agricultural crops, including canola (*Brassica napus*), soybean (*Glycine max*) and sunflower (*Helianthus annuus*) (Boland and Hall, 1994). In the field, infection of *B. napus* by *S. sclerotiorum* occurs mainly through ascospore colo-

nization of senescent flower petals that have lodged on leaves or petioles adjacent to the stem. Necrotic lesions develop on the stems, resulting in premature wilting, stem breakage and lodging of plants with subsequent yield loss. Partial resistance has been reported in semi-winter-type *B. napus* cultivars (Zhao *et al.*, 2004). Quantitative trait loci (QTLs) associated with partial resistance have been reported in two independent studies and indicate that resistant cultivars may possess different resistance genes (Zhao and Meng, 2003; Zhao *et al.*, 2006); however, the biochemical and genetic bases of resistance to *S. sclerotiorum* are not well understood.

Plants have evolved complex mechanisms to recognize and respond to various pathogens. Plant–pathogen interactions have been investigated using microarray technology in model plant species, such as *Arabidopsis thaliana*, challenged with several pathogens and pest species (AbuQamar *et al.*, 2006; Maleck *et al.*, 2000; Schenk *et al.*, 2000; Thilmony *et al.*, 2006). In crop plants, such studies have been confined to gene expression profiling based on expressed sequence tag (EST) analysis: for example, the response of rice to *Magnaporthe grisea* (Jantasuriyarat *et al.*, 2005), sorghum to aphid (Zhu-Salzman *et al.*, 2004), cotton to *Fusarium oxysporum* (Dowd *et al.*, 2004), soybean to *Pseudomonas syringae* (Zabala *et al.*, 2006) and wheat to various biotic and abiotic stresses (Chao *et al.*, 2006). As a result of the high degree of synteny in gene sequences, *A. thaliana* microarrays have been used to examine gene expression profiles in cultivated *Brassica* species (Girke *et al.*, 2000; Lee *et al.*, 2004), including transcriptional changes associated with the response of *B. napus* cultivars to *S. sclerotiorum* infection (Yang *et al.*, 2007; Zhao *et al.*, 2007). These studies have revealed that a common set of genes is induced during the later stages [48–72 h post-inoculation (hpi)] of the infection in partially resistant and susceptible cultivars. Genes expressed soon after inoculation, within 12 hpi, that might modulate the later defence response have not been examined.

An extensive degree of genome duplication in *Brassica* species (Parkin *et al.*, 2005) gives rise to expanded gene families which are indistinguishable using *A. thaliana* microarrays. A

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clear example of this is the polygalacturonase inhibitor protein (PGIP) gene family in *B. napus* which contains at least 17 members, many of which respond differently to biotic and abiotic stresses and signalling hormones (Hegedus *et al.*, 2008). The *A. thaliana* genome contains only two PGIP genes. Recently, a gene-specific oligonucleotide microarray representing 15 000 unique genes from *B. napus* has been developed (Sharpe *et al.*, 2006; Stasolla *et al.*, 2008). This paper reports the use of this *B. napus* microarray to examine the patterns of gene expression in a resistant *B. napus* cultivar, ZhongYou 821 (ZY821), and a susceptible doubled haploid line of *B. napus* cv. Westar in response to *S. sclerotiorum* inoculation. Stem tissue, the site of natural infection, was used, and five sampling time points from 6 to 72 hpi were studied, as these were most likely to reveal clues about the mechanisms underlying the resistance. The expression of specific sets of regulatory and defence-related genes was monitored following challenge with *S. sclerotiorum*, and differences in the response between resistant and susceptible cultivars were assessed. The expression of genes associated with key signalling and metabolic pathways is discussed.

RESULTS AND DISCUSSION

Disease development and expression of genes in response to *S. sclerotiorum* inoculation

Natural infection of *B. napus* stems by *S. sclerotiorum* was simulated by the inoculation of unwounded plants with mycelial

plugs at flowering. Visible lesions developed between 24 and 48 hpi. After 7 days, the lesions on stems of the resistant cultivar ZY821 were smaller than those on the susceptible cultivar Westar, and most cv. Westar plants died within 21 days of inoculation (Fig. 1).

To elucidate the mechanisms underlying the differences in the response of these two cultivars, the gene expression profiles of stem tissues were examined in the vicinity of the inoculation site over 3 days with the emphasis on the early stages of infection. Across all time points examined, the expression of 3659 and 4124 genes was altered significantly [analysis of variance (ANOVA), false discovery rate (FDR) < 0.0001] in cv. ZY821 and Westar, respectively, by inoculation. In both cultivars, the number of differentially expressed genes increased steadily up to 48 hpi and then declined thereafter, with the greatest change occurring between 12 and 48 hpi (Fig. 2). A complete list of the differentially expressed genes with at least a two-fold difference in expression across the five time points is provided in Tables S1 and S2 (see Supporting Information). All lists include the ID number of the *B. napus* oligonucleotides on the microarray and the coordinate for the corresponding *A. thaliana* orthologue and annotation for the particular gene affected. The microarray data have been deposited in Gene Expression Omnibus (Accession no. GSE13262) and can be accessed at <http://www.ncbi.nlm.nih.gov/geo/index.cgi>. It should be noted that some differences between the two cultivars examined in this study could potentially be a result of variations in hybridization caused by allelic differences between the two cultivars and *B. napus* cv. DH12075,

Fig. 1 Phenotype of *Brassica napus* cv. ZY821 and Westar, 7 and 21 days after inoculation of stems with *Sclerotinia sclerotiorum*.

Fig. 2 Dynamics of differentially expressed genes in *Brassica napus* cv. ZY821 (squares) or cv. Westar (circles) after infection of stems with *Sclerotinia sclerotiorum*. The total (broken line) number of differentially expressed genes, as well as genes that were differentially expressed in only one cultivar (full line), are shown. The data presented are the number of differentially expressed genes detected by one-way analysis of variance (ANOVA) from three biological replicates of each cultivar at each time point. The number of genes that exhibited increased or decreased transcript abundance are represented as positive or negative values, respectively, on the y axis.

which was used to generate the sequences employed to design the oligonucleotides for the array (Stasolla *et al.*, 2008). In addition, 774 unique *S. sclerotiorum* cDNAs deposited in GENBANK, representing genes expressed on pectin medium or during infection of *B. napus* (Li *et al.*, 2004a), were scanned against the 15 000 oligonucleotides on the array. Of these, only eight (CD645785, NADH:ubiquinone oxidoreductase subunit; CD645868, polyubiquitin; CD646053, histone H3; CD646110, ADP-ATP translocase; CD645779, severe depolymerization of actin; CD645744, G-protein β -subunit; CD645671, cytochrome C oxidase polypeptide Vib; CD646004, isocitrate dehydrogenase subunit I) exhibited a match better than 1×10^{-5} and have the potential to cross-hybridize.

The allotetraploid genome of *B. napus*, comprising contributions from *B. rapa* and *B. oleracea*, contains expanded gene families (paralogues) for which only a single orthologue is present in the *A. thaliana* genome. The *B. napus* microarray contains oligonucleotides that differentiate the expanded families corresponding to these orthologous *A. thaliana* genes. In cv. ZY821 alone, 1158 expanded gene families, containing two to eight members, were found to be differentially expressed 48 h after *S. sclerotiorum* inoculation (Table S3, see Supporting Information). In 767 gene families, transcripts from only one member were detected, indicating that the other members were either not differentially expressed or their transcript levels were undetectable using the microarray methodology employed. Trends in

transcript abundance indicating either an increase or decrease in response to inoculation were observed with members of 369 families, whereas the members of 22 gene families responded differently (differential orthologues; Table S3). These included genes encoding proteins implicated in the defence response, such as chitinase and peroxidase.

Verification of microarray data

Sixteen genes were selected to examine the reliability of the use of microarray analysis to determine the relative changes in gene expression after *S. sclerotiorum* inoculation (Fig. 3). In general, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis showed that the change in gene expression was greater than that indicated by microarray analysis, but the overall trend was consistent. qRT-PCR analysis was also more sensitive and was able to detect elevated levels of transcripts 6–12 h earlier than microarray analysis, indicating that pathogen recognition and induction of the response occurred very soon after inoculation. The genes selected included five that encoded defence-associated proteins, including that encoding a lectin whose transcripts increased 24 000-fold within 48 hpi as determined by qRT-PCR. Transcripts from genes encoding enzymes involved in jasmonic acid (JA) (BN15608, *BnOPR2*), ethylene (ET) (BN14781, *BnSAM3*; BN13547, *BnACS6*; BN18488, *BnACO*) and auxin (BN24462, *BnCYP79B2*; BN15682, *BnAAO1*) biosynthesis

Fig. 3 Verification of the expression of selected *Brassica napus* cv. ZY821 genes by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) (black bars) and microarray (grey bars) analysis after inoculation of stems with *Sclerotinia sclerotiorum*. Errors bars for qRT-PCR analysis show the standard deviation of three technical replicates for each of three biological replicates.

were detected within 6 hpi, as were those encoding enzymes required for the biosynthesis of glucosinolates (BN22452, *BnSOT16*) and phenylpropanoids (BN22021, *BnOPCL*; BN13060, *BnCCR*). As discussed below, there were also profound changes in the expression of genes associated with carbon metabolism, which was confirmed by qRT-PCR analysis.

Identification of genes induced in the resistant cultivar ZY821

Four genes were selected for qRT-PCR analysis from those that showed higher transcript abundance in cv. ZY821 at 6–12 hpi compared with cv. Westar based on the microarray data (Fig. 4). The genes encoded *BnLOX1* (BN22526), a lipoxygenase (LOX), *BnWRKY33* (BN17285), a WRKY-like transcription factor, *BnWRKY40* (BN23912), another WRKY-like protein, and *BnLectin*

Fig. 4 Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of four genes that were differentially expressed in *Brassica napus* cv. ZY821 (white bars) and Westar (grey bars) stems at 6 and 12 h (indicated) after inoculation with *Sclerotinia sclerotiorum*. The increase in transcript levels was relative to a mock-inoculated control after normalization to a *B. napus* reference gene (*Actin*). The error bars show the standard deviation, with means that were statistically different indicated by either ** (Student *t*-test, $P > 0.05$) or * (Student *t*-test, $P > 0.1$) below each time point.

(BN25790), a legume lectin family protein. qRT-PCR analysis confirmed that the transcript level for each gene was significantly higher in cv. ZY821 than in cv. Westar at these early time points, suggesting that the resistant cultivar responds more quickly to the pathogen.

A comparison of the lists of genes with increased transcript abundance in the two cultivars across all time points revealed 41 genes that were expressed only in cv. ZY821 (Table 1). Transcripts from four genes encoding transcription factors were elevated, two of which were induced within 12 hpi. These may be partly responsible for regulating the expression of genes involved in the stress response or metabolism. Two genes encoding annexins (BN18917 and BN15878) were induced, one within 12 hpi and the other by 48 hpi. Annexins bind to membranes in a calcium-dependent manner, but undergo cellular redistribution in response to stimuli, including environmental stress (Clark *et al.*, 2001). Some plant annexins have been shown to form calcium channels (Gorecka *et al.*, 2007) which may be important for the initiation of the signalling cascades needed to induce the expression of defence-associated genes. Eleven other genes encoded proteins involved in some aspect of the stress response. Most noteworthy was a gene encoding a concanavalin-like lectin (BN25790) that was induced five-fold at 6 hpi and 109-fold by 48 hpi based on microarray analysis, and several 1000-fold based on qRT-PCR analysis (Fig. 3). Although such proteins have not been shown to exhibit anti-mycotic activity, a gene encoding an endo- β -1,3-glucanase was induced within 6 hpi with expression increasing steadily afterwards. A gene encoding BnPGIP1 (BN12243), a member of the expanded PGIP gene family in *B. napus* that is responsive to *S. sclerotiorum* infection (Hegedus *et al.*, 2008), was also induced. PGIPs inactivate polygalacturonases, which are major virulence determinants, and several are produced by *S. sclerotiorum* (Li *et al.*, 2004b). Genes encoding a multidrug resistance protein (BN27037) and a UDP-glucosyl transferase (BN22038) involved in secondary metabolite biosynthesis were induced within 12 and 24 hpi, respectively. The expression of these genes implies that a pharmacological battle is being waged between host and pathogen.

Genes involved in hormone signalling

Auxin and gibberellin

Genes encoding products involved in aspects of hormonal signal transduction (Table S4, see Supporting Information) were identified in the response of cv. ZY821 to inoculation with *S. sclerotiorum*. Most plant defence genes are regulated by endogenous plant hormones, such as salicylic acid (SA), JA and ET. An indole-3-acetic acid amino synthetase has also been reported to be involved in the activation of basal immunity in rice (Ding *et al.*, 2008), indicating the role of auxin in plant defence. Two *B. napus* genes (BN13468 and BN22832), similar to At4g22620 and

Table 1 Classification of genes that were induced in only *Brassica napus* cv. ZY821 stems after *Sclerotinia sclerotiorum* infection.

Oligo ID*	Corresponding <i>A. thaliana</i> locus	Description	6†	12	24	48	72
Transcription							
BN11472	At3g57390	MADS-box protein	–	4.16‡	6.28	12.8	4.95
BN26939	At2g47460	Myb transcription factor	–	2.21	2.17	5.28	5.71
BN27665	At1g28360	ERF domain protein 12	–	–	3.05	9.46	7.87
BN26396	At5g57520	Zinc finger	–	–	3.30	5.37	3.57
Stress							
BN25790	At3g16530	Lectin	5.18	15.8	50.5	109	108
BN22148	At3g57260	Endo β -1,3-glucanase	2.27	3.52	6.11	9.91	7.56
BN18917	At2g38750	Annexin	–	3.65	2.66	25.1	33.5
BN27037	At4g22990	Multidrug resistance protein 2	–	2.20	2.57	8.02	9.01
BN22038	At4g34135	UDP-glucosyl transferase	–	–	3.95	8.95	3.40
BN16777	At4g15160	Protease inhibitor	–	–	2.92	3.02	18.4
BN20902	At4g27670	Heat shock protein	–	–	2.65	7.35	3.71
BN11639	At1g69530	Expansin	–	–	–	7.21	9.46
BN17923	At4g10265	Wound-responsive protein	–	–	–	6.03	7.79
BN18404	At5g27060	Leucine-rich repeat	–	–	–	5.58	5.00
BN15878	At5g65020	Annexin	–	–	–	4.26	7.39
BN13492	At2g37640	Expansin	–	–	–	3.90	7.79
BN12243	At5g06860	PGIP1	–	–	–	3.27	18.9
Metabolism							
BN22522	At4g27070	Tryptophan synthase	2.65	2.69	4.65	10.6	8.31
BN24404	At4g15417	Ribonuclease III	2.00	2.76	3.33	26.5	14.0
BN23990	At1g05680	UDP-glucosyl transferase	–	2.53	2.95	24.5	9.04
BN16989	At1g48920	Nucleolin	–	2.08	4.04	6.67	4.41
BN18865	At1g07720	β -Ketoacyl-CoA synthase	–	–	3.09	28.8	15.7
BN15489	At1g17710	Phosphoric monoester hydrolase	–	–	3.01	73.0	54.4
BN23164	At4g18950	Ankyrin protein kinase	–	–	2.10	5.80	4.61
Others							
BN14638	At1g19770	Purine permease	2.13	2.10	4.59	10.9	10.1
BN20514	At1g76790	O-Methyltransferase	2.00	7.87	12.5	25.3	31.1
BN13257	At1g54020	Lipolytic enzyme	–	3.15	14.2	–	–
BN14979	At2g30540	Glutaredoxin	–	2.16	4.74	17.4	7.81
BN13468	At4g22620	Auxin-induced protein	–	2.05	2.16	–	8.11
BN26740	At2g31990	Exostosin	–	2.04	2.17	10.4	7.10
BN23610	At5g43340	Inorganic phosphate transporter	–	–	4.66	27.5	14.8
BN20039	At1g15180	Membrane protein family	–	–	4.37	11.7	12.1
BN18437	At5g15440	Circadian clock coupling factor	–	–	3.75	13.4	11.5
BN24154	At5g01210	Transferase	–	–	3.63	5.48	7.55
BN17385	At5g10300	Hydrolase	–	–	2.14	3.91	7.93
BN26419	At5g17450	Copper homeostasis factor	–	–	–	11.1	9.69
BN19106	At5g01750	Expressed protein	–	–	–	9.41	6.90
BN20931	At5g60760	2-Phosphoglycerate kinase	–	–	–	7.38	4.21
BN24358	At3g54040	Photoassimilate-responsive protein	–	–	–	5.07	–
BN17022	At3g13857	Expressed protein	–	–	–	2.94	10.5
BN13468	At4g22620	Auxin-induced protein	–	–	–	–	9.38

*Oligonucleotide designation from the *B. napus* gene-specific array.

†Hours post-inoculation.

‡Fold changes relative to mock-inoculated control.

At5g35735, which are known to be responsive to auxin, were induced in cv. ZY821 at 12 hpi, but not in cv. Westar (Table 2). Two other genes encoding enzymes involved in the biosynthesis of indole-3-acetic acid, cytochrome P-450 CYP79B2 (BN24462) and aldehyde oxidase AA01 (BN15682), were induced at 6 and 12 hpi only in cv. ZY821 (Table S4). Gibberellins (GAs) also regulate plant growth and various developmental processes. Transcripts from a *B. napus* gene encoding gibberellin 2-oxidase

(BN19546), which is responsible for GA degradation (Thomas *et al.*, 1999), increased at 12 hpi in cv. ZY821 (Table 2), suggesting that dynamic changes in plant growth and development are occurring.

Jasmonic acid and ethylene

Of the classic defence hormones, JA and ET tend to broadly coordinate the response to mechanical attack from insects and

Table 2 Induction of regulatory genes in *Brassica napus* cv. ZY821 and Westar stems after *Sclerotinia sclerotiorum* inoculation.

Oligo ID*	Corresponding <i>A. thaliana</i> locus	Signalling pathway	Description	ZY821		Westar	
				6†	12	6	12
BN13468	At4g22620	Auxin	IAA induced	–	2.05	–	–
BN22832	At5g35735	Auxin	IAA induced	–	2.57	–	–
BN19546	At1g30040	Gibberellin	GA degradation	–	2.11	–	–
BN17492	At1g76650	Calcium	Ca ²⁺ binding	–	–	–	2.14
BN18528	At4g20780	Calcium	Ca ²⁺ binding	–	2.81	–	3.16
BN19238	At3g63380	Calcium	Ca ²⁺ transport	–	9.93	–	6.03
BN19735	At3g57330	Calcium	Ca ²⁺ transport	–	2.08	–	2.06
BN24299	At2g17290	Protein kinase		–	2.80	–	2.61
BN10050	At3g45640	MAP kinase	MPK3	2.00	–	–	–
BN25533	At1g73500	MAP kinase	MPK9	–	2.56	–	–
BN25534	At1g73500	MAP kinase	MPK9	–	–	–	2.23
BN26459	At4g17500	Transcription	ERF1	–	5.01	–	3.57
BN15727	At5g47220	Transcription	ERF2	–	–	–	3.68
BN25670	At5g47230	Transcription	ERF5	2.00	2.31	–	2.09
BN25738	At4g17490	Transcription	ERF6	–	–	–	2.17
BN17976	At5g51190	Transcription	AP2	2.97	3.11	–	–
BN11384	At2g46510	Transcription	bHLH	–	–	–	2.55
BN14283	At4g14365	Transcription	RING finger	2.53	3.13	–	2.22
BN18692	At5g27420	Transcription	RING finger	–	–	–	3.96
BN26939	At2g47460	Transcription	MYB12	–	2.21	–	–
BN14274	At3g23250	Transcription	MYB15	–	2.71	–	–
BN22570	At3g46600	Transcription	SCL11	–	3.05	–	–
BN17285	At2g38470	Transcription	WRKY33	3.72	5.69	–	4.11
BN23912	At1g80840	Transcription	WRKY40	2.98	8.05	–	2.73
BN10329	At1g27730	Transcription	ZAT10	2.45	3.94	–	–
BN10330	At1g27730	Transcription	ZAT10	4.41	8.12	2.08	6.40
BN14917	At5g59820	Transcription	ZAT12	–	3.20	–	–
BN14919	At5g59820	Transcription	ZAT12	–	5.39	–	2.70
BN22157	At3g21150	Transcription	Zinc finger	–	–	–	–

AP2, APETALA2; bHLH, basic helix–loop–helix; ERF, ethylene response factor; GA, gibberellin; IAA, indole acetic acid; MPK, mitogen activated protein kinase; MYB, myeloblastosis; RING, really interesting new gene; SCL, scarecrow-like; ZAT, zinc-finger protein from *Arabidopsis thaliana*.

*Oligonucleotide designation from the *B. napus* gene-specific array.

†Fold change relative to mock-inoculated control within each genotype at 6 and 12 hours post-inoculation (hpi), respectively.

necrotrophic pathogens, whereas SA is most often associated with the response to biotrophic pathogens (Dong, 1998; McDowell and Dangl, 2000). This classification is not absolute as these signalling pathways may act individually, synergistically or antagonistically depending on the pathogen involved (Berrocal-Lobo *et al.*, 2002; Glazebrook, 2005). For example, resistance to *A. brassicicola* in *A. thaliana* depends on JA signalling and camalexin production (Glazebrook, 2005), but is also dependent on ET signalling (Oh *et al.*, 2005). Resistance to *B. cinerea* depends on genes co-regulated by JA and ET, but SA, abscisic acid (ABA) and hydrogen peroxide-dependent defences also contribute to the resistance (Asselbergh *et al.*, 2007; Glazebrook, 2005). JA is synthesized from α -linolenic acid via four enzymatic steps (Turner *et al.*, 2002), the first of which yields 13-hydroperoxylinolenic acid via the action of LOXs. In *A. thaliana*, LOX2 is primarily responsible for this reaction in response to wounding (Bell *et al.*, 1995), but five *BnLOX* genes were induced in cv. Westar within 24 hpi (Table S4). Interestingly, only two *BnLOX* genes were

induced in cv. ZY821; however, one of these was induced more than five-fold within 6 hpi. Hydroperoxide lyase (HPL) gene expression is also induced by wounding, and this activity can divert some of the 13-hydroperoxylinolenic acid towards the synthesis of leaf alcohols and aldehydes involved in defence against insect attack (Bate *et al.*, 1998). In the current study, both cultivars exhibited a strong decrease in the abundance of transcripts for a gene encoding HPL (BN10615), which should serve to increase the pool of 13-hydroperoxylinolenic acid available for JA synthesis. Allene oxide synthase (AOS) catalyses the formation of 12,13-epoxylinolenate, which is then converted to 12-oxophytodienoic acid by allene oxide cyclase (AOC). Similar to the *BnLOX* genes, three *BnAOC* genes were induced in cv. Westar within 24 hpi, whereas cv. ZY821 expressed only two, one of which was induced within 12 hpi. The final step in JA biosynthesis is catalysed by 12-oxophytodienoate reductase (OPR). Both cultivars exhibited elevated levels of three *BnOPR* genes with similar expression patterns in this study.

ET is synthesized by three enzymatic reactions from methionine (Wang *et al.*, 2002), with the first step being catalysed by *S*-adenosyl methionine synthase (SAM). A sharp decrease in *BnSAM1* transcripts was detected in cv. Westar by 48 hpi. In contrast, *BnSAM3* was induced in cv. ZY821 within 24 hpi. Two aminocyclopropane carboxylate synthase (ACS) genes were detected, *BnACS6* and *BnACS8*; however, only *BnACS6* was induced in cv. ZY821 and, once again, earlier than in cv. Westar. The final step in ET biosynthesis is catalysed by aminocyclopropane carboxylate oxidase (ACO). In cv. Westar, three *BnACO* genes were induced within 24 hpi, but only one in cv. ZY821 with the same expression profile as in cv. Westar.

Salicylic acid

Recently, Guo and Stotz (2007) reported that defence to *S. sclerotiorum* is dependent on JA, SA and ET signalling in *A. thaliana*. Likewise, an ABA-deficient tomato line displayed an SA-dependent defence response and was less susceptible to *S. sclerotiorum* and *Erwinia chrysanthemi* (Asselbergh *et al.*, 2007). In this study, genes encoding enzymes leading to JA and ET synthesis were rapidly induced on *S. sclerotiorum* inoculation, as were the genes responsive to them, such as chitinase, β -1,3-glucanase and plant defensin 1 (PDF1), whereas transcript abundance from several genes leading to SA biosynthesis decreased. Two orthologues of isochorismate synthase 1 (ICS1) required for the synthesis of SA in *A. thaliana* (Wildermuth *et al.*, 2001) were represented on the *B. napus* microarray (BN15208/BN15209 and BN18712). Although it is recognized that changes in gene expression do not necessarily reflect changes in metabolite levels, transcripts from both genes decreased similarly in cv. Westar and ZY821 (Tables S1 and S2). The coordination of the response to necrotrophic pathogens is obviously complex, and the role of SA and other signalling molecules cannot be discounted, as genes that exclusively respond to them, for example *BnPR1*, were induced.

Genes encoding transcription factors

Rapid and directed induction of gene expression requires both a sensitive surveillance system and a highly responsive transcriptional apparatus. Several genes encoding zinc finger, WRKY, MYB-like and APETALA2 (AP2)-like transcription factors exhibited elevated levels of transcripts after inoculation with *S. sclerotiorum* (Table 2). ET response factors (ERFs) are a class of AP2 domain-containing transcription factor that recognize the ET responsive element in the promoters of ET-responsive genes (Riechmann and Meyerowitz, 1998). Transcript levels for *BnERF1* (BN26459) were similar between the two cultivars; however, *BnERF2* (BN15727) and *BnERF6* (BN25738) were elevated in cv. Westar at 12 hpi and *BnERF5* (BN25670) was elevated at 6 hpi in cv. ZY821 and in both cultivars at 12 hpi (Table 2).

The genes encoding additional AP2 domain, several zinc finger, RING finger, MYB and WRKY transcription factors were found to be induced exclusively, or at higher levels, in cv. ZY821 within 6–12 hpi. AbuQamar *et al.* (2006) identified 30 genes encoding transcription factors that exhibited increased transcript abundance in *A. thaliana* leaves within 24 h after inoculation with *B. cinerea*. Functional redundancy precluded the establishment of a readily visible phenotype for most insertion inactivation mutants; however, a few were found to show reduced resistance. These included two zinc finger proteins with ankyrin repeats (C-X₈-C-X₅-C-X₃-H-type), At3g55980 and At2g40140, which, when mutated, gave rise to plants with enhanced local susceptibility and sensitivity to ABA. In the current study, orthologues of these genes were not detected in either of the *B. napus* cultivars used, but genes encoding a similar zinc finger protein, BnZAT12 (BN14917 and BN14919), were induced soon after inoculation of cv. ZY821 (Table 2). ZAT12 is required for the expression of ascorbate peroxidase, which provides some measure of resistance to hydrogen peroxide during oxidative stress (Rizhsky *et al.*, 2004).

The genes encoding two WRKY factors similar to *A. thaliana* WRKY33 (BN17285) and WRKY40 (BN23912) were also rapidly induced in cv. ZY821 (Table 2). Several pieces of evidence attest to the importance of WRKY factors in the mediation of the resistance response. A *B. napus* WRKY33 orthologue was induced in a partially resistant cultivar on infection by *S. sclerotiorum* and mapped to a QTL for resistance on N14 (Zhao *et al.*, 2007). *Arabidopsis thaliana wrky33* mutants were more susceptible to *B. cinerea* and *A. brassicicola*, which corresponded to a reduction in the expression of the gene encoding the anti-fungal protein PDF1.2 (Zheng *et al.*, 2006). Surprisingly, over-expression of *AtWRKY33* in *A. thaliana* enhanced resistance to necrotrophic fungal pathogens, but increased susceptibility to *P. syringae*, leading to the suggestion that WRKY33 might serve as a positive regulator of JA/ET-responsive genes and a repressor of SA-responsive genes (Zheng *et al.*, 2006).

Genes encoding components of signal transduction pathways

Genes coding for products that function upstream of transcription initiation (Table 2) were identified. One gene (BN17492) encoding a calcium-binding protein was induced in cv. Westar but not in cv. ZY821, whereas the expression of other genes (BN18528, BN19238 and BN19735) involved in calcium signalling was similar. Protein phosphorylation and dephosphorylation were implicated in the mediation of the response to the necrotrophic fungus *Phoma macdonaldii* in sunflower (Alignan *et al.*, 2006). Several genes encoding protein phosphatase 2C (Tables S1 and S2) were induced after exposure to *S. sclerotiorum* in both cultivars. These enzymes are negative regulators of

mitogen-activated protein kinase (MAPK) signalling pathways (Rodriguez, 1998), including those leading to programmed cell death (PCD) (Tamura *et al.*, 2006), but are also produced after treatment with the necrosis-inducing protein, Nep1, concomitant with a calmodulin-like protein (Keates *et al.*, 2003). MAPK cascades are also required for the induction of defence responses to biotic and abiotic stresses. The inactivation of MPK6 and MPK4 by the protein phosphatase, AP2C1, in *A. thaliana* reduced innate immunity and increased susceptibility to *B. cinerea* (Schweighofer *et al.*, 2007). Two MAPK genes, *BnMPK3* (BN10050) and *BnMPK9* (BN25533/BN25334), were induced at 6 and 12 hpi, respectively, in cv. ZY821 (Table 2); microarray studies in *A. thaliana* have shown that *AtMPK3* is responsive to chitin oligomers.

Defence-related genes and pathways

Of the genes that were induced within 6–12 hpi in both cultivars, those encoding pathogenesis-related (PR) proteins and proteins involved in the response to biotic and abiotic stress were prominent (Table 3). Several of these are known to possess anti-fungal activity, such as chitinase and β -1,3-glucanase, but they may have only a limited role in providing resistance to *S. sclerotiorum*, as they were also induced in the susceptible cultivar. Plant lectins are members of a large group of carbohydrate-binding proteins (Peumans and Van Damme, 1998), some of which are induced by chitin (Ramonell *et al.*, 2005) and exhibit potent antimicrobial activity (Koo *et al.*, 2002). In this study, genes encoding concanavalin A-like (BN25790) and curculin-like mannose-binding lectins (BN24426 and BN24464) were induced at 6–12 hpi, although these lectins have not been reported to show antimicrobial activity. As indicated previously, many genes were members of expanded families, with some members being commonly expressed in the two cultivars and a subset that was exclusive to each cultivar. This feature was most clearly evident in the expression pattern of a group of 11 glutathione-S-transferase (GST) genes, 10 of which were exclusive to either cv. ZY821 or cv. Westar within 6–12 hpi.

Although PCD resulting from the release and accumulation of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, is well suited for containing biotrophic pathogens, the accumulation of ROS is directly proportional to the degree of infection for necrotrophs. Interestingly, transcript levels from two syntaxin genes, *BnSYP121* and *BnSYP122* (BN25095 and BN24260), which have been implicated in the suppression of PCD pathways (Zhang *et al.*, 2007), were elevated (Table 3). Osmotin-like PR proteins may exhibit antimicrobial activity (Shatters *et al.*, 2006), and one osmotin gene (BN24866) was induced early in the resistant cultivar and later in the susceptible cultivar. Osmotins accumulate normally in the cell apoplast during cold acclimation and provide general

Table 3 Induction of defence-related genes in *Brassica napus* cv. ZY821 and Westar stems after *Sclerotinia sclerotiorum* inoculation.

Oligo ID*	<i>A. thaliana</i>		ZY821		Westar	
	locus	Description	6†	12	6	12
BN14925	At3g57240	β -Glucanase	2.10	6.42	–	9.52
BN14926	At3g57240	β -Glucanase	–	–	8.54	–
BN15009	At2g43570	Chitinase	3.66	10.1	2.41	10.4
BN24266	At2g43590	Chitinase	7.21	10.9	10.1	20.1
BN24268	At2g43590	Chitinase	–	3.93	–	–
BN11310	At3g12500	Chitinase	–	–	–	2.35
BN10008	At2g29450	GST	–	2.78	–	–
BN14800	At1g69930	GST	–	–	–	3.00
BN15797	At4g02520	GST	2.11	6.60	2.21	–
BN15802	At4g02520	GST	–	–	–	3.82
BN16623	At2g02930	GST	–	–	–	2.59
BN19123	At1g17170	GST	–	–	–	6.20
BN19124	At1g17170	GST	–	5.95	–	–
BN24382	At1g02920	GST	–	2.96	–	–
BN25567	At1g27130	GST	–	2.58	–	–
BN25568	At1g27130	GST	–	3.47	–	–
BN26558	At2g29460	GST	–	4.12	–	–
BN19079	At1g78820	Lectin	–	–	–	2.34
BN24426	At1g78860	Lectin	–	6.31	–	4.84
BN24464	At1g78850	Lectin	–	14.8	–	6.13
BN25790	At3g16530	Lectin	5.18	15.8	–	–
BN25791	At3g16530	Lectin	–	–	2.74	17.0
BN24866	At4g11650	Osmotin	–	5.26	–	–
BN14490	At1g75830	PDF1.1	–	–	2.25	6.18
BN14537	At2g26020	PDF1.2b	–	–	–	3.15
BN11341	At2g38390	Peroxidase	–	–	–	2.93
BN23917	At4g08780	Peroxidase	2.56	8.08	–	–
BN19400	At3g49110	Peroxidase 33	2.17	–	–	–
BN24675	At3g49110	Peroxidase 33	–	–	–	2.65
BN15231	At4g37520	Peroxidase 50	–	2.88	–	–
BN12244	At5g06860	PGIP1	–	2.34	–	2.17
BN14604	At2g24810	Thaumatocin	2.77	9.63	–	4.55
BN14605	At2g24810	Thaumatocin	–	3.60	–	–
BN23765	At4g33720	PR1	–	2.08	–	–
BN25356	At2g14610	PR1	–	–	–	7.14
BN25095	At3g11820	SYP121	–	–	–	2.13
BN24260	At3g52400	SYP122	–	–	–	2.44

GST, glutathione-S-transferase; PDF, plant defensin; PGIP, polygalacturonase inhibitor protein; PR1, pathogenesis-related protein 1; SYP, syntaxin of plant.

*Oligonucleotide designation from the *B. napus* gene-specific array.

†Fold change in inoculated stems vs. control 6 and 12 h post-inoculation (hpi).

cryo- and osmotic protection. One report has indicated that osmotin, in conjunction with chitinase, forms a complex with actin, causing microtubule disruption and cytoplasmic aggregation (Takemoto *et al.*, 1997). Cytoskeletal reorganization is required for translocation of the nucleus to the site of pathogen interaction, a process that is thought to be important for rapid and targeted deployment of early defences. D'Angeli and Altamura (2007) reported that osmotin regulated the onset of PCD by blocking the Ca^{2+} signalling necessary to initiate actin micro-filament depolymerization.

ROS are generated by normal mitochondrial electron transport mediated by the cytochrome c pathway and through the

actions of oxidases (Amirsadeghi *et al.*, 2006). Several alternative oxidases are capable of serving as the terminal oxidase, accepting electrons from ubiquinone and diverting electron flow from the cytochrome *c* pathway (Finnegan *et al.*, 2004). In the current study, transcript abundance from a gene encoding an alternative oxidase (BN18515) increased early in the infection process in both cultivars (Tables S1 and S2), which should alter the rate of ROS generation. The relationship between alternative oxidase, ROS and PCD is not entirely clear as transgenic tobacco lines over-expressing alternative oxidase exhibit smaller lesions (Ordog *et al.*, 2002), whereas wheat mutants with accentuated lesions show elevated alternative oxidase levels (Sugie *et al.*, 2007).

Genes involved in secondary metabolism

Certain secondary metabolites may play a role in plant defence. Altered patterns of expression were observed for genes associated with biochemical pathways leading to the biosynthesis of phenylpropanoids and glucosinolates in this study. The phenylpropanoid pathway is responsible for the synthesis of a wide variety of secondary metabolites, including lignins, flavonoids, phytoalexins and antioxidants, which play a role in plant defence (Dixon and Paiva, 1995). Of the 11 differentially expressed genes associated with the phenylpropanoid biosynthetic pathway, most exhibited similar expression patterns in both cv. ZY821 and Westar (Table S4). Transcripts from a gene encoding a 4-coumarate-CoA ligase (BN22021), responsible for the formation of coumaroyl-CoA, were elevated. Coumaroyl-CoA is directed to several pathways, one of which leads to the formation of coniferyl aldehyde. The final step in this pathway is catalysed by cinnamoyl-CoA reductase, and transcript abundance from three genes encoding this enzyme (BN23842, BN15787 and BN13060) was elevated in both cultivars. The fate of coniferyl aldehyde is less clear, as genes encoding alcohol dehydrogenases that would result in the formation of coniferyl alcohol were induced in cv. Westar (BN17669), but repressed in cv. ZY821 (BN22074). In contrast, transcript abundance from a gene that encodes a ferulate hydroxylase (BN18532), catalysing the first step in the pathway to ferulate and sinapate, which are precursors for lignin biosynthesis, decreased in cv. Westar. Glucosinolates are plant secondary metabolites found in the Brassicaceae family and can be grouped into three classes, aliphatic, indolyl and aromatic glucosinolates, according to the amino acid precursor used for their synthesis. Tryptophan biosynthesis could be accelerated, as transcript levels from genes encoding enzymes for chorismate biosynthesis and for four of the five biochemical reactions converting tryptophan to indolylmethylglucosinolate, increased sharply in both cultivars. Indolylmethylglucosinolate might be converted into different indolyl glucosinolate derivatives.

Changes in the expression of genes associated with carbon metabolism

The expression of genes encoding key enzymes involved in carbohydrate utilization and energy production [glycolysis, tricarboxylic acid (TCA) and Calvin cycles] was altered in both cultivars (Table S4), suggesting that changes in plant metabolism may occur following inoculation with *S. sclerotiorum*. A sharp decrease was detected in transcript abundance for five genes encoding glyceraldehyde-3-phosphate (G3P) dehydrogenase (BN11845, BN14336, BN24802, BN16865 and BN18780), which is responsible for the generation of G3P from 1,3-diphosphoglycerate, the key intermediate required in carbon fixation via the Calvin cycle. Two genes further downstream in the pathway, encoding transketolase (BN25656) and ribose-5-phosphate isomerase (BN17967), were induced, and may serve to scavenge available G3P and other Calvin cycle intermediates by converting them to ribulose-1,5-bisphosphate. This compound could be directed to the photorespiration pathway, where expression of a gene encoding glycolate oxidase (BN13732) was induced by as much as 50-fold. Glycolate oxidase converts glycolate to glyoxylate and H_2O_2 , the latter being an important component of the defence response.

Transcript abundance from genes involved in the photorespiration pathways downstream of glyoxylate decreased, suggesting that glyoxylate is metabolized via a different mechanism. Glyoxylate may enter the TCA pathway by way of the glyoxylate shunt after conversion to malate. Transcript abundance from malate dehydrogenase (BN16831) and citrate synthase (BN24865 and BN15521) genes increased, although transcript abundance from two isocitrate lyase genes (BN26052 and BN16663) decreased. Many of the altered gene expression patterns appear to be directed towards increasing carbon flow to and accelerating flux through the TCA cycle. Multiple genes encoding each of the enzymes required to convert pyruvate to acetyl-CoA (such as dihydrolipoyl dehydrogenase, dihydrolipoyl lysine-residue acetyltransferase, pyruvate decarboxylase and pyruvate dehydrogenase) were induced, as were genes for several key steps in the TCA cycle.

If carbon fixation via the Calvin cycle is impeded, the plant can access carbon reserves to drive the increased flux through the TCA cycle. A potential source of TCA cycle intermediates is the degradation of fatty acids by the β -oxidation pathway. In this study, genes encoding enzymes that convert acyl-CoA to acetyl-CoA, such as acyl-CoA oxidase (BN16426 and BN22545), enoyl-CoA hydratase (BN13325) and 3-ketoacyl-CoA thiolase (BN11605), were induced; however, transcript abundance from several genes encoding long-chain fatty acid-CoA ligases (BN22268, BN13971 and BN20014), responsible for the initial stage of degradation, decreased (Table S4). Sucrose serves as a storage compound and can be transported throughout the plant.

It can be converted into UDP-glucose and fructose by sucrose synthase, or to glucose and fructose by invertase. Sucrose degradation via the latter pathway appears to be accelerated, as indicated by the induction of four genes encoding invertases (BN10428, BN18301, BN19126 and BN18327), whereas the expression of genes encoding enzymes involved in the alternative pathway, namely sucrose synthase (BN12649) and phosphoglucumutase (BN21073), was reduced in this study. In addition, the genes encoding hexokinase (BN25532) and glucose-6-phosphate isomerase (BN11549) were also induced, suggesting that a large pool of fructose-6-phosphate (F6P) is formed. F6P generated from sucrose degradation is converted to pyruvate by the glycolysis I pathway in the plant cytosol. A gene encoding a key enzyme in this pathway, phosphofructokinase (BN19519), which converts F6P into fructose-1,6-diphosphate, was induced in both cultivars. F6P can also be formed from starch degradation in plastids and is converted to pyruvate by the glycolysis II pathway; however, the means by which the carbon in F6P enters the TCA cycle does not appear to be so direct. Similar to that observed for the Calvin pathway, the generation of 3-phosphoglycerate from G3P appears to be blocked by the repression genes encoding G3P dehydrogenases. It seems more likely that G3P is directed to the photorespiration pathway, resulting in the formation of glyoxylate and H_2O_2 , with the glyoxylate entering the TCA cycle via the glyoxylate shunt.

CONCLUSIONS

In this study, a *B. napus* oligonucleotide microarray representing 15 000 unigenes was used to study the response to inoculation with *S. sclerotiorum* of stems in resistant and susceptible oilseed rape cultivars. A large number of genes were found to be differentially regulated after infection. The gene expression profiles during the latter stages of stem infection were similar to those of *B. napus* seedlings in response to *S. sclerotiorum* infection (Yang *et al.*, 2007; Zhao *et al.*, 2007) and other necrotrophic pathosystems, namely *Botrytis cinerea*—*A. thaliana* (AbuQamar *et al.*, 2006) and *Alternaria brassicicola*—*Brassica oleracea* (Cramer *et al.*, 2006). Sampling was biased towards the very early stages of the infection, as it was hypothesized that events occurring soon after the initial interaction between pathogen and host were critical for resistance to aggressive necrotrophs, such as *S. sclerotiorum*. Infected stems were examined as these are the natural site for infection in this pathosystem. The resistant cultivar ZY821 effected the induction of defence-associated genes earlier than the susceptible cultivar Westar. Differences were also observed in the patterns of a large group of regulatory genes, as well as genes involved in plant hormone synthesis and aspects of the host defence mechanisms against pathogens. Changes in the expression of genes involved in carbon metabolism suggest that carbon storage reserves (such as sucrose, starch and lipid) are

accessed and shuttled through the photorespiration pathway. This pathway leads to the formation of glyoxylate, which can enter the TCA cycle, as well as hydrogen peroxide, which may form a part of the defence response (Huckelhoven *et al.*, 1999). The activity of the TCA cycle may be greatly increased, as evidenced by the induction of many genes encoding TCA cycle enzymes. This not only generates reducing power and energy, but also precursors for amino acid biosynthesis. A sharp increase in transcript abundance for genes encoding enzymes for tryptophan biosynthesis was observed, this amino acid being a precursor for both glucosinolates and phenylpropanoids. This study has provided new insights into aspects of the defence response to necrotrophic pathogens using an experimental design that closely resembles the actual circumstances occurring in the field. Several targets are being analysed to determine their contribution to the resistance in *B. napus* cv. ZY821 and other resistant cultivars.

EXPERIMENTAL PROCEDURES

Plant material, inoculation and tissue harvest

The semi-winter-type *B. napus* cv. ZY821 and a spring-type doubled haploid line (N-o-1) derived from the cultivar Westar (hereafter referred to simply as cv. Westar) were used. *Brassica napus* cv. ZY821 is partially resistant to *S. sclerotiorum* (Zhou *et al.*, 1994), but cv. Westar is susceptible. Seeds of cv. ZY821 were sown in a Redi-Earth soil mixture (Scotts Fertilizer Company) amended with coconut fibre to improve drainage and Osmocote 15-7-12 (Scotts Fertilizer Company, Marysville, OH, USA) for the controlled release of fertilizer. The plants were maintained in a growth chamber under 200 $\mu\text{mol}/\text{m}^2/\text{s}$ light with a 16-h photoperiod provided by fluorescent and incandescent light. The temperature was maintained at 20 °C in the light and 16 °C in the dark. Two seeds were sown in each of 36 cells, and seedlings were thinned to one plant per cell 11 days after sowing. Four-week-old seedlings of the winter type were vernalized in a cold room at 5–8 °C for 4 weeks with an 8-h photoperiod and half of the previous light intensity. After vernalization, each plant was transplanted to a 6-in (15-cm) pot and returned to the growth chamber. Seeds of cv. Westar were sown directly in 6-in pots and plants were grown under the same conditions as for cv. ZY821, except for the vernalization treatment. Plants were selected for inoculation and sampling using a randomized design with three biological replicates for each cultivar. Each replicate consisted of 60 plants for five time points (6, 12, 24, 48 and 72 hpi) and two treatments (inoculated and mock-inoculated controls). An *S. sclerotiorum* isolate (321; Kohli and Kohn, 1995), obtained originally from field-sown canola plants, was used throughout this study. Plate cultures were maintained on minimal medium according to Li

et al. (2004a). When the plants were at the full flowering stage, three sites on the main stem were inoculated at every other internode with 8-mm plugs cut from the growing margin of a plate colony cultured on minimal salts-glucose agar (20.0 g glucose, 2.0 g NH_4NO_3 , 1.0 g KH_2PO_4 , 1.0 g NaOH, 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g malic acid and 20.0 g agar per 1000 mL distilled water) modified from Cruickshank (1983). Mock-inoculated plants were treated with an agar plug only. Plugs were secured to the stem with Parafilm™. Each line yielded 180 inoculation sites per biological replicate, or 540 sites in total. Epidermal stem tissues extending 10 mm beyond the inoculation site and 1 mm deep were excised at 6, 12, 24, 48 and 72 hpi with a razor blade. The tissues harvested from one biological replicate at each time point (six individual plants comprising 18 inoculation sites) were pooled as one sample. Harvested tissues were frozen immediately in liquid nitrogen and stored at -80°C .

Probe preparation and slide hybridization

Total RNA was isolated from 1 g of ground tissue using the plant RNeasy kit (Qiagen, Mississauga, Ontario, Canada) and stored at -80°C . RNase-free DNase was applied during RNA isolation to remove any DNA contamination (Qiagen). The concentration of total RNA was determined using a Nanodrop spectrophotometer (Nanodrop Technologies, Montchanin, DE, USA) and verified by agarose gel electrophoresis.

Probe preparation, hybridization and washing were performed using the methods of Stasolla *et al.* (2008). Briefly, the Amino Allyl MessageAmp II aRNA amplification kit was used for cDNA synthesis and RNA amplification (Ambion, Austin, TX, USA) with 1 μg of total RNA, following the manufacturer's instructions. *In vitro* transcription to prepare amplified RNA (aRNA) was conducted at 37°C for 14 h and then purified. aRNA was quantified using a Nanodrop spectrophotometer, and 5 μg were coupled with either cyanine-3 (Cy3) or cyanine-5 (Cy5) dye. The coupling reaction mixture was incubated at 20°C for 30 min, quenched with 4 M hydroxylamine for 15 min and purified. Cy3/Cy5-labelled aRNA was dried completely in a vacuum, dissolved in 9 μL double-distilled H_2O and fragmented by the addition of 1 μL of fragmentation reagent (Ambion, catalogue #8740), followed by incubation at 70°C for 15 min. The reaction was stopped with 1 μL of stop solution and 47 μL of hybridization solution [25% formamide, $5 \times$ standard saline citrate (SSC), 0.1% sodium dodecylsulphate (SDS), 0.1 mg/mL sheared salmon sperm DNA] was added. Hybridizations were performed at 37°C for 16 h in the dark, after which the slides were washed in $2 \times$ SSC, 0.1% SDS at 37°C for 5 min, followed by two washes in $1 \times$ SSC for 2 min each and two washes in $0.1 \times$ SSC for 1 min each. The washed slides were dried by centrifugation at 225 g for 1 min at 20°C .

Microarray experimental design and data analysis

Dye swap hybridizations were used to compare changes in gene expression between inoculated and mock-inoculated samples at each time point (from 6 to 72 hpi) for each of the two cultivars. Each dye swap experiment comprised two hybridizations: the first consisted of two samples labelled with either Cy3-dCTP or Cy5-dCTP and hybridized onto a single slide, and the second with the same two samples labelled in reverse. Thus, the experimental design included five time points, two cultivars and three biological replicates, for a total of 60 slides.

The slides were scanned using a Virtek Chip Reader (Bio-Rad Laboratories, Hercules, CA, USA). For each scan, the signal intensity of the two channels was initially adjusted on the basis of the control spots so that an average red/green signal ratio of approximately unity was achieved and the spots were not saturated. Spot intensities from scanned slides were quantified using ArrayPro 4.0 (Bio-Rad Laboratories) and the data were imported into the BioArray Software Environment (BASE version 1.0). Spots with intensities lower than the background were eliminated. Gene Spring 7.3 (Agilent Technologies, Inc., Mississauga, Ontario, Canada) was used to select spots with intensity values ≥ 50 in at least half of the samples (slides) for further analysis. Normalization was conducted using the per-spot and per-chip Lowess normalization procedure with a smooth factor of 20. A one-way ANOVA with a parametric test and variances not assumed to be equal was performed (Craig *et al.*, 2003). A multiple test correction (Benjamini and Hochberg, 1995) was applied and an FDR of ≤ 0.0001 was used. A list of genes for which the expression varied by more than 2.0-fold was generated.

qRT-PCR analysis

qRT-PCR analysis was conducted to verify some of the microarray data. Single-stranded cDNA was synthesized from 4 μg of total RNA using the Superscript First-Strand Synthesis System (Invitrogen, Burlington, ON, Canada); 6 ng of total cDNA was used in each RT-PCR reaction. qRT-PCR was performed in a DNA Engine Opticon 2 (MJ Research, Waltham, MA, USA) system with the SYBR Green qPCR kit (Finnzymes Oy, Espoo, Finland) in a reaction volume of 20 μL containing 0.5 μM of each primer. The PCR conditions were as follows: 95°C for 15 min, followed by 45 cycles of 94°C for 10 s, 60°C for 30 s and 72°C for 30 s. A melting curve of denatured double-stranded cDNA was established to test the purity of the products. Data were obtained from each biological replicate and repeated in triplicate. The PCR primer amplification efficiency was tested using a six dilution series of the template, according to Pfaffl (2001). Expression ratios in the treatments relative to the mock-inoculated controls were calculated using data corrected for PCR efficiency and normalized against the product from the *B. napus Actin* gene.

Primers were designed using PRIMERTM (<http://www.workbench.sdsc.edu>) based on the homologues in *B. napus* DH12075, and the product size was limited to 100–300 nucleotides (Table S5, see Supporting Information).

ACKNOWLEDGEMENTS

This work was funded by Saskatchewan Agriculture and Food, the Saskatchewan Canola Development Commission and the Federal Matching Investments Initiative. We thank Branimir Gjetvaj for technical assistance.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Significantly differentially expressed genes in *Brassica napus* Westar stems after inoculation with *Sclerotinia sclerotiorum*.

Table S2 Significantly differentially expressed genes in *Brassica napus* ZhongYou 821 stems after inoculation with *Sclerotinia sclerotiorum*.

Table S3 Expression of members of gene families in *B. napus* cv. ZY821.

Table S4 Metabolic pathways affected after infection of *Brassica napus* stems with *Sclerotinia sclerotiorum*: (A) hormone biosynthesis; (B) carbon metabolism; (C) secondary metabolite biosynthesis.

Table S5 Primers used for quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

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