Differential profiling of selected defence-related genes induced on challenge with *Alternaria brassicicola* in resistant white mustard and their comparative expression pattern in susceptible India mustard

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SUMMARY

The lack of availability of sources of resistance against *Alternaria brassicicola* within the family Brassicaceae has made oilseed mustard plants a target for one of the most damaging and widespread fungal diseases, *Alternaria* black spot. Of the other non-host-resistant/tolerant plants, *Sinapis alba*, white mustard, is considered to be the most important apart from *Arabidopsis*. To understand the defence response of *S. alba* upon incompatible interaction with this pathogen, a functional genomic approach using cDNA amplified fragment length polymorphism was performed. The highly reproducible bands, found to be either more amplified or uniquely present in infected *S. alba* plants compared with non-infected plants, were further subjected to comparative reverse Northern analysis in the incompatible white mustard (*S. alba*) and compatible India mustard (*Brassica juncea* L.) plants. The suppression of 46% of the genes in the compatible background indicates the possibility of effective and specific recognition of *Alternaria* in *S. alba*. Analysis of the 118 genes up-regulated specifically in infected *S. alba* compared with *B. juncea* showed that 98 genes have similarity to proteins such as receptor-like protein kinase genes, genes involved with calcium-mediated signalling and salicylic acid-dependent genes as well as other genes of known function in *Arabidopsis*. The apparent expression profile data were further confirmed for selected genes by quantitative real-time polymerase chain reaction analysis. Classification of these genes on the basis of their induction pattern in *Arabidopsis* indicates that the expression profile of several of these genes was distinct in *S. alba* compared with *B. juncea*.

INTRODUCTION

Alternaria black spot is one of the most damaging and widespread fungal diseases of the oilseed mustards *Brassica juncea*, *Brassica napus* and *Brassica rapa*. The major constraint towards introgression of disease resistance characters is unavailability of sources of resistance against the causal pathogen *Alternaria brassicicola* within the germplasm of oilseed mustards. By contrast, *Arabidopsis thaliana* and non-crossable *Sinapis alba*, which belong to the same family (Brassicaceae) as oilseed mustards, express non-host resistance against *A. brassicicola* (Pedras *et al.*, 2001; Thomma *et al.*, 1999).

Arabidopsis non-host resistance genes (Navarno *et al.*, 2004) as well as pathogen-associated molecular pattern (PAMP)-triggered genes (Thilmony *et al.*, 2006) have already been characterized and the molecular basis of basal resistance was found largely to overlap with incompatible responses to avirulent pathogens (Eulgem *et al.*, 2004). These defence mechanisms are primarily controlled by transcriptional activation of defence-related genes (Glazebrook 2001). During incompatible interactions defence-related genes are expressed with accelerated kinetics and increased magnitude relative to their induction during the expression of basal resistance (Eulgem *et al.*, 2004; Maleck *et al.*, 2000; Tao *et al.*, 2003). In the case of non-host resistance, PAMPs play a vital role in controlling pathogen invasion (Eulgem *et al.*, 2004). They can be perceived during both compatible and incompatible interactions but in the absence of resistance (R) gene-mediated recognition, basal resistance can be suppressed by effector proteins from virulent pathogens (Caldo *et al.*, 2004). Conversely, incompatible interactions through specific recognition of avirulence (AVR) proteins are robust and negate any pathogen-mediated suppression by accelerated expression of defence responses (Tao *et al.*, 2003).

Key modulators of the plant defence response, which control signalling process locally as well as systematically, are salicylic acid (SA), jasmonic acid (JA), ethylene (ET) (Glazebrook 2005;

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Grant and Loake 2007), reactive oxygen intermediates (ROIs) (Grant and Loake, 2000) and nitric oxide (NO) (Feechan *et al.*, 2005; Hong *et al.*, 2008), with several independent pathways found to be instrumental. In the JA-insensitive mutant *coi-1* of Arabidopsis, resistance against *A. brassicicola* is compromised and systemic expression of the JA-inducible PDF1.2 gene is also reduced significantly within 12 h of infection with *A. brassicicola*. This indicates the requirement of JA-mediated responses for the expression of this trait (Glazebrook 1999). By contrast, the fact that the SA-insensitive mutant *npr-1* and SA-depleted *nahG* line have no effect on the resistance phenotype (Thomma *et al.*, 1998) indicates no direct involvement of SA as a signalling molecule.

Additionally, phytoalexins may also play a major role in conferring resistance against *A. brassicicola* as the *pad3-1* mutant of Arabidopsis, which affects the production of the indole-type phytoalexin camalexin, is more susceptible than wild-type Col-0. The role of camalexin was found to be in the inhibition of destruxin B production (a major phytotoxin produced by *A. brassicicola*) (Pedras *et al.*, 2003). In the ET-insensitive *ein-2* mutant and *nahG* plants, although camalexin biosynthesis is lowered upon infection (Thomma *et al.*, 1999), resistance against *A. brassicicola* is not compromised. However, induction of PR1, a marker of SA signalling, and enhanced biosynthesis of the antifungal compound camalexin upon infection with *A. brassicicola* in Arabidopsis raises the possibility of cross-talk between these different signalling networks. Furthermore, camalexin biosynthesis has been found to be positively controlled by SA, ROI and ET but not by JA. Hydroxylated destruxin B, which acts as an inducer of phytoalexin biosynthesis in resistant *S. alba*, is found to be generated by detoxification through hydroxylation of destruxin B. This step is dominant in *S. alba* compared with *B. juncea* (Pedras *et al.*, 2001, 2003) but no information is available so far as to whether this is a part of a signalling network.

Recently, mutations in *asymmetric leaves 1 (as1)*, resulting from a forward genetic screen, were found to convey increased disease resistance against both *A. brassicicola* and *Botrytis cinerea* in Arabidopsis (Nurmburg *et al.*, 2007). AS1 encodes an MYB transcription factor that has a well-established role in leaf dorso-ventral pattern formation (Waites *et al.*, 1998) and *as1* is a classical mutation in Arabidopsis (Rédei and Hirono, 1964). While AS1 functions in combination with AS2 and other transcription factors to control leaf development, AS1 operates independently of AS2 and cognate components in disease resistance (Nurmburg *et al.*, 2007). Furthermore, this novel function for AS1 is conserved in plant species with a divergence time of ~150 Myr.

Microarray analysis has shown that a large number of genes induced by *A. brassicicola* in Arabidopsis (Saskia *et al.*, 2003) were also co-induced or co-repressed after treatment with SA, JA or ET (Schenk *et al.*, 2000) and many of them were induced systemically (Schenk *et al.*, 2003). Seok *et al.* (2005) found that one ET-sensitive GDSL lipase, also induced by SA, was involved in

resistance against *A. brassicicola*. In addition, proteomic analysis of two *B. napus* lines suggested a role for ROI-mediated auxin signalling as part of the tolerance mechanism towards this pathogen (Sharma *et al.*, 2007). The collective evidence indicates that a coordinated network prevails among SA, JA, ET and ROI signalling pathways engaged during the establishment of resistance against *A. brassicicola*. Although significant transcriptome analysis data from Arabidopsis and to a lesser extent *B. napus* are available, comparisons of data between genetically close compatible and incompatible species are missing. In this context, a combination of PCR-based and hybridization-based transcript profiling of genes through techniques such as cDNA-AFLP (amplified fragment length polymorphism) and reverse Northern analysis, respectively, have been carried out to understand similarities and differences at the molecular level through comparing the gene expression pattern between resistant *S. alba* and susceptible *B. juncea* plants, which are closely related. Comparison of the expression profiles during these plant-microbe interactions created the possibility of studying the identified genes in two genetic backgrounds that are apparently equipped with the same set of basal resistance genes but with altered signal perception capacity.

RESULTS

Disease progression

Visible lesion formation was observed on infected leaves of *S. alba* as well as *B. juncea*; Fig. 1A shows leaves excised from intact plants after infection at different time points. It appeared that the rate of hyphal growth was increased in *B. juncea* compared with *S. alba*. Lesion formation appeared to be faster in *B. juncea* as it started to develop after 48 h and spread over the entire leaf within 72 h. In an identical situation, *S. alba* did not develop lesions at the infection site until after 72 h and the rate of increase in lesion diameter was considerably slower compared with in *B. juncea*. Trypan blue (TB) staining of the challenged leaves (data not shown) of both *S. alba* and *B. juncea* at different time points showed that this fungus started sporulating within 24 h in *B. juncea* leaves but only at 36 h in *S. alba*. Upon transferring the plants to sunlight (both infected and control plants of *S. alba* and *B. juncea*) it was observed that infected *S. alba* plants survived and grew well for more than 15 days though the infected leaves were shed within 7 days and showed some growth retardation compared with control *S. alba* (uninfected) plants. In comparison, infected *B. juncea* plants died within a week (Fig. 1B).

cDNA-AFLP

cDNA-AFLP (Bachem *et al.*, 1996; Durrant *et al.*, 2000; Vos *et al.*, 1995) is an extremely efficient, reproducible and high-throughput

A

B

Fig. 1 Challenge of *S. alba* and *B. juncea* with *A. brassicicola*. (A) Leaves of *S. alba* and *B. juncea* were detached from the plant at 48 and 72 h following infection with *A. brassicicola*. Control leaves were detached from plants without fungal inoculation. (a) *Brassica juncea* leaf—control; (b) *Brassica juncea* leaf—48 h after infection; (c) *Brassica juncea* leaf 72 h after infection; (d) *Sinapis alba* mock control; (e) *Sinapis alba* leaf 48 h after infection; (f) *Sinapis alba* leaf 72 h after infection. (B) *S. alba* and *B. juncea* plants infected with an *A. brassicicola* spore suspension. (a) Left, control *Brassica juncea*; right *Brassica juncea*—5 days after infection. (b) *Sinapis alba* mock control. (c) *Sinapis alba* 15 days after infection. Arrows indicate the *S. alba* leaves which were shed after infection.

PCR-based mRNA fingerprinting method in which prior knowledge of the sequences is not a prerequisite. This approach can be applied when genome-wide microarrays are not available (Reijans *et al.*, 2003). However, analysis of all the transcripts at a particular time point through cDNA-AFLP is limited by the availability of restriction sites. Thus, as well as using the two restriction sites *Eco*RI and *Mse*I, an *Eco*RI site was incorporated into the oligo dT primer to ensure that all the cDNA was at least restricted with *Eco*RI.

Comparisons of the cDNA-AFLP profiles with 100 possible primer combinations were carried out between infected and non-infected *S. alba*. The auto-radiograms (Fig. 2) are representatives of many such gels. During the process, about 5500 bands were scored. The 223 highly reproducible bands (100–800 bp) were either uniquely present in infected plants or amplified to a greater extent in *A. brassicicola* challenged compared with mock challenged plants. All of these 223 bands were reamplified after elution from gels with specific cDNA-AFLP primer combinations and the sizes of the fragments were checked in agarose gels.

Differential screening of the cDNA-AFLP fragments by reverse Northern analysis

In total, 223 cDNA-AFLP fragments were reamplified and subjected to reverse Northern analysis using α - P^{32} -dCTP-labelled first-strand cDNA of non-infected *S. alba* and those infected for 48 h. This was undertaken by a dot blot method in order to judge the apparent expression pattern and eliminate false positive bands obtained from the cDNA-AFLP experiment. Furthermore, a second reverse Northern analysis using α - P^{32} -dCTP-labelled first-strand cDNAs of non-infected *B. juncea* and those infected for 48 h was performed to check the expression pattern of the 223 cDNA-AFLP fragments in the susceptible species. The hybridization pattern of two sets of 32 fragments each are represented in Fig. 3. No hybridization in 11 fragments out of 223 (5%) in both *S. alba* and *B. juncea* indicated high levels of positive amplification in cDNA-AFLP as no hybridization does not rule out the possibility of weakly expressed genes. The intensity of hybridization of the spots was analysed using 'Phosphor imager' for comparative analysis of the transcript level of the 212 products between *S. alba* and *B. juncea*. Among the 177, the 118 fragments (67%) which were up-regulated in *S. alba* compared with *B. juncea* upon infection are presented in Supporting Information Table S3. In *B. juncea* the status of these 118 fragments were: 54 fragments (46%) down-regulated in the infected compared with non-infected *B. juncea* and 19 fragments (16%) equally expressed in both control and infected plants. Another 45 fragments (38%) were up-regulated both in *B. juncea* and in *S. alba* on infection, but the induction was greater in *S. alba*. The remaining 59 (33%) of the 177 fragments were up-regulated more in infected *B. juncea* compared with *S. alba* and were not included for further study, except for a few randomly selected genes. It is important to

Fig. 2 cDNA-AFLP profile of *Sinapis alba* transcripts on infection with *A. brassicicola*. Representative autoradiogram of cDNA-AFLP gels: C, non-infected *S. alba* cDNA; I, infected *S. alba* cDNA; M, labelled 100-bp ladder. Arrows indicate some of the up-regulated or uniquely expressed amplified cDNA-AFLP fragments found in infected *S. alba*.

mention that all the 177 fragments were hybridized with three different batches of non-infected and infected *S. alba* and *B. juncea* labelled cDNA in a highly reproducible and consistent manner.

Distribution of the 118 fragments in terms of fold induction in *S. alba* were: 46 (39%) showed at least a two-fold increase, 22 (18%) showed a greater than two-fold but less than five-fold induction, 33 (28%) showed a greater than five-fold but less than

ten-fold induction, and 17 fragments (15%) showed a greater than ten-fold induction compared with the control. Although this kind of approach is sufficient for the elimination of false positive bands and classification of genes in terms of expression, the quantification of transcript levels may vary with some genes due to inevitable cross hybridization.

Cloning of reamplified fragments and sequence analysis

Using the novel strategy detailed in the experimental procedures below, the cDNA fragments which were up-regulated in infected *S. alba* compared with infected *B. juncea* were cloned and sequenced. Additionally, a few cDNA fragments which were expressed more in infected *B. juncea* compared with infected *S. alba* were randomly selected and cloned in the same fashion and sequenced.

Of the 118 cDNA-AFLP fragments, 98 (83%) which showed high homology with known plant genes present in the database and no or very little homology with genes of other organisms were considered. The remaining 20 fragments showed homology with expressed proteins whose functions are not yet known (Supporting Information Table S3). In summary, 23 were either plant defence-related or stress response-related, 12 encoded transcription factor-related proteins, ten were calcium-signalling-related, seven were protein kinase-related, 17 were photosynthesis and metabolic pathway-related genes, 13 were transporter-related and intra-membrane protein genes, and 16 were involved with transcription and translation. Among the genes which were expressed more in infected *B. juncea*, six showed high homology to an anti-fungal protein (AAY15221.1), cytochrome P450 (AAD03415.1), an MLO family protein (DQ988610), pectin esterase inhibitor (DQ988555), peroxidase 33 (DQ988543) and no apical meristem (NAM) genes (Supporting Information Table S3). All the sequences were submitted in batch to the NCBI database by Sequin, a standalone software tool developed by NCBI for submitting large numbers of sequences to GenBank. The accession numbers provided were DQ988528–DQ988620, EF107052–EF107090 and EF595983. Although no information about *A. brassicicola*-induced genes was available, a few genes from *S. alba* such as the antifungal protein (AAY15221.1), cytochrome P450 (AAD03415.1) and chalcone synthase (CAA32496.1) were already present in the database and their presence was further confirmed by our analysis.

Relative quantification of transcript levels by real-time PCR

The expression pattern of 12 of the genes was analysed by real-time quantitative RT-PCR (Fig. 4) to validate the results obtained from reverse Northern analysis and also for quantitative assessment of the relative abundance of the transcripts at different time points (48 and 64 h) in the localized leaves of both *S. alba*

Fig. 3 Reverse Northern analysis of selected cDNA-AFLP fragments. Autoradiogram representing dot blot hybridization of purified and reamplified cDNA fragments of two sets of 32 fragments each with α - 32 P dCTP labelled first-strand cDNA. The spots marked with circles indicate the internal control, GADPH.

and *B. juncea* (48 h). The expression patterns of eight of these genes were monitored in systemic leaves of *S. alba* at 48, 72 and 96 h after *A. brassicicola* infection (Fig. 5). The GADPH gene was used as an internal standard to normalize any variation in the quantity and quality of the starting template cDNA. In the case of *B. juncea* the 64-h samples were not included in this study as the huge down-regulation of the GADPH gene evidently indicated the possible onset of necrosis of the entire leaf (data not shown). Primers of all the 12 genes including GADPH were designed from *S. alba* cDNA sequences (Supporting Information Table S4) and tested by normal RT-PCR to ensure the specificity of the primers as well as for optimization of cDNA concentration in both *S. alba* and *B. juncea*. The bar diagrams (Figs 4 and 5) represent the average fold induction values of infected *S. alba* localized and distal uninoculated leaves as well as localized *B. juncea* leaves compared with the control after calculating the standard error where $n = 3$ (n represents the number of biological replicates). It was observed that the overall pattern of expression (i.e. up-regulation and down-regulation) of these 12 genes reflected the same trend and also corroborated the result obtained by reverse Northern blotting. Among these genes, enhanced expression of anti-

fungal protein (AAY15221.1), cytochrome P450 (AAD03415.1) and MLO family protein (DQ988610) in infected *B. juncea* compared with *S. alba* was also reflected in reverse Northern data. However, comparison of the fold-induction values between reverse Northern and real-time quantitative RT-PCR indicated some variation. The likely reason was that real-time quantitative RT-PCR is more specific and accurate for quantification compared with reverse Northern analysis, as the possibility of cross-hybridization among the homologous genes cannot be ruled with reverse Northern blotting.

DISCUSSION

Deciphering the resistance mechanism of Arabidopsis and *S. alba* against the fungal pathogen *A. brassicicola* has immense potential for increasing the production of rapeseed mustard by minimization of yield loss. In Arabidopsis, *A. brassicicola*-induced genes have been documented by Schenk *et al.* (2003). However, information on *A. brassicicola*-induced genes in *S. alba* is not yet available. Thus, the present approach of expression profiling of *S. alba* on infection with *A. brassicicola* and comparative expression analysis with *B. juncea* is a significant step in this direction.

Fig. 4 Real-time relative quantitative estimation of the genes induced in *B. juncea* and *S. alba* leaves following local challenge with *A. brassicicola*. Bar diagrams representing the relative changes in expression of twelve genes with respect to respective controls. Error bars represent SE ($n = 3$). Open bars, control; closed bars, 48 h localized; shaded bars, 64 h localized leaves of *S. alba* and *B. juncea*.

Comparative expression analysis indicates that a significant number of genes (33%) are over-expressed during compatible interactions compared with during incompatible interactions. A similar phenomenon has been reported for *B. napus* infected with *Sclerotinia sclerotiorum* (Zhao *et al.*, 2007). Furthermore, the phenomenon of gene suppression in a compatible background indicates that, in the absence of suppressed genes, the genes which were up-regulated in the compatible background may not be directly involved with resistance. Thus, comparative transcript profiling through reverse Northern analysis comparing the resistant *S. alba* and susceptible *B. juncea* has been carried out to eliminate

the genes induced more in compatible interactions than in incompatible interactions. Several of the gene classes up-regulated in *S. alba* overlapped with non-host resistance genes (Navarro *et al.*, 2004) as well as PAMP-triggered genes (Thilmony *et al.*, 2006). On the other hand, suppression of 46% of genes in the compatible background indicates the possibility of effective and specific recognition of the pathogen in *S. alba*, which shows for the first time, at the molecular level, that the interaction of *S. alba* with *A. brassicicola* may be an incompatible interaction. Expression of a number of genes both in *S. alba* and in *B. juncea* on infection demonstrated that apparently compatible and

Fig. 5 Real-time relative quantitative estimation of genes induced in systemic leaves of *S. alba* following challenge with *A. brassicicola*. Bar diagrams representing the relative change in systemic expression (i.e. expression in distal uninoculated leaves) of eight genes with respect to controls. Error bars represent SE ($n = 3$). Open bars, control; closed bars, 48 h systemic; pale shaded bars, 72 h systemic; dark shaded bars, 96 h systemic leaves of *S. alba*.

incompatible interactions may not be attributable to largely distinct molecular mechanisms, but rather that many of these mechanisms may be shared. In summary, the percentage of different classes of genes which are induced more in *S. alba* compared with *B. juncea* on infection are: 19.5% encode plant defence-related or stress response-related, 10% encode transcription factor-related proteins, 8.5% are calcium-signalling-related, 6% are protein kinase-related, 14.5% are photosynthesis and metabolic pathway-related genes, 11% are transporter-related and intra-membrane protein genes, 13.5% are involved with transcription and translation, and 17% show homology with expressed proteins whose functions are not yet known. Similar

induction patterns of genes by using cDNA-AFLP-mediated differential profiling of transcripts in the case of infection with other organisms on different resistant plants have also been reported (Polesani *et al.*, 2008; Torres *et al.*, 2003).

However, the contribution of genetic differences between *S. alba* and *B. juncea*, which belong to the same family, requires further investigation.

Early signal perception components

In plants, RLKs (Receptor like kinase) are localized in the plasma membrane and act as high-affinity binding sites for PAMPs and

play a vital role within multi-component protein complexes for the activation of basal resistance. Several leucine-rich repeat (LRR)-containing RLK genes are found to be up-regulated at the transcriptional level in *Arabidopsis* in response to different PAMPs as well as by phytohormones and wounding. For example, RLK3 (Czernic *et al.*, 1999), FRK1 (Asai *et al.*, 2002), various different types of RLKs (Liquin and Zhixiang, 2000) and ADR1 (Grant *et al.*, 2003) have already been documented. In *S. alba*, two RLK genes (DQ988582, DQ988587), which have very high homology with *Arabidopsis* homologues AT3G14350.3 and AT2G31880.1, respectively, were found by reverse Northern blotting to be marginally up-regulated on infection with *A. brassicicola*. The real-time quantification of DQ988582 showed that expression was marginally induced or remained at the basal level in infected *S. alba* but that in *B. juncea* this gene was down-regulated significantly on infection.

The LRR containing lectin protein kinases LECRK1 and LRK1 in *Arabidopsis* and Pi-d2 in rice, which confers resistance to *Magnaporthe grisea* (Xuewei *et al.*, 2006), are capable of recognizing oligosaccharide elicitors derived from the breakdown of the cell wall (Herve *et al.*, 1996). One such RLK gene with a legume lectin binding site showed transient up-regulation in *S. alba* but down-regulation in *B. juncea* by reverse Northern and real-time quantitative RT-PCR analysis (Fig. 5).

Another very important participant in this cascade are the mitogen-activated protein kinases (MAPKs). Comparison of the expression of the MPK6-like gene by real-time PCR showed that in *B. juncea* this gene was down-regulated significantly compared with *S. alba* at 48 h in localized infected leaves (Fig. 5). The systemic induction of MPK6 was also observed in *S. alba* with an increase in expression after a longer infection time. MPK6 is known to be an SA-induced protein kinase (SIPK) (Zhang and Klessig, 2001) and plays a role in both *R* gene-mediated resistance and basal resistance (Asai *et al.*, 2002; Frank *et al.*, 2004). The MPK6-mediated downstream signalling in *S. alba* against *A. brassicicola* is perhaps a very important step, as up-regulation of an NDR1/HIN-1- (Varet *et al.*, 2002) like gene NHL25, and a beta 1–3 glucanase-like PR gene was evident in *S. alba* on infection. Both these genes were previously found to be downstream targets of MPK6 in *Arabidopsis* and to be controlled by SA (Desikan *et al.*, 1999).

Calcium-mediated signalling

An increase in cytosolic free calcium derived from the extracellular medium or internal stores has been found to be associated with many stress responses, including the defence response. The immediate Ca^{2+} sensor proteins which undergo structural alteration or changes in catalytic activity upon Ca^{2+} binding are calmodulin, calcineurin-B-like (CBL) proteins (Yong *et al.*, 2003) and Ca^{2+} -dependent protein kinases (CDPKs). We have identified

CBL interacting protein kinases 9 and 3 by reverse Northern analysis, which were up-regulated in *S. alba* compared with *B. juncea* upon infection. Moreover, significant up-regulation of a vacuolar $\text{Ca}^{2+}/\text{H}^{+}$ antiporter gene (similar to *Arabidopsis* CAX2) was identified by both reverse Northern and real-time PCR quantification, and up-regulation of a plasma membrane (PM)-type ATP-driven Ca^{2+} -ATPase gene (with calmodulin binding site) was shown by reverse Northern analysis in localized *S. alba* leaves following *A. brassicicola* infection. Down-regulation of both these genes in *B. juncea* indicates that sequestration of free cytosolic calcium in the intracellular organelles in addition to cytosolic pH changes may be important factors in signal perception. This observation can be correlated with the preferential up-regulation of calcium binding C2 domain-containing protein genes in *S. alba* compared with *B. juncea* on *A. brassicicola* challenge. This class of proteins operates in Ca^{2+} -mediated signal transduction and membrane trafficking (Kim *et al.*, 2003) and acts as an important contributor to the plant defence response. Another important gene in this context, MLO, was found to be up-regulated significantly in localized *S. alba* and *B. juncea* leaves on infection, both by reverse Northern analysis and by real-time quantitative RT-PCR. Although MLO is reported to be a calmodulin-dependent membrane-associated protein and in *Arabidopsis* is involved in resistance against the necrotrophic pathogens *A. alternata* and *A. brassicicola* (Consonni *et al.*, 2006), interpretation of the exact role of MLO in *S. alba* resistance against *A. brassicicola* needs further investigation.

Genes involved in indole biosynthesis

An increase in the biosynthesis of indole-containing compounds is an important step in the plant defence response as both indole-type glucosinolates and the phytoalexin camalexin have antimicrobial properties. Up-regulation of indole-3-glycerolphosphate synthase, a very important enzyme in the indole biosynthetic pathway, was shown in localized leaves of both *S. alba* and *B. juncea* on *A. brassicicola* infection by reverse Northern analysis, suggesting the activated synthesis of indole derivatives. Furthermore, up-regulation of the gene coding for the enzyme CYP79B2, involved in synthesis of indole-3-acetaldoxime (a common intermediate), in the localized leaves of *B. juncea* compared with *S. alba* on infection (Fig. 5), indicates the dual possibilities of enhanced biosynthesis of both indole-type glucosinolates and phytoalexins (Glawischnig *et al.*, 2004).

Expression pattern of *Arabidopsis* phytohormone-induced gene homologues in *S. alba*

In *Arabidopsis*, Schenk *et al.* (2000) have shown that infection with *A. brassicicola* caused induction of JA-, SA- and ET-mediated activation of defence response genes, although SA and ET were

Fig. 6 Classification of the genes induced in *S. alba* on the basis of phytohormone-dependent/independent expression in *Arabidopsis*. Venn diagram representing 98 phytohormone-induced overlapping, non-overlapping and unknowingly induced genes of *S. alba*.

not directly involved with resistance. This indicates crosstalk among the pathways in *Arabidopsis*. In the present study, preferential expression of already identified SA-induced genes in *S. alba* compared with *B. juncea* on infection, as shown in Fig. 6, signifies the same possibility. It remains to be determined whether the phytohormone-dependent/independent expression pattern of the genes in *Arabidopsis* follows the pattern in *S. alba*.

In conclusion, the overall results indicate that the combination of cDNA-AFLP and reverse Northern blotting, followed by real-time PCR not only supplemented each other but also proved to be very consistent in differential expression profiling of the *A. brassicicola*-induced genes. Furthermore, comparative expression profiling has shown differences in signal perception capabilities between *B. juncea* and *S. alba* on infection with *A. brassicicola*. The data obtained through this work are important as a subset of the genes were not up-regulated in *Arabidopsis* under similar conditions (Schenk *et al.*, 2000). Hence, we think this information will be helpful in the future development of resistant mustard plants through either genetic manipulation or plant breeding.

EXPERIMENTAL PROCEDURES

Disease progression

Seeds of *Sinapis alba* (collected from Bet Dagan, Israel) were obtained from Professor Zohara Yaniv (Israeli Gene Bank for Agricultural Crops). *S. alba* and *Brassica juncea* plants (8- to 12-leaf stage) were grown in a controlled environment at 25 °C with a 12-h photoperiod (100 $\mu\text{E}/\text{m}^2/\text{s}$). Spores of *Alternaria brassicicola* strain MUCL 20297 (obtained from Professor Willem Broekaert, Katholieke Universiteit Leuven, Belgium) were grown on 2.4% potato dextrose agar (Himedia, India) medium at 25 °C in the dark for 15 days. Two to four leaves of each intact plant of *S. alba* (resistant) and *B. juncea* (susceptible) were inoculated with 5 μL of spore suspension of *A. brassicicola* in water at a concentration of 4×10^5 spores/mL. Control plants were not inoculated, but were otherwise treated in the same way. To identify systemic expression, upper uninoculated distal leaves of a plant were taken whose two bottom most leaves were inoculated. Plants (including the uninfected plants) were kept in a box with a Plexiglas cover at 25 °C at high humidity for monitoring disease progression at different time points. Development of disease symptoms and rate of hyphal growth were measured on the basis of lesion formation around the site of infection and by trypan blue staining, respectively, at different time points.

Isolation of RNA, c-DNA synthesis and c-DNA AFLP analysis

Total RNA was isolated from 2 g of control and 48-h infected *S. alba* frozen leaves using a CONCERT™ plant RNA reagent kit method (Invitrogen). mRNA was isolated from about 1 mg of total RNA using an m-RNA purification kit (Amersham Biosciences, UK). Double-stranded cDNAs were synthesized using a SMART™ cDNA synthesis kit (BD Biosciences) according to the manufacturer's instructions with modification in the sequences of the 3'CDS primer where one *Eco*RI site was incorporated (5'-AAG-CAGTGGTAACAACGCAGAGAATTCT₍₃₀₎N₋₁N-3', where N = A,C,G or T; N₋₁ = (A,G,C) and PCR Primer IIE (5'-AAGCAGTGGTAT-CAACGCAGAG-3'). Then, 250 ng of purified double-stranded c-DNA was digested with *Eco*RI and *Mse*I and the fragments were ligated to *Eco*RI and *Mse*I adapters as described in the AFLP core kit manual (Invitrogen). Pre-amplification was carried out using 1/10 volume of the template, according to the protocol of the kit using pre-amplification primers (Supporting Information Table S1). The *Eco*RI site-specific cDNA-AFLP primers with two selective nucleotide extensions in different combinations at the 3' end were radiolabelled using $\gamma\text{P}^{32}\text{-ATP}$ (6000 Ci/mmol from Perkin-Elmer) and polynucleotide kinase (Invitrogen). Selective amplifications were carried out individually with 100 different combinations (ten *Eco*RI and ten *Mse*I) of labelled *Eco*RI and *Mse*I primers

(Supporting Information Table S2). The PCR cycles comprised 14 touchdown cycles with a 0.7 °C decrease per cycle with initial annealing temperature at 68 °C and normal 28 cycles according to the AFLP kit instructions. Amplification products were resolved in 6% polyacrylamide sequencing gels (BioRad Laboratories) according to standard protocols (Bachem *et al.*, 1996; Vos *et al.*, 1995). The cDNA-AFLP pattern was detected after exposing the dry gel to X-ray film (Kodak bio max, India) at –70 °C overnight for autoradiography.

Isolation and reamplification of cDNA-AFLP fragments

The bands of cDNA-AFLP which were up-regulated more in the infected compared with the non-infected *S. alba* were cut from the gel and eluted according to Michael and Guggenheim (1999). The 4 µL of eluted product was then reamplified in 100 µL volume using the same primer combination and PCR conditions as for selective amplification without touchdown cycles. Then each reamplification product was purified using a QIAquick PCR product purification kit (Qiagen Inc.). All the PCR reactions were performed in a PTC-200 programmable thermal cycler (MJ Research).

Reverse Northern analysis

Purified PCR-reamplified product (10 µL) was mixed with water (40 µL) and samples were heat denatured at 95 °C for 5 min and quickly chilled on ice. One volume of 10× SSC was added and the samples were spotted on a Hybond-N⁺ membrane using a 96-well vacuum manifold. The membranes were denatured and neutralized by soaking in denaturing solution and neutralizing solution (Dong-Chul *et al.*, 1998). Membranes were air dried and cross linked using a UVC 500 UV cross linker (Amersham Biosciences, UK). Radiolabelled first-strand cDNA probes were synthesized from 1 µg of polyA RNA using α -P³²-labelled dCTP (10 µCi/µL) and purified using spun columns packed with Sephadex G-50 according to the protocol of Sambrook *et al.* (1989). Specific activity of the probes was measured using a scintillation counter (Beckman). Membranes were hybridized with an equivalent amount of α -³²P-labelled single-stranded cDNA (specific activity, 1.3×10^9 cpm/µg) probe at 65 °C in the presence of rapid hybridization buffer solution (Amersham Biosciences, UK), washed at successive stringency of 2×, 1× and 0.5× SSC with 0.1% SDS at 65 °C and exposed to X-ray film followed by a phosphor imaging screen overnight. The experiments were repeated three times with three different biological replicates each containing leaves of 30 individual plants. The intensity of the spots was calculated (after background subtraction) in OD/mm² using GS-525 phosphor imaging software (BioRad Laboratories). The RT-PCR-mediated amplified cDNA of the *S. alba* housekeeping gene GADPH, blotted in equal amounts on each membrane, was used as the

internal control to normalize the hybridization. The mean spot intensities from three individual experiments were calculated. Fold induction or repressions of each gene were calculated by dividing the average hybridization intensity values of the infected plant cDNA by that of the control plant cDNA. Genes that showed an induction ratio of at least two-fold and a *P* value of at least 0.05 (calculated using GraphPad Prism version 2.01 by one-way ANOVA using the Newman–Keuls multiple comparison test) were considered for the present study.

Cloning of the differentially expressed cDNA-AFLP fragments

Cloning of all the cDNA fragments was done in a batch process to make it economical. In this strategy, each batch consisting of a maximum of six PCR-amplified bands from two different primer combinations, each having three different sizes of fragments, were mixed together for each ligation with pGEM-T Easy vector (Promega Corp.). The ligation mix was then transformed into DH5 α and the white colonies were then checked by colony PCR-derived band size using different cDNA AFLP primer combinations and also by restriction analysis. Sequencing of the positive clones was done in an Automated DNA sequencer 3130XL Genetic analyser (Applied Biosystem) using M13 forward primer. The sequences of cDNA-AFLP-derived fragments were analysed using the BLASTN and BLASTX software of the NCBI database (Altschul *et al.*, 1990) and TAIR (The Arabidopsis Information Resources).

Real-time quantitative RT-PCR analysis of the transcripts

One microgram of purified polyA mRNA was isolated from each of the samples and cDNA synthesis was carried out using powerscriptTM (BD Biosciences) according to the protocol of the kit. RT-PCR was done using different cDNA concentrations and gene-specific primers designed from the coding region of each gene using the Primer 3 software (v 0.4.0) (Supporting Information Table S4) and *Taq* DNA polymerase (Promega Corp.). PCR cycling conditions comprised an initial denaturation step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 35 s, 53 °C for 45 s and 72 °C for 1 min. Amplification of the products was monitored by running in 1.2% agarose gel containing ethidium bromide to check the efficiency of each set of primers for individual genes and also to optimize the cDNA concentration in the exponential phase of amplification over 35 cycles.

Quantitative real-time PCR was carried out using the 7500 Fast real-time PCR sequence detector and SYBR Green Master mix (Applied Biosystems) using primers at a final concentration of 0.28 µM each and 3 µL of diluted cDNA template (optimized by RT-PCR) in 20 µL total volume. PCR cycling conditions comprised an initial polymerase activation step at 95 °C for 10 min,

followed by 40 cycles at 95 °C for 15 s and 59 °C for 1 min, and a final stage of 15 s at 95 °C, 15 s at 60 °C and 15 s at 95 °C to determine dissociation curves of the amplified products. Real-time cDNA amplification was monitored and analysed using the Sequence Detection system software (version 1.4, Applied Biosystems). Variations in cDNAs of the samples were normalized using GADPH as an internal standard. Fold induction was calculated for 48- and 64-h localized infected leaves of *S. alba*, 48-h localized infected leaves of *B. juncea* and 48-, 72- and 96-h systemic leaves of *S. alba*. Fold changes in gene expression between inoculated and mock-inoculated *S. alba* and *B. juncea* leaves were based on ΔCt calculation ($2^{-\Delta\Delta\text{Ct}}$ method). ΔCt corresponded to Ct of one selected gene subtracted from Ct of GADPH, and fold-change expression was based on calculation of the $\Delta\Delta\text{Ct}$ (Livak and Schmittgen, 2001) that corresponded to ΔCt in inoculated leaves subtracted from the ΔCt in mock-inoculated tissues. Quantitative RT PCR experiments of all the 12 genes were performed in triplicate. The average fold induction values were calculated after considering the standard error, where $n = 3$ (where n represents the number of biological replicates and leaves of 30 individual plants were pooled together for each biological replicate).

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SUPPORTING INFORMATION

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

Table S1 Primers used for preamplification reaction. Sequences of the *Eco*R1 and *Mse*I adapter-specific primers used for preamplification.

Table S2 Primers used for selective amplification reaction. Sequences of the ten *Eco*R1 and ten *Mse*I primers used for selective amplification. The two selective nucleotides at the 3' end of each primer are indicated by bold letters.

Table S3 Homology-based classification of *S. alba* genes induced upon infection and their differential expression pattern in susceptible and resistant genetic backgrounds. The tables represent BLAST X results of the cloned *S. alba* genes induced by *A. brassicicola* after sequence analysis using TAIR (The Arabidopsis

Information Resources). Additionally, DEC represents the degree of expression change calculated after phosphor imaging analysis. Spots represented as (+) are up-regulated in expression whereas (−) are down-regulated in expression of genes with respect to control: (+) two-fold; (++) > two-fold to < five-fold; (+++) > five-fold to < ten-fold; (+++++) > ten-fold up-regulation in expression; (=) genes equally expressed in control and infected population. Genes which showed probability values $P < 0.05$ were included in this table.

Table S4 Primers used for real-time quantitative RT-PCR. Sequences of 12 gene-specific primers designed from the coding region of *S. alba* using Primer3 software (v 0.4.0).

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