

# The glutaredoxin ATGRXS13 is required to facilitate *Botrytis cinerea* infection of *Arabidopsis thaliana* plants

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## SUMMARY

*Botrytis cinerea* is a major pre- and post-harvest necrotrophic pathogen with a broad host range that causes substantial crop losses. The plant hormone jasmonic acid (JA) is involved in the basal resistance against this fungus. Despite basal resistance, virulent strains of *B. cinerea* can cause disease on *Arabidopsis thaliana* and virulent pathogens can interfere with the metabolism of the host in a way to facilitate infection of the plant. However, plant genes that are required by the pathogen for infection remain poorly described. To find such genes, we have compared the changes in gene expression induced in *A. thaliana* by JA with those induced after *B. cinerea* using genome-wide microarrays. We have identified genes that are repressed by JA but that are induced by *B. cinerea*. In this study, we describe one candidate gene, *ATGRXS13*, that encodes for a putative glutaredoxin and that exhibits such a crossed expression. In plants that are infected by this necrotrophic fungus, *ATGRXS13* expression was negatively controlled by JA and TGA transcription factors but also through a JA-salicylic acid (SA) cross-talk mechanism as *B. cinerea* induced SA production that positively controlled *ATGRXS13* expression. Furthermore, plants impaired in *ATGRXS13* exhibited resistance to *B. cinerea*. Finally, we present a model whereby *B. cinerea* takes advantage of defence signalling pathways of the plant to help the colonization of its host.

**Keywords:** glutaredoxin, *Botrytis cinerea*, jasmonic acid, salicylic acid, necrotrophic pathogen.

## INTRODUCTION

Resistance of plants to pathogens is a combination of constitutive barriers and inducible defence reactions that can be induced after an encounter with a potential pathogen. They include production of antimicrobial metabolites and proteins, histological barriers or the induction of a cell-death response. This innate immunity is activated upon recognition of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) or upon recognition of damage inflicted by insects or wounds (damage-associated molecular patterns; DAMPs) by corresponding plant receptors. This is rapidly followed by changes in ion fluxes, production of

reactive oxygen species and nitric oxide, and by the activation of protein phosphorylation involving mitogen-activated protein (MAP) kinase cascades, finally leading to localized defence responses (Boller and Felix, 2009; Nürnberger and Kemmerling, 2009). Resistance can also be induced in tissues remotely located from the infection site (Vlot *et al.*, 2008) and comprises systemic acquired resistance (SAR) in which resistance is induced by a pathogen infection on a lower leaf (Durrant and Dong, 2004) and induced systemic resistance (ISR) triggered by beneficial root-associated microorganisms (Van Loon *et al.*, 1998).

An ongoing question concerns the understanding how virulent pathogens cope with such a sophisticated defence system. Avoidance and tolerance of antimicrobials constitutes an important mechanism displayed by virulent pathogens (Van Etten *et al.*, 2001). Interference with host defence by a pathogenic effector was long postulated before its existence was demonstrated experimentally. Effectors produced by virulent pathogens can directly interact and inhibit host defences. Their actions have been described for specialized hemibiotrophic or biotrophic bacterial, oomycete pathogens (Chisholm *et al.*, 2006; da Cunha *et al.*, 2007) or nematodes (Davis *et al.*, 2008). New insights start to gain understanding in the specific interactions of plants with fungi (Métraux *et al.*, 2009) but to date little information is known about the action of effectors of fungal pathogens characterized by a broad host range.

The signalling for innate immunity is relayed by hormones such as SA, JA or ethylene (ET) and leads to changes in gene expression that are associated with enhanced resistance (Bari and Jones, 2009). For instance, JA is generally associated with basal levels of defence against the necrotrophic fungus *B. cinerea* in *A. thaliana* (Glazebrook, 2005). However, some classical studies have shown how pathogens can also use the power of hormones to their advantage. Examples include the infections by *Gibberella fujikuroi*, *Agrobacterium tumefaciens* or *Pseudomonas savastanoi* that all result in hormonal imbalances with consequences of uncontrolled growth of the invaded tissue favoring pathogen invasion (Robert-Seilaniantz *et al.*, 2007). These observations provide the conceptual basis for the experimental approach used here to find host genes that are required by virulent pathogens such as the necrotrophic fungus *Botrytis cinerea* to infect plants.

To find such plant genes we compared the changes in gene expression in *A. thaliana* induced by JA with those induced by *B. cinerea* using genome-wide microarrays and have identified a group of genes that exhibit a crossed expression pattern: they are repressed by JA but induced by *B. cinerea*. We hypothesized that this expression would be indicative for genes whose expression is altered by *B. cinerea* to its advantage. On one hand, the increased expression of such genes after infection by *B. cinerea* could therefore help the pathogen in its colonization of the host. On the other hand, JA-repressed genes might be part of a protective reaction of the plant towards *B. cinerea*. Thus, a JA-dependent signalling pathway might repress plant genes required for susceptibility to *B. cinerea*. We would predict that inactivation of such genes should alter the interaction of *B. cinerea* with the plant. We describe one candidate gene, *ATGRXS13*, obtained from such a search for crossed expression. The expression of *ATGRXS13* that encodes a putative glutaredoxin is repressed by JA and induced by *B. cinerea*. Plants impaired in *ATGRXS13* expression exhibit partial resistance to *B. cinerea*. We present a model whereby

*B. cinerea* takes advantage of defence signalling pathways of the plant to help its colonization of the host.

## RESULTS

### *Botrytis cinerea* strain BMM induces SA and JA production in Arabidopsis plants

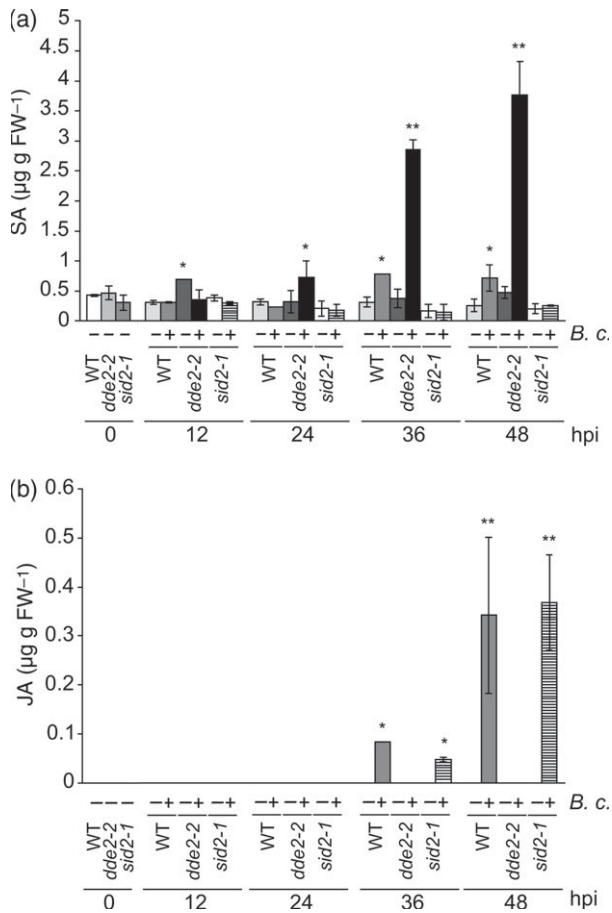
After inoculation of wild-type (WT) Arabidopsis with *B. cinerea* strain BMM, the concentration of SA (both free and conjugated) increased from 0.31 to 0.78  $\mu\text{g g}^{-1}$  of fresh weight (FW) at 36 hours post inoculation (hpi) compared with uninfected controls in which the SA level remained low and stable from 0 to 48 hpi. In the *dde2-2* mutant defective in JA biosynthesis (von Malek *et al.*, 2002), an increase in SA concentration was already detected after 24 hpi and reached 3.8  $\mu\text{g g}^{-1}$  FW at 48 hpi in comparison with 0.7  $\mu\text{g g}^{-1}$  FW in the WT at the same time-point (Figure 1a). Accordingly, the SA marker gene *PR1* was more expressed in *dde2-2* in comparison with WT plants after infection (Figure S1a). The SA level in the SA defective *sid2-1* mutants (Nawrath and Métraux, 1999) remained low and unchanged after *B. cinerea* inoculation (Figure 1a).

An increase in JA accumulation was observed at 36 hpi in WT (0.08  $\mu\text{g g}^{-1}$  FW) and *sid2-1* plants (0.05  $\mu\text{g g}^{-1}$  FW) whereas JA was not detected in mock-inoculated plants. At 48 hpi, the level of JA was similar in WT (0.34  $\mu\text{g g}^{-1}$  FW) and in *sid2-1* plants (0.37  $\mu\text{g g}^{-1}$  FW). However, JA was not detected in *dde2-2* after inoculation with *B. cinerea* (Figure 1b).

These results show that the inoculation with *B. cinerea* is followed by both an increase in SA and JA at 36 hpi and highlight the JA/SA crosstalk; mutants impaired in JA synthesis accumulated more SA and expressed more *PR1* after *B. cinerea* inoculation.

### *AtGRXS13* is induced and alternatively spliced after *B. cinerea* infection

Our goal was to identify *A. thaliana* genes that are required for the colonization by a virulent pathogen. We have used a simple concept of crossed expression to find potential candidates. As basal resistance to the virulent fungus *B. cinerea* depends on JA (Ferrari *et al.*, 2003; Rowe *et al.*, 2010) and because JA is induced upon infection by *B. cinerea*, we have used microarray data available on public databases to compile genes whose expression is changed after inoculation with *B. cinerea* and treatment with JA using the Excel macro FiRe (Garcion *et al.*, 2006). As expected, the expression of most of the genes either increased or decreased after *B. cinerea* or JA. Interestingly, a small group of genes departed clearly from this pattern and showed an increase in expression after *B. cinerea* inoculation but also decrease in expression after JA treatment. We have focused on one such gene, *ATGRXS13* (*AT1G03850*), encoding a putative glutaredoxin. Glutaredoxins are involved in redox



**Figure 1.** Accumulation of SA and JA in WT Arabidopsis plants and mutants infected with *B. cinerea*. Total salicylic acid (SA) (a) and jasmonic acid (JA) (b) accumulation in wild type (WT), *dde2-2* and *sid2-1* mutants. All plants were sprayed at time 0 with the mock buffer or a spore suspension of *B. cinerea*. Two replicates of leaves from at least four plants were collected at various time points after treatment. The data represent the mean of two independent experiments ( $\pm$ SD). No star or different stars represent groups which were significantly different from one another as determined by a one-way analysis of variance (ANOVA) followed by a multiple comparison with the Student–Newman–Keuls method ( $P < 0.05$ ). hpi: hour post inoculation. *B. c.*: *Botrytis cinerea*.

regulation of proteins (Rouhier *et al.*, 2004). As *B. cinerea* infection is accompanied by oxidative stress, we suspected that this protein might be a target for altered regulation upon *B. cinerea* infection.

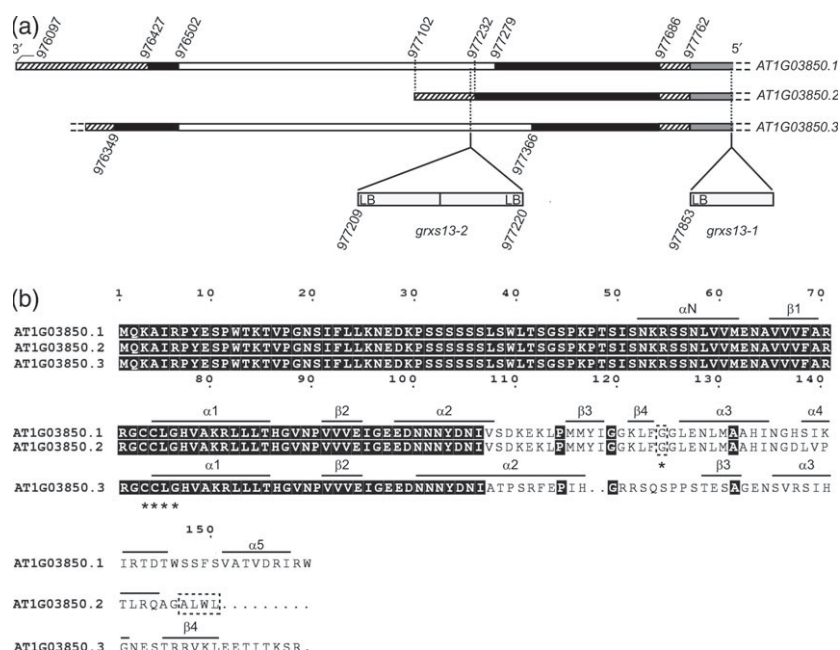
We have isolated three mRNA corresponding to *ATGRXS13* indicating that *ATGRXS13* was alternatively spliced in *A. thaliana* plants infected with *B. cinerea*. Only two transcripts, *AT1G03850.1* and *AT1G03850.2*, are described in the TAIR (The Arabidopsis Information Resource) database. An alignment of the three transcript sequences and the genomic sequence of *ATGRXS13* is shown (Figures S2 and 2a). *AT1G03850.2* is composed of a unique exon and is predicted to encode a protein of 150 amino acid residues (Figure 2). However, *AT1G03850.1* and

*AT1G03850.3* are composed of two exons surrounding one intron (Figure 2a). They are predicted to encode two proteins of respectively 159 and 158 amino acid residues (Figure 2b). *AT1G03850.3* is produced by the use of an alternative splice donor site in the first exon. This splicing event causes a frame-shift that generates a different stop codon leading a different C-terminal of the protein (Figures 2b and S2). Although *AT1G03850* is alternatively spliced, the three predicted *AT1G03850* proteins maintained the glutaredoxin putative catalytic site CCLG (Figure 2b). The characteristic amino acid sequence that defines a GRX domain (Couturier *et al.*, 2009) was only found in *AT1G03850.1* and *AT1G03850.2*. More precisely, the glycyl residue located at position 124 in the putative glutathione binding site was absent in *AT1G03850.3*. Furthermore, the prediction of the secondary structures of the proteins from primary amino acid sequence indicated that only *AT1G03850.1* and *AT1G03850.2* possess a secondary structure typical of a thioredoxin fold, a characteristic feature of GRX (Martin, 1995). Finally, the ALWL motif present in the C-terminal end of many GRX from the group III (Li *et al.*, 2009) was only present in isoform 2 (Figure 2b). The induction of expression of each splice variant after *B. cinerea* inoculation was confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using specific primers (Table S1) and was normalized against a constitutive gene as described by Czechowski *et al.* (2005). Primers used to detect *AT1G03850* splice variants have the same PCR efficiency (data not shown). In non-treated and mock-treated WT plants, each splice variant was expressed at low basal levels, but the transcripts accumulated within 24 h after infection with *B. cinerea* (Figure 3). Interestingly, by comparing the normalized expression values of *AT1G03850* splice variants, it can be seen that *AT1G03850.2* was much more abundant than *AT1G03850.3* and *AT1G03850.1* (Figure 3).

#### Mutation in *ATGRXS13* alters the susceptibility of *A. thaliana* to *B. cinerea*

To determine the biological importance of *ATGRXS13*, mutants homozygous for T-DNA insertions (Alonso *et al.*, 2003) in *ATGRXS13* were identified by PCR and the genomic DNA surrounding each T-DNA insertion was sequenced to precisely localize the insertions (Figure 2a). A residual expression of *ATGRXS13* splice variants was observed in *grxs13-1* following mock and *B. cinerea* treatment but *ATGRXS13* was no longer inducible after *B. cinerea* inoculation (Figure 4). These results can be explained by the localization of the T-DNA-insertion in the promoter region of the gene. In *grxs13-2*, the expression of *ATGRXS13* transcripts was barely detectable.

To test the susceptibility of *grxs13* mutants to *B. cinerea*, plants were drop-inoculated with a solution of conidia of *B. cinerea* and lesion diameters (LD) were measured after



**Figure 2.** Structure of the *ATGRXS13* gene and protein.

(a) Schematic representation of *ATGRXS13* splice variants. Slanted numbers indicate the coordinates of *AT1G03850* splice variants on the chromosome 1 (<http://www.arabidopsis.org>). The positions of T-DNA insertions in *grxs13* mutants are displayed. Grey: promoter; hatching: 5'- and 3'-untranslated region (UTR); black: exon; white: intron, LB: left border of T-DNA.

(b) Alignment of the deduced amino acid sequence from the three *AT1G03850* splice variants. Sequences were aligned with ClustalW (Thompson *et al.*, 1994) and displayed using ESPript (Gouet *et al.*, 2003). Secondary structures were predicted using Psi-Pred (Jones, 1999). Stars indicate the conserved residue CCLG in the putative active site at position 73–76 and the putative glutathione (GSH)-binding site at position 124 (dashed box and star). The ALWL motif specific to the second isoform is surrounded by a dashed box.

3 days. WT plants were susceptible to the *B. cinerea* strain BMM (Figures 5a and S3). A decrease in LD in *grxs13* mutants was observed indicating that both mutants were more resistant to *B. cinerea* than WT plants (Figure 5a). More small (LD < 2 mm) and less large (LD > 6 mm) lesions were observed in the two *grxs13* mutants in comparison with WT plants (Figure S3). To confirm this result, we quantified fungal growth in both WT and *grxs13* mutants. Fungal growth was reduced at 48 hpi in both *grxs13-1* and *grxs13-2* mutants in comparison with WT plants (Figure 5b). These results indicate that *ATGRXS13* is required to facilitate *B. cinerea* infection of *A. thaliana* plants.

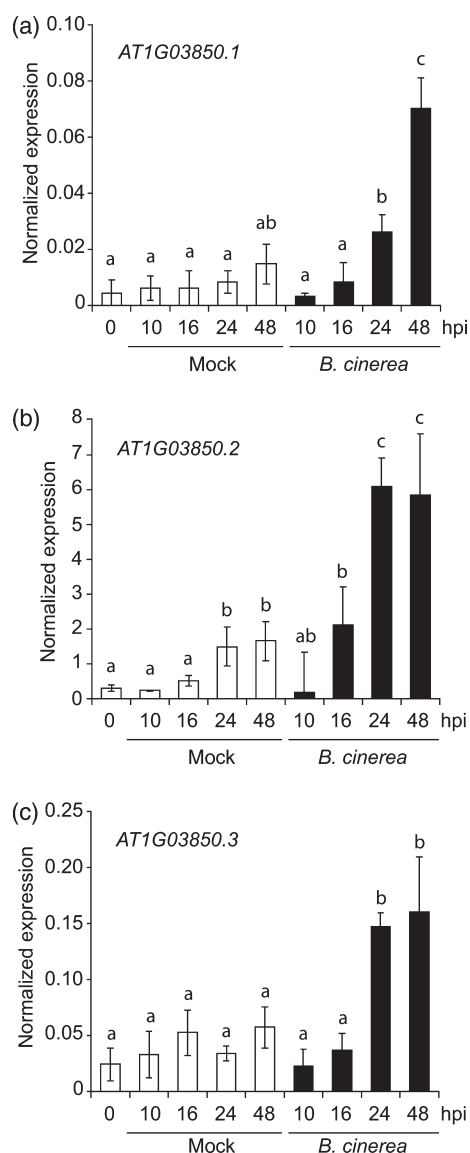
As three *ATGRXS13* mRNA accumulate during *B. cinerea* infection (Figure 3), the role of each *ATGRXS13* isoform was tested separately during infection. To this end, the cDNAs corresponding to each of the three *ATGRXS13* mRNA were introduced into *grxs13-1* under the control of 1500 bp of the native promoter of *ATGRXS13* (Figure 5c). *AT1G03850.2* was able to complement *grxs13-1* resistance phenotype whereas *AT1G03850.1* and *AT1G03850.3* were not (Figure 5c). Furthermore, transgenic plants constitutively expressing *ATGRXS13* splice variants were generated. The cDNA corresponding to each *ATGRXS13* mRNA were placed under the control of the CaMV35S promoter. Over-expression of *AT1G03850.2* in WT plants resulted in an increased

susceptibility to *B. cinerea*, whereas over-expression of *AT1G03850.1* and *AT1G03850.3* had no effect (Figure 5d). A recent study has indicated that over-expression of *ATGRXC7/ROXY1* in Arabidopsis results in a strong accumulation of reactive oxygen species (ROS) in transformed plants (Wang *et al.*, 2009) that correlates with an enhanced susceptibility to *B. cinerea* of these plants. By contrast, plants over-expressing each *GRXS13* splice variants do not accumulate more ROS (Figure S4). In conclusion, among the three isoforms of *ATGRXS13*, only *AT1G03850.2* is likely to be required for infection of Arabidopsis by *B. cinerea*. Thus, we focused our attention on the role of *AT1G03850.2* in this context.

#### Induction of *AtGRXS13* expression after *B. cinerea* infection depends on both SA and JA signalling pathways

We determined the contribution of signalling pathways commonly associated with plant defence responses to the induction of *ATGRXS13* (Figure 6). We tested mutants defective either in the SA (*sid2-1*, *eds5-3*; Heck *et al.*, 2003; Nawrath and Métraux, 1999) or in the JA pathway (*dde2-2*, *opr3*, *coi1-16*; Stintzi and Browse, 2000; von Malek *et al.*, 2002; Xie *et al.*, 1998) and the *npr1-1* mutant, a key positive regulator of SA-mediated gene expression (Spoel *et al.*, 2003). After *B. cinerea* infection, the accumulation of

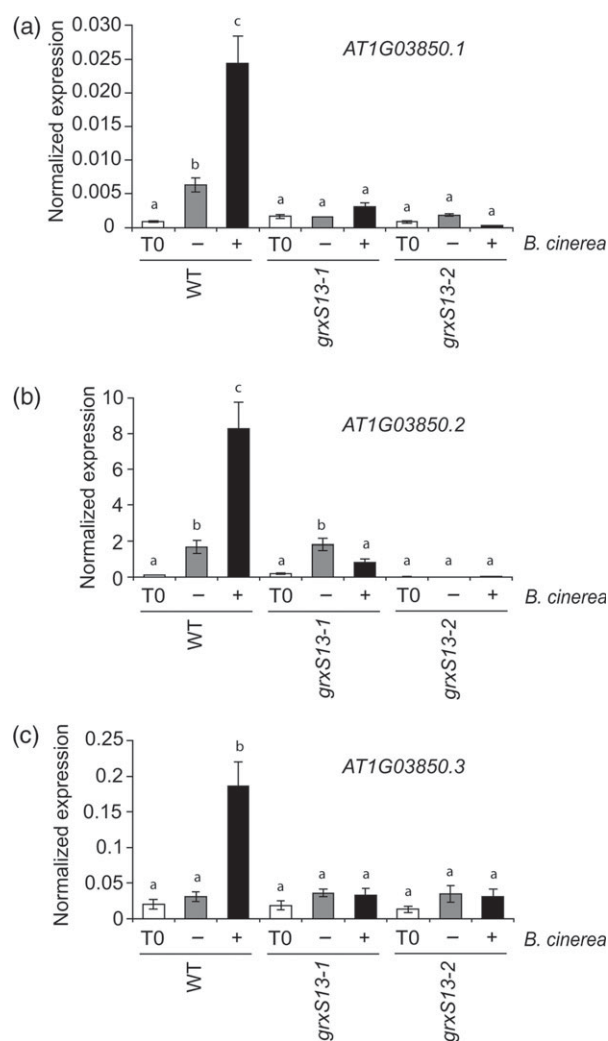




**Figure 3.** *ATGRXS13* mRNAs accumulation in wild-type (WT) Arabidopsis plants infected with *B. cinerea*. WT Col plants were sprayed with mock solution (white bars) or *B. cinerea* spores (black bars). Leaves from four to six plants were harvested at different time points for each treatment. *AT1G03850.1* (a), *AT1G03850.2* (b), *AT1G03850.3* (c) transcripts levels were quantified by real-time polymerase chain reaction (PCR). Data are expressed as normalized expression (no unit) to the plant reference gene *AT4G26410* transcript level (Czechowski *et al.*, 2005) and represent the mean of four independent experiments ( $\pm$ SD). Different letters represent groups which were significantly different from one another as determined by a one-way ANOVA followed by a multiple comparison with the Student–Newman–Keuls method ( $P < 0.05$ ).

*AT1G03850.2* mRNA was strongly increased in JA-related mutants but was strongly impaired in SA-related mutants in comparison with WT plants (Figure 6).

Furthermore, *ATGRXS13* was induced after exogenous treatment of Arabidopsis plants with 1 mM of SA at all time points analyzed confirming that this gene is positively



**Figure 4.** *ATGRXS13* mRNAs accumulation in WT and *grxs13* mutants infected with *B. cinerea*. The leaves from six wild-type (WT) Col and *grxs13* plants were not sprayed (white bars), sprayed with mock solution (grey bars) or *B. cinerea* spores (black bars) and were harvested after 24 h. *AT1G03850.1* (a), *AT1G03850.2* (b), *AT1G03850.3* (c) transcripts levels were quantified by real-time PCR and expressed as normalized expression (no unit). The data represent the mean of four independent experiments ( $\pm$ SD). Different letters represent groups that were significantly different from one another as determined by a one-way analysis of variance (ANOVA) followed by a multiple comparison with the Student–Newman–Keuls method ( $P < 0.05$ ).

regulated by SA (Figure 7a). The JA-related mutants are more susceptible to *B. cinerea* than WT plants (Figure S1b; Ferrari *et al.*, 2003). To exclude the possibility that the over-induction of *ATGRXS13* in these mutants is the consequence of a stronger infection, we tested whether JA could negatively regulate the induction of *ATGRXS13* by SA. We sprayed plants with 100  $\mu$ M of MeJA 3 h before application of SA and analyzed *ATGRXS13* gene induction 6 h after SA treatment. MeJA pretreatment reduced the induction of *ATGRXS13* by SA indicating that JA negatively controls *ATGRXS13* expression (Figure 7b).

According to these results, we can conclude that JA and SA regulate the induction of *ATGRXS13* in opposing ways after inoculation by *B. cinerea*.

#### **GRXS13 is negatively regulated by TGA transcription factors that also bind to GRXS13 protein**

TGA2 clade of transcription factors has been shown to be mediator of plant defence in Arabidopsis plants challenged with *B. cinerea* (Zander *et al.*, 2010). We identified the TGACGTCA sequence in the promoter of *AT1G03850* corresponding to the perfect binding site for TGA transcription factor (Figure S5; Qin *et al.*, 1994). Furthermore, the TGACG sequence sufficient for TGA recognition (Spoel *et al.*, 2003) was found eight times within this promoter. We tested the hypothesis that this class of TGA transcription factors modulates *ATGRXS13* expression after *B. cinerea* infection. The expression of *ATGRXS13.2* was stronger in the *tga2tga5tga6* triple mutant in comparison with WT plants indicating that the TGA2 clade of transcription factors negatively regulates *ATGRXS13* gene expression during infection with *B. cinerea* (Figure 8a). Members of the Arabidopsis group III GRX can directly interact with TGA transcription factors (Ndamukong *et al.*, 2007; Li *et al.*, 2009). We tested whether this can also be true for *ATGRXS13* and TGA2 in a yeast two-hybrid assay. The  $\beta$ -galactosidase activity was induced only when both *ATGRXS13* and TGA2 proteins were introduced in yeast cells indicating that both proteins interact (Figure 8b).

#### **Classical defence genes are not controlled by *AtGRXS13***

To explain the resistance phenotype of *grxs13* mutants, we tested whether the expression of the JA marker *PDF1.2* and the SA marker *PR1* were affected in *grxs13-1* after infection by *B. cinerea*. *PDF1.2* was induced at 24 hpi (Figure S6b) whereas *PR1* was strongly induced 48 hpi (Figure S6a) as previously described (Chassot *et al.*, 2007). However, neither *PDF1.2* nor *PR1* induction were impaired in *grxs13-1* mutant after *B. cinerea* inoculation. Furthermore, we tested the hypothesis whether the induction of *ATGRXS13* by *B. cinerea* can inhibit the production of the phytoalexin camalexin, a major component of Arabidopsis defence against this fungus (Ferrari *et al.*, 2003; Kliebenstein *et al.*, 2005). *PAD3* catalyzes the last step of camalexin biosynthesis and *PAD3* expression correlates with camalexin production in response to *B. cinerea* (Schuhegger *et al.*, 2006). The expression of *PAD3* and the accumulation of camalexin were similar in WT and *grxs13-1* plant inoculated with *B. cinerea* (Figure S6c,d). These results indicate that the induction of such classical defence genes or camalexin can not explain the resistance to *B. cinerea* observed in *grxs13*.

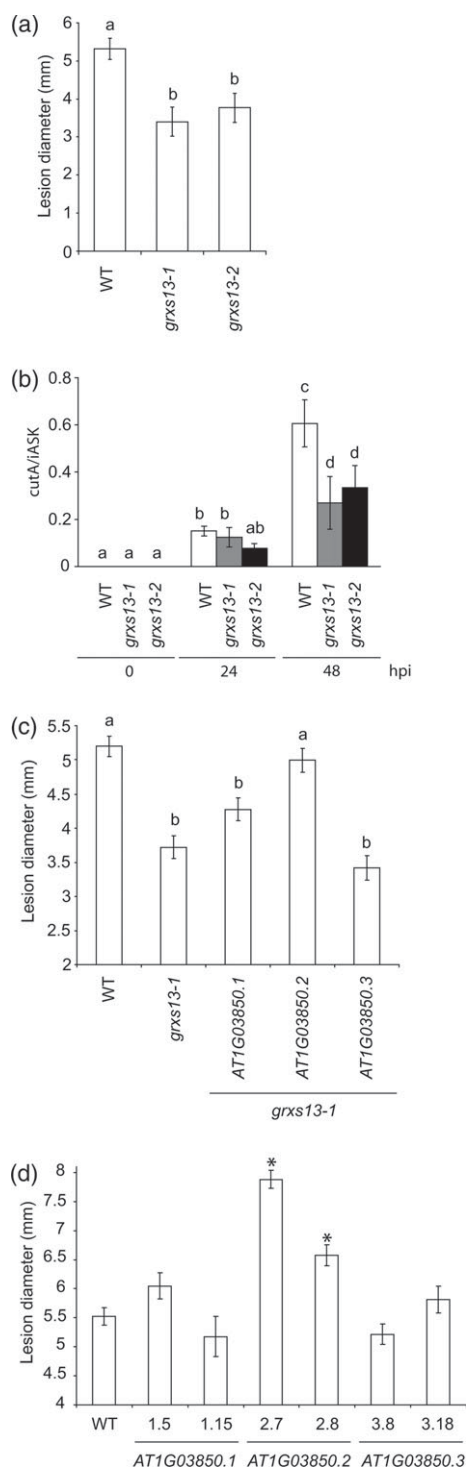
#### **DISCUSSION**

Hormones such as SA and JA are key regulators of the plant defence machinery. During plant infection by pathogens, these hormones control a highly complex network of sig-

nalling cascades that lead to gene expression and improved resistance. SA is mainly associated with resistance to biotrophic pathogens whereas JA is associated with resistance against necrotrophic pathogens such as *B. cinerea* (Glazebrook, 2005). Here we show that infection of Arabidopsis by *B. cinerea* led to an increase in the level of both JA and SA detected at 36 hpi (Figure 1). Interestingly, a lack of JA production in the allene oxide synthase mutant *dde2-2* (von Malek *et al.*, 2002) resulted in a strong increase in SA accumulation and *PR1* gene expression (Figures 1 and S1a). Furthermore, this mutant is hyper-susceptible to *B. cinerea* (Figure S1b). However, in *sid2-1* plants, that do not accumulate SA in response to *B. cinerea*, the level of JA is similar to that in WT plants. These results indicate the existence of a negative effect of JA on the production of SA and SA-dependent events in Arabidopsis plants infected with *B. cinerea*. Although important, the repressive effect of JA on SA accumulation in WT plants is not fully efficient as SA accumulated and *PR1* was expressed (Figures 1 and S5).

Virulent pathogens can infect and invade plants despite attempted defence mechanisms deployed by their hosts. JA is a key regulator of basal plant defence against necrotrophic pathogens (Glazebrook, 2005) and it is produced in response to *B. cinerea* (Figure 1). Furthermore, plants affected in JA responses are more susceptible to necrotrophic fungi including *B. cinerea* (Figure S1b; Ferrari *et al.*, 2003; Rowe and Kliebenstein, 2010; Thomma *et al.*, 1998; Yang *et al.*, 2007). We have identified a small number of genes, including the putative glutaredoxin *ATGRXS13* presented here, that are repressed by JA and yet induced by *B. cinerea*. We postulate that such genes could potentially interfere with the metabolism of the plant and favor growth of the pathogen *in planta*. Supporting our assumption, mutations in *ATGRXS13* resulted in an increased resistance to *B. cinerea* strain BMM. Both T-DNA mutants *grxs13-1* and *grxs13-2* exhibited reduced macroscopic symptoms of infection and a reduced pathogen growth (Figure 5). Therefore, it should be noted that there is a great diversity in *B. cinerea* strain virulence (Rowe and Kliebenstein, 2010) and we can not rule out the possibility that the phenotype we observed could be different with other *B. cinerea* strains. Glutaredoxins are small redox proteins of the thioredoxin superfamily the major functions of which are to reduce intra- and intermolecular disulfide bridges of target proteins or to reduce glutathionylated proteins. They can also serve as scaffold proteins for the assembly and delivery of [2Fe-2S] clusters most likely independently of their thiol-reductase activity (Rouhier *et al.*, 2004, 2010; Bandyopadhyay *et al.*, 2008). In plants, the role of GRX has mainly been studied *in vitro* and some publications indicate a role in the response to oxidative stress *in vivo* (Cheng *et al.*, 2006) or in the development of petals and anthers (Xing *et al.*, 2005; Xing and Zachgo, 2008). Recent studies have suggested an involvement of these proteins in plant defence. Over-expression of

ATGRXC7/ROXY1 in Arabidopsis enhanced susceptibility to *B. cinerea* that correlates with an increased oxidative stress (Wang *et al.*, 2009). However, over-expression of *ATGRXS13* splice variants does not result in an increased oxidative stress (Figure S4). Furthermore, *ATGRXC9/GRX480* has been shown to suppress the JA-responsive PDF1.2 expression (Ndamukong *et al.*, 2007). We show now that



**Figure 5.** Phenotype of the *grxs-13* mutants infected with *B. cinerea* and role of *ATGRXS13* splice variants in Arabidopsis plants infected by *B. cinerea*.

(a) Lesion diameter observed on wild-type (WT) Col and *grxs13* mutants 3 days after inoculation with *B. cinerea*. The data represent the mean lesion diameters from 12 (*grxs13-1*) and six (*grxs13-2*) independent experiments. In each experiment, 12–16 plants per genotype were infected with *B. cinerea*. Different letters represent groups which were significantly different from one another as determined by a one-way analysis of variance (ANOVA) followed by a multiple comparison with the Student–Newman–Keuls method ( $P < 0.05$ ).

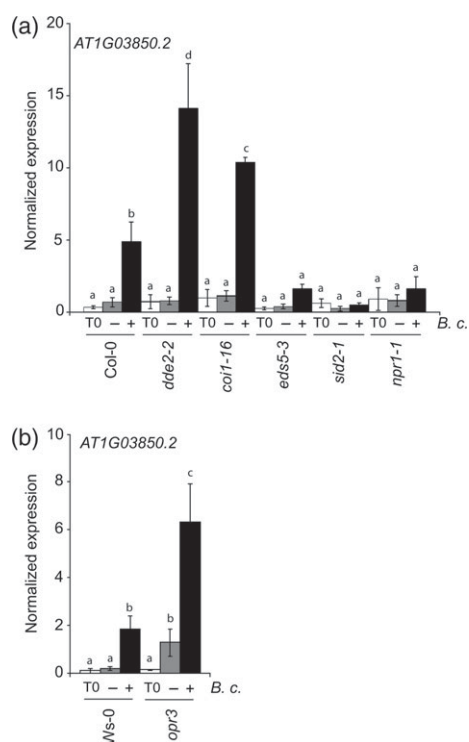
(b) Fungal growth quantification on WT Col and *grxs13* mutants infected with *B. cinerea*. The abundance of genomic DNA of *B. cinerea* and *A. thaliana* was estimated by the quantification of *CUTINASE-A* gene from *B. cinerea* and *IASK* gene from *A. thaliana* and represented by the *CUTA/IASK* ratios. In each experiment, eight plants per genotype were infected with *B. cinerea* and the data are the mean of three independent experiments ( $\pm$ SD). Different letters represent groups which were significantly different from one another as determined by a one-way ANOVA followed by a multiple comparison with the Student–Newman–Keuls method ( $P < 0.05$ ).

(c) Infection phenotype of *grxs13-1* mutant complemented with *AtGRXS13* splice variants under the control of *ATGRXS13* native promoter. In each experiment, sixteen plants per genotype were infected with *B. cinerea* and lesion diameters were measured after 3 days. The means of lesion diameter data from five independent experiments are shown ( $\pm$ SD). Different letters represent groups which were significantly different from one another as determined by a Kruskal–Wallis one-way ANOVA on ranks followed by a multiple comparison with the Dunn’s method. ( $P < 0.01$ ).

(d) Infection phenotype of WT plants over-expressing *ATGRXS13* splice variants. In each experiment, sixteen plants per genotype were infected with *B. cinerea* and lesion diameter was measured after 3 days. Two lines over-expressing *AT1G03850.1* (1.5; 1.15), *AT1G03850.2* (2.7; 2.8) and *AT1G03850.3* (3.8; 3.18) were tested. The means of lesion diameter data from three independent experiments are shown ( $\pm$ SD). Significant differences (\*) from WT plant were determined by a Kruskal–Wallis one-way ANOVA on ranks followed by a comparison with the Dunn’s method (\* $P < 0.01$ ).

*ATGRXS13*, another glutaredoxin, is required for a successful colonization of Arabidopsis by the necrotrophic fungus *Botrytis cinerea*.

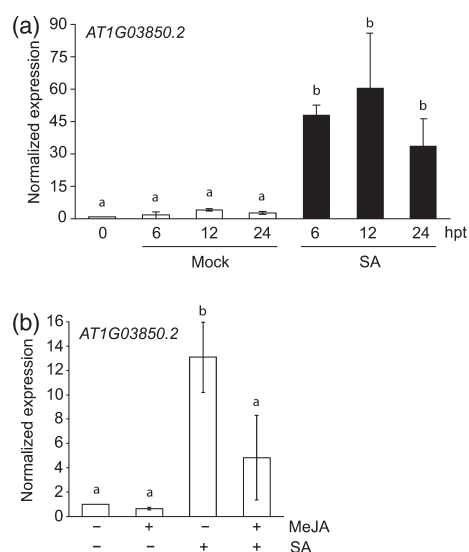
GRX from photosynthetic organisms are grouped into six different classes based on sequence identity of both the active site and a conserved motif involved in glutathione (GSH)-binding. Based on protein homology sequence, *ATGRXS13* belongs to the class III (CCxx active site) that is specific to terrestrial plants (Couturier *et al.*, 2009). However, no typical glutaredoxin activity has been shown for this protein so far. *ATGRXS13* gene is induced and alternatively spliced into three different mRNAs (Figures 2, 3 and S2) in plants infected with *B. cinerea*. Only the *AtGRXS13.2* cDNA sequence under the control of the native *ATGRXS13* promoter was able to complement the mutant *grxs13-1* resulting in normal susceptibility to *B. cinerea* (Figure 5c). This is further supported by our data showing that over-expression of *ATGRXS13.2* but not of *ATGRXS13.1* and *ATGRXS13.3* in WT plants resulted in a greater susceptibility to *B. cinerea* (Figure 5d). Finally, *ATGRXS13.2* exhibited the highest amount of transcripts after fungal infection compared with the other splice variants (Figure 3). Thus, only *AtGRXS13.2* is likely to be crucial during the colonization of Arabidopsis by *B. cinerea*. Whereas the three predicted glutaredoxin proteins



**Figure 6.** *ATGRXS13.2* mRNA accumulation in various signalling mutants infected with *B. cinerea*.

The leaves from sixteen plants were not sprayed (white bars), sprayed with mock solution (grey bars) or *B. cinerea* spores (black bars) and were harvested after 24 h. *AT1G03850.2* transcript levels were quantified by real-time polymerase chain reaction (PCR) and expressed as normalized expression (no unit) to the plant reference gene *AT4G26410* transcript level (Czechowski *et al.*, 2005). Data represent the mean of three independent experiments ( $\pm$ SD) and different letters represent groups which were significantly different from one another as determined by a one-way analysis of variance (ANOVA) followed by a multiple comparison with the Student-Newman-Keuls method ( $P < 0.05$ ).

have the same N-terminal sequence and the same putative catalytic site CCLG, only *ATGRXS13.1* and *ATGRXS13.2* display the typical thioredoxin fold characteristic of GRX (Figure 2b; Martin, 1995). Furthermore, the putative glutathione binding site is missing in *AtGRXS13.3* (Figure 2b). Therefore, *ATGRXS13.3* is unlikely to encode a functional GRX. The C-terminal part of the three isoforms is different and only *ATGRXS13.2* contains the ALWL motif that is shared by many group III glutaredoxins from plants (Figure 2b). This motif is essential for the protein function of *ATGRXC7* (*ROXY1*) during petal development (Li *et al.*, 2009). Furthermore, only glutaredoxins possessing the ALWL motif can complement the altered petal development phenotype of *roxy1-2* mutant (Li *et al.*, 2009). Thus, our results show that during infection of *A. thaliana* the expression of what appears to be the most active splice variant of the *ATGRXS13* gene is induced and required by *B. cinerea* for optimal infection.



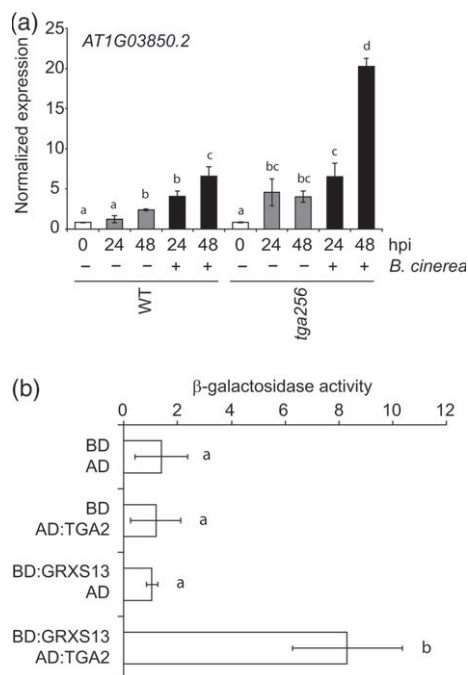
**Figure 7.** *ATGRXS13.2* mRNA accumulation in wild-type (WT) plants treated with SA and MeJA.

(a) Accumulation of *ATGRXS13.2* mRNA in response to salicylic acid (SA) treatment. The leaves from 10 plants were sprayed with Silwet-L77 0.015% (white bars) or with 1 mM of SA (black bars) and harvested at the indicated time points.

(b) SA-induced accumulation of *ATGRXS13.2* mRNA in plants pre-treated with MeJA. The leaves from 10 plants were sprayed with EtOH or 100  $\mu$ M of MeJA and sprayed 3 h later with Silwet-L77 0.015% or 1 mM of SA. Leaves were harvested 6 h after SA treatment. For (a) and (b), *AT1G03850.2* transcript levels were quantified by real-time polymerase chain reaction (PCR). Data are the mean of three independent experiments and are expressed as normalized expression (no unit) to the plant reference gene *AT4G26410* transcript level (Czechowski *et al.*, 2005). Different letters represent groups which were significantly different from one another as determined by a one-way analysis of variance (ANOVA) followed by a multiple comparison with the Student-Newman-Keuls method ( $P < 0.05$ ). hpt: hour post treatment.

*AT1G03850* was negatively controlled by JA-dependent pathways during infection but positively regulated by NPR1- and SA-dependent pathways as shown using various mutants (Figure 6). These data were confirmed by ectopic treatments of WT plants with SA and JA. SA induced the expression of *ATGRXS13* and application of MeJA before SA resulted in a decreased expression of *ATGRXS13* compared with a treatment with SA alone (Figure 7). These results indicate that the induction of *ATGRXS13* is under the control of both hormones. Thus, our results indicate that JA produced after *B. cinerea* infection negatively controls the production of SA and the expression of the SA-responsive gene *ATGRXS13*. While most reports on the SA/JA antagonism have documented a negative interference of SA on JA-dependent responses, some studies also reported a negative crosstalk of JA on SA-mediated signalling (Bostock, 2005; Koornneef and Pieterse, 2008). For instance, SA-dependent responses are not expressed in the *cev1* mutant that constitutively activates JA responses (Ellis *et al.*, 2002a,b). Plants mutated in *COI1*, a central regulator of the JA signalling,





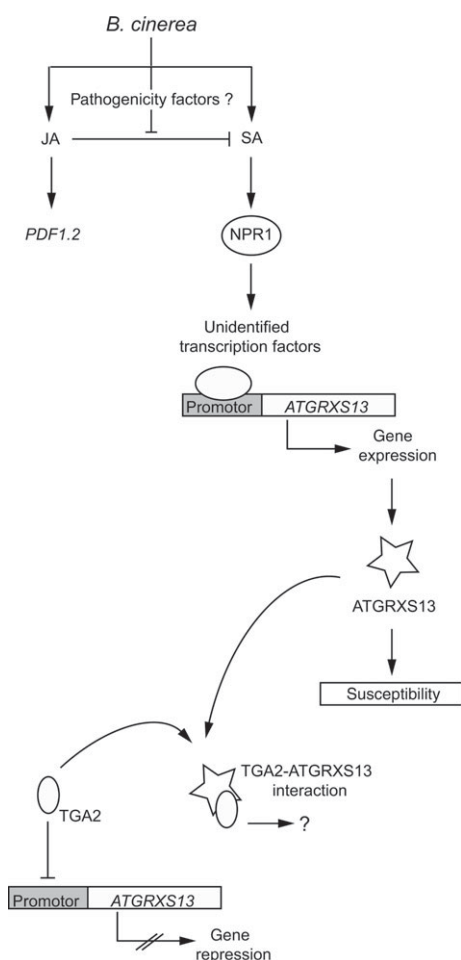
**Figure 8.** Relationship between ATGRXS13.2 and TGA. (a) *AT1G03850.2* mRNA accumulation in *tga256* triple mutant infected with *B. cinerea*. The leaves from sixteen plants were not sprayed (white bars), sprayed with mock solution (grey bars) or *B. cinerea* spores (black bars) and were harvested at the indicated time points. *AT1G03850.2* transcript levels were quantified by real-time polymerase chain reaction (PCR) and expressed as normalized expression (no unit) to the plant reference gene *AT4G26410* transcript level (Czechowski *et al.*, 2005). Data are the mean of three independent experiments. (b) Analysis of the interaction of ATGRXS13 with TGA2 in a quantitative yeast two-hybrid assay. The coding region of ATGRXS13 (*AT1G03850.2*) was fused to the GAL4 DNA-binding domain (BD:GRXS13), and that of TGA2 was fused to the GAL4 activation domain (AD:TGA2). After transformation of the respective plasmids in yeast MAV203 cells, β-galactosidase activity was measured. Three to seven independent clones were used for each construct in a single experiment. The results represent the average of three independent experiments. For (a) and (b), different letters represent groups which were significantly different from one another as determined by a one-way analysis of variance (ANOVA) followed by a multiple comparison with the Student–Newman–Keuls method ( $P < 0.05$ ).

are more resistant to *Pst* DC3000 due to an elevated level of SA and SA-dependent defences (Kloek *et al.*, 2001). Furthermore, the *jin1* (*jasmonate insensitive-1*) mutant that is mutated in the ATMYC2 transcription factor also accumulates more SA after challenge with *Pst* DC3000 (Nickstadt *et al.*, 2004). One may still wonder how relatively high levels of expression of *ATGRX13* can be induced after *B. cinerea* infection despite the JA/SA crosstalk. A possible explanation might be that *B. cinerea* interferes with the existing crosstalk between JA and SA through the release of various pathogenicity factors, making possible that the crosstalk is not complete and would thus allow for some expression of *ATGRXS13*. Alternatively, such a pathogenicity factor might affect the repression of *ATGRXS13*

by JA. In summary, infection of Arabidopsis by *B. cinerea* leads to an SA-dependent increase in the expression of the second isoform of *ATGRXS13* despite a negative crosstalk of JA on the SA-dependent responses. The details of the upregulation of *ATGRXS13* by *B. cinerea* in the general context of a negative interference by JA remain now to be elucidated.

The role of SA in the interaction between Arabidopsis and *B. cinerea* warrants a comment. We show that SA-dependent expression of *ATGRXS13* is required for full infection by *B. cinerea*. The *bik1* mutant affected in a membrane-anchored kinase is more susceptible to *B. cinerea* due to an increased accumulation of SA (Veronese *et al.*, 2006). Furthermore, the triple mutant *wrky18wrky40wrky60* exhibits a higher susceptibility to *B. cinerea* that correlates with an enhanced expression of the SA marker *PR1* (Xu *et al.*, 2006). In contrast, some reports indicate that SA is required for local resistance in Arabidopsis (Govrin and Levine, 2002; Ferrari *et al.*, 2003) making it likely that SA has a dual role in the response to *B. cinerea*. When a low dose of SA (e.g. 0.5 mM) is sprayed on Arabidopsis plants before inoculation, a greater susceptibility to *B. cinerea* is observed (Yang *et al.*, 2007) whereas a high dose of SA (e.g. 5 mM) induces resistance (Ferrari *et al.*, 2003). These apparent discrepancies on the role of SA during the interaction between Arabidopsis and *B. cinerea* could also be explained by differences in experimental conditions and *B. cinerea* isolates used between studies as discussed recently by Rowe and Kliebenstein (2010).

Members of the TGA transcription factor family such as TGA2, TGA5 and TGA6 that act redundantly regulate SA-dependent defence responses (Zhang *et al.*, 2003). It has been shown that these transcription factors contribute to basal resistance of Arabidopsis plants to *B. cinerea*. Thus, the triple mutant *tga2tga5tga6* is more susceptible to the infection by this fungus (Zander *et al.*, 2010). Here we show that *ATGRXS13.2* over-accumulated in *tga2tga5tga6* (Figure 8a) indicating that members of the TGA2 clade of transcription factors act as repressors of *ATGRXS13* gene expression in response to *B. cinerea*. The presence of TGA binding elements in the sequence of *ATGRXS13* promoter suggests that members of this TGA2 clade could directly bind to the promoter (Figure S5). However, we cannot rule out the possibility that this effect is indirect. It has been shown that the TGA2 clade is required for basal repression of SA-dependent gene expression such as *PR1* in the absence of SA production (Zhang *et al.*, 2003; Rochon *et al.*, 2006). However, TGA2 was also shown to be an activator of SA-dependent gene expression (Zhang *et al.*, 2003; Rochon *et al.*, 2006). Based on our results, we propose a hypothetical model for the induction of *ATGRXS13* and its role in plants infected with *B. cinerea* (Figure 9). JA-related mutants (in which SA over-accumulated, Figure 1) behave in the same way as the *tga2tga5tga6* mutant with regard to the



**Figure 9.** Hypothetical model of the regulation of *ATGRXS13* during the infection of Arabidopsis by *B. cinerea*.

In plants infected with *B. cinerea*, *ATGRXS13* is expressed and jasmonic acid (JA) and salicylic acid (SA) are produced. The repression of SA biosynthesis and SA-dependent gene expression by JA is indicated. However, this repression is only partial, SA is produced and the SA-dependent gene *ATGRXS13* is expressed. *B. cinerea* might release some unknown pathogenicity factors that interfere with the crosstalk from JA to SA. *ATGRXS13* expression is repressed by members of the TGA2 clade of transcription factors. During infection, *ATGRXS13* expression is derepressed through a SA- and NPR1-dependent pathway and some unidentified transcription factors can drive the expression of *ATGRXS13* that is required by *B. cinerea* to fully infect Arabidopsis plants. Finally, the interaction between *ATGRXS13* and TGA2 suggests that *ATGRXS13* could be involved in post-translational modification of TGA2 that would affect TGA2 activity.

expression of *ATGRXS13* (Figures 6 and 8a). These data suggest that the enhanced expression of *ATGRXS13* observed in JA-related mutants (Figure 6) can be the consequence of an overaccumulation of SA. In turn, SA could induce *ATGRXS13* gene expression that was repressed by TGA2. We observed that the induction of *ATGRXS13* is strongly impaired in SA-related mutants infected by *B. cinerea* (Figure 6). Thus, the expression of *ATGRXS13* is negatively regulated by JA-dependent pathways in the absence

of a stimulus such as an infection that stimulates SA accumulation. The triple *tga* mutant is deleted in the genes coding these three transcription factors (Li *et al.*, 2001; Zhang *et al.*, 2003). Thus, TGA2 is absent in this mutant but *ATGRXS13* is over-expressed in response to *B. cinerea* (Figure 8a). We can speculate that a positive regulation of *ATGRXS13* expression by TGA2 is unlikely in plants infected by *B. cinerea*. Possibly, other transcription factors can positively regulate *ATGRXS13* expression.

We have shown a direct interaction between *ATGRXS13* and TGA2 suggesting that *ATGRXS13* could be involved in post-translational modification of TGA2 that could affect TGA2 activity (Figures 8b and 9). One of the consequences might be the induction of the expression of the *ATGRXS13* gene. But the interaction between TGA2 and *ATGRXS13* is unlikely to control *PR1* and *PDF1.2* expression in response to *B. cinerea*. Indeed, expression of both genes is unaffected by the *grxs13-1* mutation (Figure S6). Other reports highlighted an interaction of *ATGRXs* with TGA transcription factors in plants (Ndamukong *et al.*, 2007; Li *et al.*, 2009; Murmu *et al.*, 2010). However, the mechanisms by which Arabidopsis glutaredoxin could regulate TGA transcription factors remains to be determined.

In conclusion, we have shown that virulence of *B. cinerea* on *A. thaliana* depends on the SA-dependent expression of a putative active splice variant of the glutaredoxin *ATGRXS13* gene. This expression escapes a negative interference by JA that is produced in response to *B. cinerea* infection. *ATGRXS13* gene expression is also negatively regulated by TGA2 clade of transcription factors and both proteins can interact. These results illustrate and further our knowledge on the subtle changes mediated by virulent pathogens in their hosts that allow a successful colonization.

## EXPERIMENTAL PROCEDURES

### Plant materials and growth conditions

*A. thaliana* seeds were grown on a pasteurized soil composed of humus and perlite (3:1) in a growth chamber under a 12 h light/12 h dark cycle, 60–70% humidity, 16–18°C during the night and 20–22°C during the day. The *ATGRXS13* mutant was *grxs13-1* (SALK\_046205) and *grxs13-2* (SALK\_088910).

### Pathogen infection

*B. cinerea* strain BMM (Zimmerli *et al.*, 2000) was grown on Difco potato dextrose agar (Becton Dickinson). Spores were harvested in water and filtered through glass wool and then diluted in quarter-strength Difco potato dextrose broth (PDB) for inoculation. For disease assays, 6  $\mu$ L of spore solution ( $5 \times 10^4$  spores  $\text{mL}^{-1}$ ) were deposited on three leaves of 5 weeks old plants. Lesion diameters were measured after 3 days. Fungal growth was measured as described previously (Gachon and Saindrenan, 2004). Plants were drop-inoculated and leaf discs were harvested at the indicated time points. For SA or JA measurements and total RNA isolation, plants were sprayed with spores ( $2 \times 10^5$  spores  $\text{mL}^{-1}$ ) and control plants with quarter-strength PDB. Entire leaves were harvested at the indicated time points and frozen in liquid nitrogen. The inoculated

plants were kept under high humidity in a tray closed with a water-sprayed transparent lid.

### Plant treatment

Plants were sprayed with 1 mM of SA sodium salt (Sigma-Aldrich) dissolved in water containing 0.015% of Silwet L-77 (Koorneef *et al.*, 2008). MeJA was dissolved as a 100 mM stock solution in absolute ethanol. Plants were sprayed with 100  $\mu$ M of MeJA diluted in water. Control plants were sprayed with water containing the same volume of Silwet L-77 or absolute ethanol. Entire leaves were harvested at the indicated time points and frozen in liquid nitrogen.

### Characterization of *grxs13* mutant alleles

Plants homozygous for *grxs13-1* and *grxs13-2* mutations were identified by polymerase chain reaction (PCR) (Table S1). The positions of the T-DNA insertions were determined by DNA sequencing of the PCR products obtained with primers Lba1 and S13-1RP (for *grxs13-1*), Lba1 and S13-2 RP or S13-2 LP (for *grxs13-2*).

### RNA extraction and quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, <http://www.invitrogen.com>) and further used for retrotranscription into cDNA (Omniscript RT kit; Qiagen AG, <http://www.qiagen.com>). Quantitative real-time PCR was performed using Absolute QPCR SYBR Green Mix (ABgene, Thermo Fisher Scientific, <http://www.thermofisher.com>). Gene expression values were normalized to the expression of the plant gene *AT4G26410*, previously described as a stable reference gene (Czechowski *et al.*, 2005). The primers are listed in Table S1.

### Cloning procedures

cDNA made from RNA extracted from *B. cinerea*-treated Arabidopsis plants were used to amplify *ATGRXS13* splice variants by PCR (Phusion Taq Polymerase, Finzymes). Products were cloned into the pENTR-D-TOPO vector (Invitrogen), sequenced and LR-recombined with pB2GW7 (Karimi *et al.*, 2002) to express *ATGRXS13* splice variants under the control of the CaMV35S promoter. WT Col plants were transformed and two independent transgenic T2 plant lines per splice variant were selected for further infection experiments with *B. cinerea*. To obtain vectors in which *ATGRXS13* splices variants were driven by *ATGRXS13* native promoter, the 1500 bp before the start codon of *ATGRXS13* were amplified by PCR and then cloned into pDONRP4P1R (Invitrogen). A LR-recombination was performed using pENTR-D-TOPO containing *ATGRXS13* splice variants, pDONRP4P1r and the destination vector pH7m24GW,3 (Karimi *et al.*, 2002). *Grxs13-1* mutants were transformed and one independent transgenic T2 plant line per splice variant was selected for further infection experiments with *B. cinerea*. The primers are listed in Table S1.

### Plant transformation

Binary plasmids described above were electroporated into *Agrobacterium tumefaciens* strain GV3101 (pMp90) and selected on LB-agar plates containing gentamicin (25  $\mu$ g mL<sup>-1</sup>), rifampicin (50  $\mu$ g mL<sup>-1</sup>) and spectinomycin (100  $\mu$ g mL<sup>-1</sup>). Plants were transformed using the floral dipping method (Clough and Bent, 1998). Transgenic plants were selected either on glufosinate, hygromycin or by PCR.

### Yeast two-hybrid analysis

The coding region of *ATGRXS13.2* was amplified by PCR, cloned in pDONR223 and LR-recombined in the yeast vector pDEST-GBKT7

(Invitrogen). The TGA2 coding region (Ndamukong *et al.*, 2007) was LR-recombined in the destination yeast vector pDEST-GAD. The activity of  $\beta$ -galactosidase was measured after transformation of the respective plasmids in yeast MAV203 cells.

### Camalexin, SA and JA quantification

SA and camalexin were quantified as described by Nawrath and Métraux (1999) using 200 mg of leaves. JA quantification was adapted from Schmelz *et al.*, 2004 and Gundlach *et al.*, 1992. About 500 mg of leaf material was extracted twice with 1 ml of extraction buffer (water/propan-1-ol/HCl:1/2/0.005, v/v) at 70°C and using a homogenizer (Polytron, Kinematica) with 100 ng of the internal standard dihydro-JA. Samples were mixed 15 sec with 2 ml of dichloromethane and centrifuged 2 min (10 000 g). The lower organic phase was dried by the addition of anhydrous Na<sub>2</sub>SO<sub>4</sub>. Carboxylic acids including JA were methylated 30 min to their corresponding methyl esters at room temperature by adding 20  $\mu$ l of 2 M bis-trimethylsilyldiazomethane and 200  $\mu$ l of MeOH. Methylation was stopped 30 min by adding 20  $\mu$ l 2 M acetic acid (room temperature). Solvent was evaporated under a nitrogen stream and the sample was dissolved in 1 ml of dichloromethane before loading onto a silica column (Chromabond; Macherey-Nagel AG, <http://www.mn-net.com>) prewashed with hexane. After washing with hexane (3  $\times$  2 ml), the column was eluted with 4 ml of hexane/diethyl ether (2:1). The eluate was evaporated and dissolved in 25  $\mu$ l of hexane before its separation on a capillary column (25 m  $\times$  0.2 mm) of BP-225 (SGE) fitted to a Hewlett Packard 5980GC coupled to a 5970 mass specific detector. The methyl esters of JA and dihydro-JA were detected by selective ion monitoring at m/z 151 and 156, respectively. JA amount (measured as methyl JA) was quantified by reference to the amount of internal standard. The results are expressed in  $\mu$ g.g<sup>-1</sup> FW of plant tissue.

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### SUPPORTING INFORMATION

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

**Figure S1.** Responses of the allene oxide synthase mutant *dde2-2* to *B. cinerea*.

**Figure S2.** Alignment of the genomic sequence of AT1G03850 to the cDNA of the three ATG1G03850 splice variants.

**Figure S3.** Phenotype of the *grxs13* mutants infected with *B. cinerea*.

**Figure S4.** Histochemical detection of ROS accumulation by diaminobenzidine (DAB) staining in wild type Col plants and in plants over-expressing *GRXS13* splice variants.

**Figure S5.** Sequence of the 1500 bp upstream of the transcriptional starting site of *ATGRXS13*.

**Figure S6.** Role of *ATGRXS13* in the accumulation of mRNA coding classical defence genes induced after *B. cinerea* infection and in the accumulation of the phytoalexin camalexin.

**Table S1.** List of primers used.

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