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## A compatible interaction of *Alternaria brassicicola* with *Arabidopsis thaliana* ecotype DiG: evidence for a specific transcriptional signature

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### Abstract

**Background:** The interaction of *Arabidopsis* with *Alternaria brassicicola* provides a model for disease caused by necrotrophs, but a drawback has been the lack of a compatible pathosystem. Infection of most ecotypes, including the widely-studied line Col-0, with this pathogen generally leads to a lesion that does not expand beyond the inoculated area. This study examines an ecotype, Dijon G (DiG), which is considered sensitive to *A. brassicicola*.

**Results:** We show that the interaction has the characteristics of a compatible one, with expanding rather than limited lesions. To ask whether DiG is merely more sensitive to the pathogen or, rather, interacts in distinct manner, we identified genes whose regulation differs between Col-0 and DiG challenged with *A. brassicicola*. Suppression subtractive hybridization was used to identify differentially expressed genes, and their expression was verified using semi-quantitative PCR. We also tested a set of known defense-related genes for differential regulation in the two plant-pathogen interactions. Several known pathogenesis-related (PR) genes are up-regulated in both interactions. *PR1*, and a monooxygenase gene identified in this study, *MO1*, are preferentially up-regulated in the compatible interaction. In contrast, *GLIPI*, which encodes a secreted lipase, and *DIOX1*, a pathogen-response related dioxygenase, are preferentially up-regulated in the incompatible interaction.

**Conclusion:** The results show that DiG is not only more susceptible, but demonstrate that its interaction with *A. brassicicola* has a specific transcriptional signature.

### Background

*Alternaria brassicicola*, the agent of black spot disease of crucifers, is able to infect *Arabidopsis*. Different ecotypes and genetic backgrounds show variation in susceptibility to this necrotrophic pathogen. Defenses against necro-

trophs and biotrophs employ different mechanisms [1]. Programmed cell death and production of reactive oxygen species (ROS) are hallmarks of the hypersensitive response (HR) that is a means of plant defense against biotrophs [2,3]. Perception of the pathogen leads to rapid

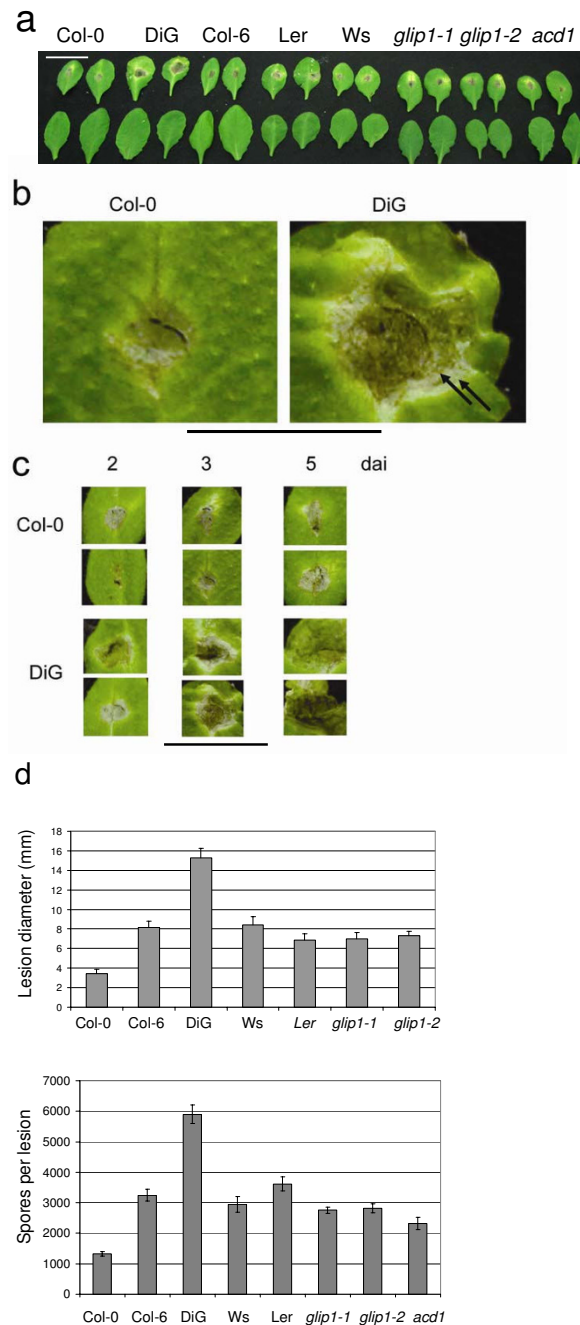
changes in expression of genes including receptor-like protein kinases, followed by cell death and HR-related defense [4-6]. Necrotrophs, in contrast, assimilate nutrients from dead host tissue, and actually benefit from ROS production and programmed cell death [7,8]. It was found, for example, that oxalic acid is apparently a virulence factor for *Sclerotinia sclerotiorum* because it signals for increased ROS production and programmed cell death in the plant [9]. Studies with *Arabidopsis* mutants in different hormone-dependent defense pathways showed that defense against necrotrophs primarily employs jasmonic acid and ethylene-dependent pathways [10,11]. Integration with SA-dependent pathways is also important, and there is cross-talk between the SA and JA pathways [12-14]. An estimated 0.48% of the *Arabidopsis* transcriptome was induced two-fold or more in response to infection with the wide host-range necrotroph *Botrytis cinerea*, and the expression of these genes depends on ethylene, jasmonate and SA pathways [15]. Defense against necrotrophs thus does not necessarily follow the gene-for-gene pattern in which successful recognition implies triggering of the HR. Inoculation of *Arabidopsis* leaves with *A. brassicicola* generally leads to an incompatible interaction in which the lesion does not spread significantly beyond where the fungus was inoculated. In contrast, *Brassica oleracea* is a compatible host, and spreading necrotic lesions are formed. Extensive gene expression data are available for incompatible interactions between *Arabidopsis* and *A. brassicicola* [16-18]. Incompatible and compatible interactions with the bacterial pathogen *Pseudomonas syringae* were compared in a genome-wide study, and the conclusion was that the distinction is mainly a quantitative and kinetic one [19].

The molecular basis for the extent to which the plant can limit infection by necrotrophic fungi is of obviously of great interest, but the use of *Arabidopsis* genetics to investigate this question has been limited by the need to study compatible and incompatible pathosystems for the same pathogen species. Efforts to overcome this gap have begun for *Colletotrichum-Arabidopsis* and *Leptosphaeria-Arabidopsis* pathosystems [20,21]. Interactions with species of *Botrytis* have been studied at the cellular level, leading to a model in which resistance depends on the balance between cell death and survival [22]. *A. brassicicola* is an attractive system because of the considerable amount of work already done with this pathosystem. As for *Leptosphaeria* and *Botrytis*, there is a genome project for *A. brassicicola* [23], which is currently in the manual curation stage (Dothidiomycete group and the Joint Genome Institute, US Department of Energy, unpubl.). *Arabidopsis* mutants defective in biosynthesis of the antimicrobial compound camalexin are more susceptible to *A. brassicicola* [24-26]. The Dijon-G (DiG) ecotype is one of the most susceptible, and is a low-camalexin ecotype [27]. Additional factors

are involved, because disease resistance did not directly correlate with camalexin levels in the 24 ecotypes studied [27]. Indeed, *A. brassicicola* infection of wild type and camalexin-deficient *pad3* mutant plants resulted in a generally similar transcriptional pattern [17]. A secreted lipase encoded by the *Arabidopsis* gene *GLIP1* is important for resistance to *A. brassicicola* [28]. Analysis of the *A. brassicicola-Brassica oleracea* interaction led to the identification of a collection of *A. brassicicola* EST sequences characteristic of this compatible interaction [29]. The DiG-*A. brassicicola* pair, chosen to investigate the role of the *A. brassicicola* non-ribosomal peptide synthase gene *NRPS6* as a virulence factor [30], has a compatible appearance. This led us to consider the question of whether there is merely a continuous range of susceptibility among ecotypes, or rather a fundamental difference between compatible and incompatible interactions. If one postulates that the DiG and Col-0 interactions with *A. brassicicola* differ merely in the extent of sensitivity of the plant to the pathogen, the transcriptional profiles should be very similar in the two interactions. The aim of this study was to ask whether the transcriptional profile of this particular *A. brassicicola-Arabidopsis* interaction differs from that of an incompatible interaction. Differential cDNA screening and a candidate gene approach led to the identification of specific markers for the two types of interaction. The hypothesis that the interactions are identical can thus be excluded.

## Results

A total of five wild type ecotypes, Col-0, Col-6, DiG (Dijon G), Ler (Landsberg erecta), Ws (Wassilewskija) and three mutants (*glip1-1*, *glip1-2* and *acd1*) were screened against *A. brassicicola* (Fig. 1a). These mutants were tested initially, because *glip1* mutants were shown to be susceptible to *A. brassicicola* [28]. The mutant *acd1* was chosen because we reasoned that, as *LLS1* in maize and its ortholog *ACD1* in *Arabidopsis* are required to limit the spread of cell death [31,32], loss of this gene might increase the spread of a necrotrophic pathogen. Inoculum amount and sampling times after inoculation were calibrated in preliminary experiments so that the different plant-fungal pairs could be compared under non-saturating conditions (data not shown). Lesion diameter and spore production were measured (Fig. 1b) to assess the disease progression. The Col-0 accession showed an incompatible interaction, in which the lesion did not progress beyond the boundaries of the inoculated region. Accession DiG was most susceptible, showing larger lesions than either of the *glip1* mutants or Col-6, which are relatively susceptible as compared to Col-0 [28]. The lesion-mimic mutant *acd1*, was no more susceptible to the pathogen than was Col-0 (Fig. 1a, d). The lesions on DiG leaves continued to spread (Fig. 1c) and often show concentric rings (Fig. 1b), as seen in the interaction with the



**Figure 1**

**Characterization of *Arabidopsis-Alternaria brassicicola* pairs.** a) Symptoms in different ecotypes and genotypes, 3 days after inoculation of intact leaves. Top row, inoculated; bottom row, control. *glip1-1* and *glip1-2* are two mutants at the *glip1* locus encoding a secreted lipase [28]; *acd1* is a lesion mimic mutant [32]. Scale bar indicates 2 cm. b) Magnification of images of leaves from (a) showing the ring-like pattern in the progression of the lesion on a DiG leaf (arrows). The innermost dark, thin, arc (no arrow) is material from the inoculum. Scale bar indicates 1 cm. c) Size of lesions on Col-0 and DiG leaves at different times post-inoculation. Representative infected leaves are shown, photographed at the indicated times after inoculation. Scale bar indicates 2 cm. d) Quantitative analysis of lesion size and spore production. Top panel, lesion diameter was measured 5 days after inoculation. Error bars indicate standard errors of the mean for 7 replicate lesions (for Col-0, 9 and DiG, 10 replicates). Lower panel, lesions were excised 5 days after inoculation, the conidia suspended in water, and counted under the microscope in a hemocytometer chamber. Values are means of two independent experiments, consisting of 12 and 4–5 replicates, respectively; the error bars indicate the standard error of the mean of the combined data from the two experiments.

compatible host *Brassica oleracea* but not in incompatible interactions with *Arabidopsis*. To test the possibility that the DiG-*A. brassicicola* interaction has a unique transcriptional signature, two approaches were followed: differential library screening and a candidate gene approach.

A suppression-subtractive hybridization (SSH) library was constructed to compare *A. brassicicola*-infected to mock-inoculated leaves of ecotype DiG. In order to limit the set of ESTs selected, to the extent possible, to plant transcripts, cDNA from RNA isolated from a saprophytic culture of *A. brassicicola* was added to the driver population. In the SSH procedure, the driver competes with the differentially expressed transcripts. Furthermore, the library was constructed at 72 h post-infection, when most of the leaf was still green in both plant-fungal pairs (Fig. 1). Sequence was obtained for 116 clones (Table 1). The library was of high diversity: a chitinase clone, for example, was represented 5 times, and a glycosyl hydrolase three times in the sequences, but most transcripts were represented only once (Table 1). Almost all the sequences were identified in the *Arabidopsis* genome database. 14 of the genes in this set were already known to be up-regulated in response to *A. brassicicola* infection [17], and an additional transcript, *PDIOX1*, was also known to be up-regulated in incompatible interactions [33]. Most of the genes, however, had not been identified previously in the interaction of *A. brassicicola* with Col-0 and its mutants. To determine whether these are all false positives or marginally up-regulated genes, or rather, represent a class of genes up-regulated in the compatible interaction, a set of primer pairs was designed based on sequences of nine clones representing transcripts that were not identified [17] in the interactions with Col-0 and its mutants, and which have annotated functions (Table 2). In an additional, candidate gene, approach, a set of known defense-response related genes (Table 2) was tested.

Semi-quantitative RT-PCR analysis of the abundance of the corresponding transcripts is shown in Fig. 2. An actin gene (*ACT2*), a ubiquitin-conjugating enzyme gene (*UBC*) and cap-binding protein 20 (*CBP20*) were used as "housekeeping" genes (Table 2). The false-positive library clones also serve as additional controls for overall efficiency of the RT PCR procedure (Fig. 2). The fold-induction by infection relative to Col-0 is shown in Fig. 3. The transcripts detected by three of the test primer pairs: *PDIOX*, *RD21A* and *MO1F* (Table 2) showed clearly differential expression in the compatible interaction. The transcripts corresponding to *GS*, *MDH* and *PEX42* did not, although even a slight differential expression might have led to inclusion in the library. The transcript corresponding to primer pair *PDIOX* is more highly expressed in the incompatible interaction. The relatively low proportion of differential SSH clones in the test set (3 out of

9) may reflect the choice of test primer pairs, which included only genes that were not previously annotated as pathogen-response dependent (Table 1). Among the candidate genes tested, *PR1*, *PR3*, *PR4* and *PDF1.2* were strongly up-regulated in both Col-0 and DiG interactions. The transcript levels of these genes were very low in uninfected plants, with the exception of *PR1* (Fig. 2), so that induction ratios could not be estimated. Transcripts of the other candidates (Table 1), including three ethylene-response related genes, were not detectable under these conditions and it can be inferred that they are not strongly induced. The infection-related lipase gene *GLIPI* is expressed at a higher level (about 4-fold) in the incompatible than in the compatible interaction, while *PR1* is expressed about 4-fold higher in the compatible than in the incompatible interaction.

## Discussion

The *A. brassicicola*-DiG pathosystem has the features of a compatible interaction, producing expanding necrotic lesions. This suggests that there may be a fundamental difference between this interaction and an incompatible one, rather than merely a graded increase in sensitivity relative to Col-0. If this is so, the defense responses of the plant should differ between the compatible and incompatible interactions. As extensive transcriptional profiling has already been reported for incompatible *A. brassicicola*-*Arabidopsis* interaction (incompatible), an initial study of the *A. brassicicola*-DiG pathosystem was performed. The set of transcripts detected overlaps partially with those induced in resistant (Col-0) or relatively sensitive (*pad3* in Col-0 background) interactions, but most of the SSH clones represent transcripts that had not been identified before as defense-related. Of a test set of 9 genes from the SSH library tested by RT-PCR, three were differentially expressed at 72 h in the DiG-*A. brassicicola* interaction: the primer pair *PDIOX* (Fig. 2) corresponds to a gene which encodes an alpha-dioxygenase involved in protection against oxidative stress and cell death, and induced in response to salicylic acid and oxidative stress.

This gene, *DIOX1*, is preferentially induced in the incompatible interaction with *A. brassicicola*, in agreement with previous data for *Arabidopsis*-bacteria interactions [33]. Induction of the monooxygenase/aromatic-ring hydroxylase gene *MO1* is specific to the compatible interaction. In Col-0 *MO1* is not induced, and is present already in non-infected plants. This suggests that Col-0 may be "primed" in some way to initiate the defense response that is characteristic of infection with *A. brassicicola*. *MO1* shares homology with monooxygenases that degrade SA. Overexpression of the SA-degrading enzyme NahG has been used to test the involvement of SA in defense responses, but the immediate product, catechol, may contribute to the phenotypes of NahG expressors [34]. The reaction cat-

**Table 1: Randomly isolated SSH clones.**

<b>Annotation</b>	<b>TAIR number</b>	<b>inc</b>	<b>primers</b>	<b>x</b>
60S ribosomal protein L13A	<a href="#">AT3G24830.1</a>	no		
SIR sulfite reductase	<a href="#">AT5G04590.1</a>	yes		3
PSAL photosystem I subunit L	<a href="#">AT4G12800.1</a>	no		
Cobalamin-independent methionine synthase	<a href="#">AT5G17920.2</a>	no	MTH	
glutamine synthase	<a href="#">AT1G66200.1</a>	no	GS	2
alpha dioxygenase I	<a href="#">AT3G01420.1</a>	no	PDIOX	
NPQ4 non-photochemical quenching	<a href="#">AT1G44575.1</a>	no		
protein phosphorylated amino acid binding	<a href="#">AT5G10450.2</a>	no		2
FF domain-containing protein 14-3-3 stress related	<a href="#">AT3G19670.1</a>	no		2
sorbitol dehydrogenase	<a href="#">AT5G51970.2</a>	no		2
26S proteasome AAA-ATPase subunit	<a href="#">AT5G19990.1</a>	no	PATP	2
no hit				
APX1 ascorbate peroxidase	<a href="#">AT1G07890.7</a>	yes		
acyl-CoA oxidase	<a href="#">AT5G65110.2</a>	no		
chloroplastic drought-induced stress protein	<a href="#">AT1G76080.1</a>	no		
chloroplast-encoded 23S ribosomal	<a href="#">ATCG01180.1</a>	no		2
no hit				
SHM3 serine hydroxymethyltransferase	<a href="#">AT4G32520.1</a>	no		
no hit				
PDF1.2 defensin	<a href="#">AT5G44420.1</a>	yes		
Chitinase	<a href="#">AT2G43590.1</a>	yes		6
no hit				
lipid binding	<a href="#">AT1G04970.2</a>	no		
sugar transporter	<a href="#">AT1G77210.1</a>	no		
no hit				
LHCA4 Photosystem I light harvesting complex gene 4	<a href="#">AT3G47470.1</a>	no		
ribosomal L6	<a href="#">AT1G74050</a>	no		
dehalogenase hydrolase	<a href="#">AT2G32150.1</a>	no		
expressed protein	<a href="#">AT1G07040.1</a>	no		
protein kinase	<a href="#">AT2G23450.1</a>	no		
NADPH cyt P450 reductase	<a href="#">AT4G24520.1</a>	no	P450	
no hit				
GTP binding	<a href="#">AT1G17470.1</a>	no		
GST	<a href="#">AT1G65820.1</a>	no		
RuBisCo	<a href="#">AT5G38410.1</a>	no		2
cobalamin independent methionine synthase	<a href="#">AT3G03780.2</a>	no		
inorganic carbon transport, small stretch	<a href="#">AT4G32340.1</a>	no		
malate dehydrogenase	<a href="#">AT5G09660.2</a>	no	MDH	
lipase class 3	<a href="#">AT5G24210.1</a>	no		
no hits				
glycosyl hydrolase family 17	<a href="#">AT4G16260.1</a>	yes		4
no hits				
peroxidase 42 (PER42)	<a href="#">AT4G21960.1</a>	no	PRX42	
speckle-type POZ protein-related	<a href="#">AT3G48360.1</a>	no		
UDP-glucose 4-epimerase	<a href="#">AT1G12780.1</a>	no		
cysteine proteinase (RD21A)/thio	<a href="#">AT1G47128.1</a>	no	RD21A	
chlorophyll A-B binding	<a href="#">AT3G61470.1</a>	no		
amino acid transporter family	<a href="#">AT3G56200.1</a>	no		
expressed protein	<a href="#">AT5G23040.2</a>	no		
glutamate:glyoxylate aminotransfer	<a href="#">AT1G23310.2</a>	no		
expressed protein	<a href="#">AT5G02020.2</a>	no		
cysteine synthase, putative/O-ac	<a href="#">AT5G28030.1</a>	no		
cytochrome b5 domain-containing	<a href="#">AT3G48890.1</a>	yes		
protein kinase family protein	<a href="#">AT3G51550.1</a>	no		
chlorophyll A-B binding	<a href="#">AT1G61520.2</a>	no		
sugar transport protein (STP4),	<a href="#">AT3G19930.1</a>	no		
expressed protein	<a href="#">AT5G54540.1</a>	no		
glycine hydroxymethyltransferase	<a href="#">AT4G37930.1</a>	no		
similar to gamma-glutamylcysteine	<a href="#">AT4G23100.1</a>	yes		

**Table 1: Randomly isolated SSH clones.** (Continued)

no hits			
autophagy 7 (APG7)	<a href="#">AT5G45900.1</a>	no	
expressed protein	<a href="#">AT3G15450.3</a>	no	
expressed protein	<a href="#">AT4G19160.3</a>	no	
expressed protein	<a href="#">AT1G02475.1</a>	no	
serine-rich protein-related	<a href="#">AT5G25280.2</a>	no	
GST	<a href="#">AT4G02520.1</a>	yes	
expressed protein	<a href="#">AT1G26110.1</a>	no	
GSH1	<a href="#">AT4G23100.1</a>	yes	
meprin and TRAF homology domain	<a href="#">AT2G32870.1</a>	no	
PSBO2	<a href="#">AT3G50820.1</a>	no	
actin-depolymerizing factor 1	<a href="#">AT3G46010.1</a>	no	
spermidine synthase 2	<a href="#">AT1G70310.1</a>	no	
cytochrome P450 (CYP83B1)	<a href="#">AT4G31500.1</a>	no	2
2-oxoacid-dependent oxidase	<a href="#">AT3G49620.1</a>	no	
dehydrin (RAB18)	<a href="#">AT5G66400.1</a>	no	
expressed protein	<a href="#">AT1G64360.1</a>	no	
auxin-responsive protein	<a href="#">AT3G07390.1</a>	no	
3-oxoacyl-(acyl-carrier protein)	<a href="#">AT1G24360.1</a>	no	3OA
cysteine proteinase	<a href="#">AT5G60360.2</a>	no	
no hits			
peroxisomal membrane 22 kDa family	<a href="#">AT5G19750.1</a>	no	
CBL-interacting protein kinase 6	<a href="#">AT4G30960.1</a>	no	
coronatine-responsive protein	<a href="#">AT1G19670.1</a>	yes	2
hevein-like protein	<a href="#">AT3G04720.1</a>	yes	
monooxygenase (MO1),	<a href="#">AT4G15760.1</a>	no	MOI
KELP transcriptional coactivator p15	<a href="#">AT4G10920.1</a>	no	
peroxidase 42 (PER42)	<a href="#">AT4G21960.1</a>	no	
ubiquitin-conjugating enzyme 1	<a href="#">AT1G14400.2</a>	no	
mannitol transporter	<a href="#">AT4G36670</a>	yes	
aspartate aminotransferase 3	<a href="#">AT5G11520.1</a>	no	
basic helix-loop-helix (bHLH) fami	<a href="#">AT5G46760.1</a>	no	
expressed protein	<a href="#">AT4G25030.2</a>	yes	
no apical meristem (NAM) family	<a href="#">AT1G69490.1</a>	no	
expressed protein	<a href="#">AT2G15890.1</a>	no	
TMS membrane family protein	<a href="#">AT1G16180.1</a>	no	
expressed protein	<a href="#">AT5G54730.1</a>	no	
60S ribosomal protein L23A	<a href="#">AT2G39460.1</a>	no	
CBL-interacting protein kinase 6	<a href="#">AT4G30960.1</a>	no	
Calmodulin	<a href="#">AT2G41410.1</a>	yes	
RER1B	<a href="#">AT2G21600.1</a>	no	
glycine-rich RNA-binding protein	<a href="#">AT4G13850.2</a>	no	
chlorophyll A-B binding	<a href="#">AT2G05070.1</a>	no	
cytochrome C	<a href="#">AT1G22840</a>	yes	

The nucleotide sequences were used to search the TAIR database by BLASTN. A brief annotation and link to the TAIR database are given for each sequence. "No hits" indicates that no *Arabidopsis* gene was identified; these transcripts might represent fungal genes. The test primer pairs chosen are listed (names refer to Table 2), as well as the number of times (x) that the same gene was identified in the set of cDNA clones sequenced (if more than once). Clones marked "yes" were previously reported [17] in an incompatible *Arabidopsis-Alternaria* interaction (inc).

alyzed by Mo1 is not known, but one possibility is that this enzyme produces SA-derived aromatic compounds that could have signaling roles. Another possibility is that Mo1 might be involved in the suppression of the SA pathway in Col-0. It is worthy of note that *PR1* is more highly expressed in DiG, the opposite of what would be expected if Mo1 acts like NahG. *GLIP1* shows the reverse pattern, and is induced less in the compatible interaction. This extracellular lipase-related protein contributes to resistance to *A. brassicicola* [28]. The decreased ability of DiG to upregulate *GLIP1* in response to *A. brassicicola* may there-

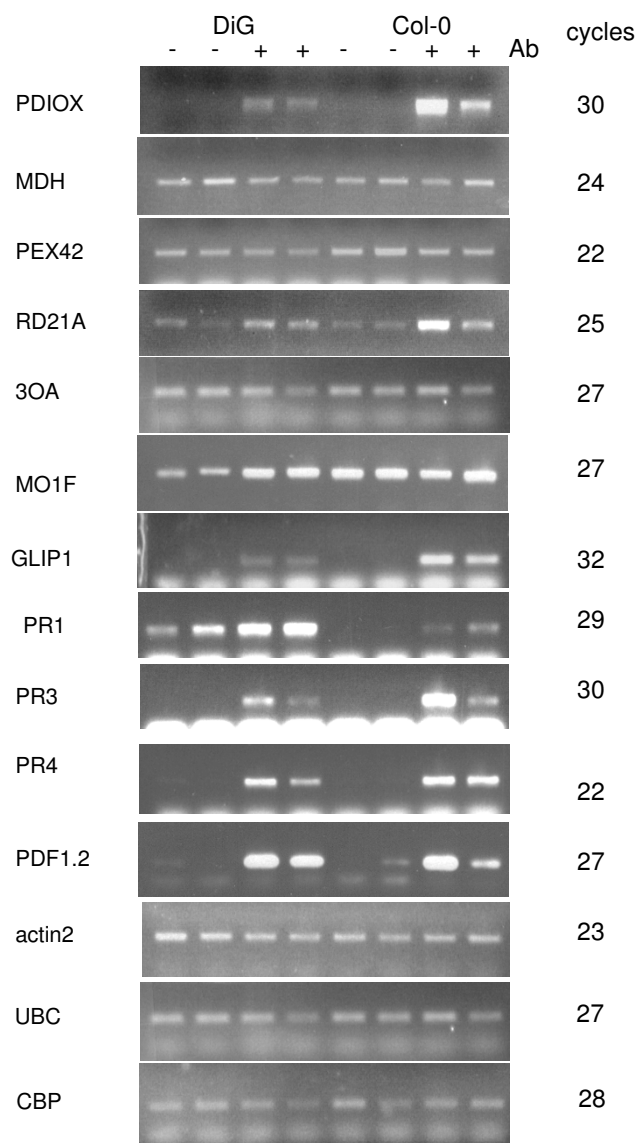
fore directly contribute to its sensitivity to the pathogen. DiG is a low producer of camalexin but this is probably not the only reason for its sensitivity, since other susceptible ecotypes produced up to several fold more camalexin than Col-0 [27]. Furthermore, the expression profiles of camalexin-lacking *pad3* and wild type (Col-0) were similar and the data sets were indeed combined [17]. This contrasts with what was found here for DiG, suggesting that additional heritable traits are involved. Segregation of incompatible interaction – related traits in crosses between DiG and Col-0 may identify loci other than those

**Table 2: Test primer pairs used for semi quantitative RT-PCR amplification: names, TAIR database numbers, and predicted product sizes in bp are listed.**

name	function	TAIR ID	sense primer	antisense primer	bp
PATP	proteasome subunit	AT5G19990	GGCGTCTGAGACAGCGATGGAG	CAGGCCTGAGAAGAGCTTGATCCAG	938
GS	cytosolic glutamine synthetase	AT1G66200	GAAGGATGTGAAGTGGCCTCTTG	GTAAGGGTCCATGTTTGAAGCTG	615
PDIOX	pathogen inducible dioxygenase	AT3G01420	GTATGCGACGCCCTCAAGGATG	CCTTGAGACTCTCTGTAGTATCCACC	936
MTH	homocysteine methyltransferase	AT5G17920	GCTGATCTCAGTTCATCTCTG	GATTGAGCTTCTTCTGCTGAGCATC	1170
MDH	malate dehydrogenase	AT5G09660	GGAAAACCTGCAGAGCTAAAGGTGG	CCAAGCTGATACACTTCTCTGTC	878
PRX42	peroxidase 42	AT4G21960	GACCACAACGAGAGTATCTCCGTC	CAAGCAGAGAAGCTCAACACACCAGAG	544
RD21A	cysteine proteinase (RD21A)	AT1G47128	GTGAGAGAAGGACTAGCCTACGGTAC	CACAAAACCGGGTACTCGTGAG	914
3OA	3-oxoacyl-(acyl-carrier protein)	AT1G24360	GATGAAAACCGCTCTTGACAATG	CATCAATGGTGAATCGCTGTCC	511
MO1	aromatic-ring hydroxylase	AT4G15760	GTTTGCTTGTGCTGCGGTGAGAG	CTGCACCGAAAGCCCGAGTAATC	555
PR1	PR1 pathogen related	AT2G14610	TCGTCTTTGTAGCTCTTGAGGTG	TAGATTCTCGTAATCTCAGCTCT	590
GLIP1	lipase, defense response	AT5G40990	CGATTGTGCACCAGCCTCATTGGTT	CAGCGCTTTGAGATTATAGGGTCC	429
PR2	PR2 pathogen-related, cellulase	AT3G57260	CGTTGGCTCTTTACAAAACAACAAAC	GAAATTAACCTCATACTTAGACTGTCGAT	870
PR3	PR3 defense response chitinase	AT3G12500	CGGTGGTACTCCTCTGGACCCACCGGC	CGGGCGCACGGTGGCGTCTGAAGGCTG	583
PR4	PR4 hevein-like defense protein	AT3G04720	GACAACAATGCGGTCTGCAAGG	AGCATGTTTCTGGAATCAGGCTGCC	552
PR5	PR5 thaumatin-like	AT1G75040	ATGGCAAATATCTCCAGTATTCACA	ATGTCCGGGCAAGCCGCTTGAGG	484
PDF1.2	defensin	AT5G44420	GCTAAGTTTGCTTCCATCATCACCTT	AACATGGGACGTAACAGATACACTTGT	237
EIN2	ethylene signal transduction	AT5G03280	TGCAGCTCGCATAAGCGTTGTACTGGTA	CGCTCTCTCCATTTAACCGAGTTAACAC	379
EIN3	ethylene signal transduction	AT3G20770	GATGTTGATGAATTGGAGAGGAGGATG	ACGTCTCTGAGGAGGATCACAGTGT	470
ERF1	ethylene response factor	AT3G23240	CGGCTTCTCACCGGAATATTCTATCG	TCTCCGAAAGCGACTCTTGAACCTCT	415
ACT2	actin 2	AT3G18780	TACCACAACAGCAGAGCGGG	GGACCTGCTCATCATACTCGG	257
UBC	ubiquitin conjugating	AT5G25760	GGCATCAAGAGCGGACTGTT	CTTTCTTAGGCATAGCGGGGAG	217
CBP20	Cap-binding protein 20	AT5G44200	TTGTGGCTTTTGTTCGTCCTG	CGTGGGTTCTTCCGGTCTC	409

encoding camalexin biosynthesis genes. One candidate is *GLIP1*, and it would be of interest to construct a mutant lacking both *GLIP1* and camalexin. Another candidate is the alpha dioxygenase *DIOX1*; the oxylipin signals produced by the alpha-DOX1 fatty acid dioxygenase encoded by this gene promote protection from ROS and cell death [33]. *PR1*, in contrast, is expressed at higher levels in DiG, despite the fact that this is a gene strongly induced by the SA pathway. This suggests that in DiG, the SA pathway might be induced upon infection with a necrotroph. Induction of the SA and JA pathways is coordinated, with induction of one pathway at the expense of the other [13,14]. Infection by a biotroph suppresses defense against the necrotroph *A. brassicicola*. Furthermore, the application of SA resulted in suppression of defense against the necrotroph, and high expression of the SA-dependent defense gene *PR1* [14]. The JA pathway is most important for defense against *A. brassicicola* [10]. Thus, induction of the SA pathway might be an important factor responsible for the development of a compatible interaction with DiG. It is possible that the extent of the trade-off between SA and JA-dependent pathways [14] has been modulated by selection in different plant ecotypes. It is striking that application of SA [14] closely mimicked the appearance of the compatible-type lesions that we observed in ecotype DiG (Fig. 1A–C). Likewise, we found strong induction of *PR1* expression (Fig. 2). Expression of the JA-dependent defensin gene *PDF1.2*, however, was not strongly suppressed in DiG (Fig. 2), while application of SA strongly suppressed *PDF1.2* expression over the entire 3-days post-inoculation period studied in [14]. Thus, suppression of JA-mediated defense may be only

part of the explanation of the susceptible phenotype of DiG. An alternative explanation is that the reciprocal regulation of the SA and JA pathways might fail as a result of successful infection by the pathogen, making this an effect, rather than cause, of the difference between the ecotypes. Genes induced in infected as compared to control leaves (in both ecotypes) may have roles in defense responses, or be induced as a result of tissue damage and cell death. The set of ESTs identified by SSH includes known defense-regulated genes. These were not further tested for differential expression here, since they have been previously studied. These ESTs include: chitinase (At2g43590), glycosyl hydrolase family 17 (AT4G16260.1), sulfite reductase/ferredoxin (At5g04590.1), *PDF2.1a* (At5g44420.1), *APX1* – ascorbate peroxidase (At1g07890.1), cytochrome b5 domain-containing (AT3G48890.1), *GST* (AT4G02520.1), *GSH1* (AT4G23100.1), coronatine responsive protein (chlorophyllase, methyl jasmonate induced, AT1G19670.1), hevein-like protein (HEL) (AT3G04720.1), mannitol transporter (AT4G36670), calmodulin (AT2G41410.1), and cytochrome c (AT1G22840) (Table 1). Our identification of 15 known defense-regulated genes (about 15%, taking into account redundancy in the set of sequenced clones) in the library (Table 1) shows that the SSH comparison was robust, despite only one third of the test set showing clearly differential expression between Col-0 and DiG (Fig. 2). We note that known *A. brassicicola* induced genes were excluded from the test set (Table 2). Although some sequences were redundant in the sample of more than 100 clones, the number of SSH-derived ESTs apparently was not saturated with respect to



**Figure 2**  
**Semi-quantitative RT-PCR analysis of transcript levels of selected genes.** - and + indicate samples from control and inoculated intact plants, respectively. RNA was isolated by harvesting the entire leaf at 72 h after inoculation. Duplicate lanes indicate two independent experiments on different sets of plants; the number of amplification cycles is indicated at the right.

the number of clones sequenced. PDF1.2, for example, was strongly induced (Fig. 2) but not found among the sequenced clones.

### Conclusion

The DiG-*Alternaria brassicicola* pathosystem shows all the characteristics of a compatible interaction between the necrotroph *A. brassicicola* and *Arabidopsis*. This initial

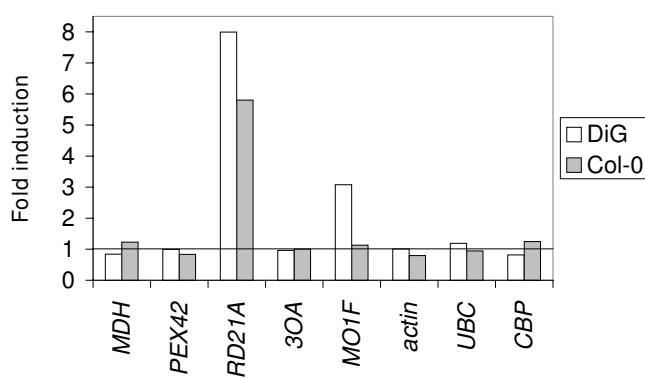
study demonstrates that the transcriptional profile of the compatible interaction is not identical to that of the well-studied incompatible interaction with Col-0. Induction of the monooxygenase gene *MO1*, and high expression and induction of *PR1*, are characteristic of the compatible interaction. *GLIP1* and *DIOX1*, in contrast, are expressed more strongly in the incompatible interaction. These particular genes are not necessarily those whose expression levels define whether the plant is able to limit lesion spread or not, but are candidates for further study. The similarity between the compatible interaction and the result of exogenous application of SA provides a clue to the mechanism. Full-scale genome-wide studies are being done for the interaction of the hemibiotroph *Magnaporthe oryzae* with rice cultivars providing compatible or incompatible interactions [35,36]. This approach can now be fully developed for the *Arabidopsis-Alternaria* pathosystem defined in this study.

### Methods

#### Plant material, growth conditions and RNA extraction

Seeds of the ecotypes Col-0, Col-6, WS and DiG were obtained from ABRC <http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrhome.htm>. Mutants *glip1-1*, *glip1-2* and *acd1* were from the same collection, and homozygous lines were selected by screening progeny of selfed plants by PCR on genomic DNA samples using appropriate diagnostic primer pairs. Seeds were sown in "cookies" (approximately 4 cm diameter soil packaged in netting, purchased locally) held at 4°C for two days to promote germination, and plants were grown in a temperature-controlled room at 23°C under continuous or 16 h/8 h fluorescent lighting (cool-white tubes). Rosette leaves were inoculated with a conidial suspension of *Alternaria brassicicola* (MUCL20297, [10]). Inoculation was as described in [17], except that in preliminary experiments we found that a higher inoculum was needed to obtain reproducible disease development on both ecotypes, and so used 5 µl of a 6 × 10<sup>6</sup> spore/ml suspension (3 × 10<sup>4</sup> conidia per drop) throughout this study. To prepare spores for inoculation, fungal cultures were grown in a growth chamber on potato dextrose agar (Difco) plates under continuous white light at 25°C for 7 days, and spores suspended in water and counted in a haemocytometer. Plants were inoculated in a biosafety laminar flow chamber, placed in large sealed containers and incubated for up to 5 days at 23–24°C in a growth chamber. Conidia production was assayed after 5 days following inoculation by suspending the conidia from lesions excised from 10 infected leaves as described [17]. Leaf material was harvested, frozen, and kept at -80°C until further use. RNA was extracted from control (mock inoculated) and inoculated leaves of Col-0 and DiG using Tri-Reagent (MBC or Fluka) according to the manufacturer's protocol, except that the starting material was leaf tissue ground in liquid





**Figure 3**  
**Expression of the monooxygenase gene *MO1* is preferentially up-regulated in the compatible interaction.** Relative transcript levels were calculated from the intensity of the RT-PCR signals shown in Figure 2, as follows. Infected Col-0 was chosen as the reference treatment. The band intensities of the three reference genes (*ACT2*, *UBC* and *CBP20*) then showed similar expression patterns as a function of experiment and replicate, over the entire data set, with no clear trend as a function of treatment. This indicates that the transcript levels of these genes varied with the amount of RNA and efficiency of the reactions, rather than with the treatment. All data for the reference genes were therefore combined, and the entire data set normalized to the combined reference values to obtain the signal plotted as "fold induction" (y-axis).

nitrogen. Each sample for RNA extraction consisted of about 20 leaves from at least 10 plants. (All the experiments were repeated at least three times with three replications) RNA concentrations were determined using a Nanodrop spectrophotometer and quality was checked by electrophoresis on denaturing agarose gels.

#### Suppression-subtractive hybridization (SSH)

4 week old DiG plants were inoculated with *A. brassicicola* with at least three replications and three biological repetitions. The infected leaves were collected after 6 h (Ast-1), 12 h (Ast-2), 24 h (Ast-3), 48 h (Ast-4) and 72 h (Ast-5) of inoculation. Total RNA was isolated from each stage of disease development along with control (Ast-0). The leaf samples were pooled for each stage from each replication and repetition of the experiment. For the SSH analysis the mRNA was enriched from the total RNA of Ast-0, Ast-3, Ast-4 and Ast-5 using the Qiagen Oligotex mRNA isolation Kit. SSH was performed using the Clontech PCRSelect cDNA subtraction kit following the manufacturer's protocol and the driver population consisted of mRNA from Ast-0 and *A. brassicicola* in the ratio of 3:1. *A. brassicicola* RNA was from a culture grown for 60 hours in shake culture on PDB. Amplification using primer pairs specific for the defense response gene *PR1* and an actin gene confirmed that the SSH procedure functioned as expected

(data not shown). For this analysis, the following primers were used, spanning regions without *RsaI* sites: *PR1* primer, sense direction, see Table 2; *PR1* antisense, GAT-CACATCATTACTTCATTAGTATG. *ACT2* primers: sense GCTGGATTCTGGTGATGGTG, antisense GATCCAG-CAGCTTCCATTC.

An enrichment of 64 fold for *PR1* relative to *ACT2* was estimated from these amplification data; it should be noted that this is likely to be the combined effect of suppression of *ACT2* cDNA abundance and/or enrichment of *PR1*, as expected from the design of the SSH subtraction method (see PCRselect manual, Promega, and literature cited therein). The fragments amplified in the second PCR reaction were cloned into pTZ57R/T (Fermentas), transformed into *E. coli* DH5 $\alpha$  (HIT, Real Biotech), 130 positive clones were picked and the inserts amplified from the bacterial colonies using M-13 forward and reverse primers. Sequence was obtained for 116 clones (Macrogen, Seoul, Korea).

#### Semiquantitative RT PCR analysis

cDNA was synthesized and assayed as follows. 2  $\mu$ g of total RNA from rosette leaves of DiG or Col-0 plants inoculated as described above were treated with 2 units of RQ1 RNase-free DNase (Promega) in a volume of 10  $\mu$ l. After addition of stop solution and incubation for 10 min at 65°C, the sample was denatured in the presence of 0.5  $\mu$ g of oligo dT primer, cooled, 200 units of MMLV reverse transcriptase (Promega), 24 units of PRI RNase inhibitor (PRI, TaKaRa) and dNTPs to a final concentration of 0.5 mM each were added, and the reaction volume adjusted to a total of 25  $\mu$ l in 1 $\times$  MMLV reaction buffer. cDNA synthesis was for 1 h at 42°C. All reactions were carried out in a thermal cycler (Biometra). A set of three "housekeeping" gene primer pairs (Sigma) was used to calibrate template amount (Table 2). The cDNA samples were diluted such that similar signal intensity was obtained upon amplification with the *Actin 2* (*ACT2*) primer pair (Table 2), and the number of cycles was calibrated for each primer pair in order for the amplification level to remain below saturation.

#### Abbreviations

dai: days after inoculation; hai: hours after inoculation; ROS: reactive oxygen species; HR: hypersensitive response; SA: salicylic acid; JA: jasmonic acid

#### Authors' contributions

AKM conceived of the study together with the other authors, and carried out the major part of the experiments. SL brought the compatible interaction phenotype of DiG to the attention of AKM and BAH, and participated in library construction and data analysis. SG participated in coordination and analysis of the results. BAH drafted

the manuscript and carried out some of the gene expression experiments. All authors participated in writing the final manuscript. All authors read and approved the final manuscript.

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