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Arabidopsis thaliana class-II TGA transcription factors are essential activators of jasmonic acid/ethylene-induced defense responses

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SUMMARY

The three closely related Arabidopsis basic leucine zipper (bZIP) transcription factors TGA2, TGA5 and TGA6 are required for the establishment of the salicylic acid (SA)-dependent plant defense response systemic acquired resistance, which is effective against biotrophic pathogens. Here we show that the same transcription factors are essential for the activation of jasmonic acid (JA)- and ethylene (ET)-dependent defense mechanisms that counteract necrotrophic pathogens: the tga256 triple mutant is impaired in JA/ET-induced PDF1.2 and b-CHI expression, which correlates with a higher susceptibility against the necrotroph Botrytis cinerea. JA/ET induction of the trans-activators ERF1 and ORA59, which act upstream of PDF1.2, was slightly increased (ERF1) or unaffected (ORA59). PDF1.2 expression can be restored in the tga256 mutant by increased expression of ORA59, as observed in the tga256 jin1 quadruple mutant, which lacks the transcription factor JIN1/AtMYC2 that functions as a negative regulator of the JA/ET-dependent anti-fungal defense program. Whereas JA/ET-induced PDF1.2 expression is strongly suppressed by SA in wild-type plants, no negative effect of SA on PDF1.2 expression was observed in the tga256 jin1 quadruple mutant. These results imply that the antagonistic effects of TGA factors and JIN1/AtMYC2 on the JA/ET pathway are necessary to evoke the SA-mediated suppression of JA/ET-induced defense responses.

Keywords: cross-talk, ethylene, jasmonic acid, PDF1.2, salicylic acid, TGA transcription factors.

INTRODUCTION

Plants are light-driven factories that supply organic carbon to our ecosystem. In the course of evolution, microorganisms have developed mechanisms to gain access to plant-fixed carbon resources by either killing the plant (necrotrophic lifestyle) or establishing structures to retrieve nutrients from living cells (biotrophic and symbiotic lifestyles) (Glazebrook, 2005). Still, most plants are immune to the majority of pathogens and susceptible to only a relatively small number of adapted microbes. This is because of the efficient activation of inducible defense responses upon recognition of pathogen-associated molecular patterns (PAMPs) or (a)virulence effectors (Chisholm et al., 2006).

Although being a simplified concept, it is generally recognized that defense responses mediated by the phytohormone salicylic acid (SA) are effective against biotrophic pathogens, whereas jasmonic acid (JA) and ethylene (ET) act as crucial signaling molecules that induce responses against necrotrophs (Glazebrook, 2005). Moreover, SA is necessary and sufficient for the inducible defense response systemic acquired resistance (SAR) that is established throughout the plant upon local infection with either avirulent or virulent pathogens (Durrant and Dong, 2004; Mishina and Zeier, 2007). This defense response is long lasting and effective against a broad spectrum of (hemi)biotrophic pathogens, including viruses, bacteria, oomycetes and fungi.

To investigate the mechanisms of the signaling cascades and their mutual interactions, the induction of marker genes in the model plant Arabidopsis thaliana is often used as a

quantifiable response. A crucial step leading to the activation of the SA marker gene PATHOGENESIS-RELATED-1 (PR-1) and the establishment of SAR involves the SA-induced nuclear translocation of the ankyrin repeat protein NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1; Mou et al., 2003), which interacts with TGA transcription factors (Zhang et al., 1999). NPR1 is required to counteract the negative regulator SNI1 (SUPPRESSOR OF NPR1, INDUCIBLE1), as revealed by the re-establishment of SA-induced PR-1 expression and SAR in the sni1 npr1 double mutant (Li et al., 1999). Moreover, TGA2 and NPR1 have been postulated to form an enhanceosome at the PR-1 promoter (Rochon et al., 2006), with NPR1 acting as a transcriptional co-activator (Spoel et al., 2009). Only simultaneous deletion of the closely related class-II TGA factors TGA2, TGA5 and TGA6 impaired SAR, indicating that these factors act redundantly (Zhang et al., 2003). Conflicting data have been reported with respect to their role for the induction of PR-1, ranging from a complete loss of PR-1 induction (Zhang et al., 2003) to slightly delayed induction kinetics (Blanco et al., 2009) upon treatment with either the SA analogue 2,6-dichloro-isonicotinic acid or SA, respectively. Increased basal PR-1 transcript levels were observed in the tga256 mutant in both reports (Zhang et al., 2003; Blanco et al., 2009). TGA2, but not TGA6, suppresses PR-1 expression in the absence of SA (Rochon et al., 2006; Kesarwani et al., 2007).

Studies initiated to understand the mechanism of JA-induced gene expression have often made use of the genes VSP2, LOX2, PDF1.2 and b-CHI (Lorenzo et al., 2004). Expression of these genes requires COI1 (CORONATINE INSENSITIVE1), an F-box protein that can form a ternary complex with JA-isoleucine and members of the JAZ repressor proteins (Thines et al., 2007; Yan et al., 2009). JAZ repressors inactivate transcription factor AtMYC2 at the protein level by direct protein–protein interactions (Chini et al., 2007). After COI1-mediated ubiquitinylation and subsequent degradation of JAZ proteins, AtMYC2 can activate its own gene, VSP2 and LOX2. At the same time, AtMYC2 is a negative regulator of PDF1.2 and b-CHI (Lorenzo et al., 2004).

In contrast to VSP2 and LOX2, induction of PDF1.2 and b-CHI requires a functional ET signaling cascade, even if only JA is applied (Penninckx et al., 1998). In plants grown in soil, PDF1.2 can be induced by either JA or ET, whereas in agar-grown plants, strong induction is only observed upon simultaneous application of both hormones. Upon activation of the ET signaling cascade the key ethylene response transcription factors EIN3 and EIN3-like 1 (EIL1) are no longer degraded through the 26S proteasome pathway (Kendrick and Chang, 2008). EIN3 and EIL1 regulate downstream targets of the ET signaling pathway, such as the ethylene response factor 1 (ERF1) (Solano et al., 1998). Ectopic expression of ERF1 and the related APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) ORA59 is sufficient for PDF1.2 expression (Solano et al., 1998; Pre et al., 2008).

Activation of the SA or JA/ET pathway is not always initiated exclusively in response to either biotrophs or necrotrophs. For example, the hemi-biotrophic bacterial leaf pathogen Pseudomonas syringae pv. tomato DC3000 can simultaneously induce synthesis of both SA and JA. However, elevated levels of SA eventually suppress JA accumulation so that the SA pathway is prioritized (Spoel et al., 2003). Pharmacological studies (Spoel et al., 2003; Koornneef et al., 2008; Leon-Reyes et al., 2009), analysis of pathogen-challenged mutants defective in either of the signaling pathways (Kloek et al., 2001), and experiments with plants simultaneously infected with biotrophs and necrotrophs (Spoel et al., 2007; Koornneef et al., 2008) have led to the concept that SA strongly antagonizes the JA and JA/ET pathways in A. thaliana. As revealed by mutant analysis, NPR1 and class-II TGA factors are important for the SA–JA crosstalk (Spoel et al., 2003; Ndamukong et al., 2007). However, in the presence of elevated levels of ET, NPR1 becomes dispensable (Leon-Reyes et al., 2009). In addition, experiments with ectopically expressed proteins suggest the TGA-interacting glutaredoxin GRX480 (Ndamukong et al., 2007) and transcription factor WRKY70 (Li et al., 2004) are involved in the SA-mediated suppression of JA-induced genes. However, the molecular mechanisms set in motion by these regulatory proteins have remained elusive.

Here, we show that class-II TGA transcription factors are essential for the induction of PDF1.2 transcription after infection with either Botrytis cinerea or P. syringae, or in JA/ET-induced plants. This result establishes a so-far unknown role for TGA factors in defense responses against necrotrophic pathogens. Moreover, we provide evidence that the integration of TGA factors into the JA/ET pathway establishes a molecular link that connects the SA and the JA/ET signaling networks.

RESULTS

ET introduces the requirement of TGA factors for PDF1.2 expression

TGA2, TGA5 and TGA6 form one clade within the TGA family of transcription factors (Xiang et al., 1997), and their simultaneous knock-out was required to detect their function as crucial activators of SAR (Zhang et al., 2003). The tga256 mutant has resulted from the cross of two mutants obtained after fast neutron-deletion mutagenesis, lacking the coding regions of TGA6 and the two closely linked TGA2 and TGA5 alleles (Zhang et al., 2003). When infecting wild-type plants with the necrotrophic fungus B. cinerea, almost 80% of the lesions were in the 3–8-mm range in diameter, and only 10% were larger than 8 mm. In contrast, 40% of the lesions on tga256 mutant plants were larger than 8 mm (Figure 1a), demonstrating that the fungus inflicts significantly more

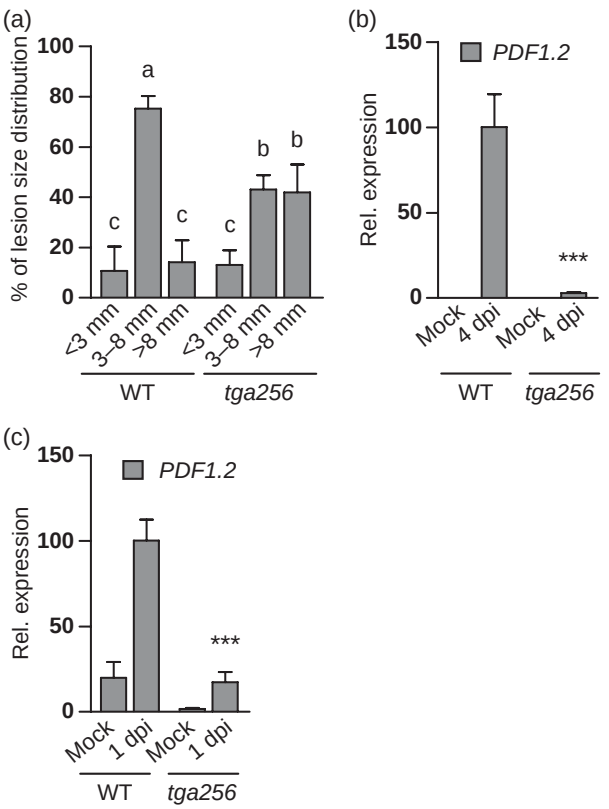

Figure 1. Symptom development and PDF1.2 expression in wild-type and tga256 mutant plants after pathogen infection.

(a) Distribution of lesion sizes on wild-type and tga256 mutant plants after 3 days of infection with Botrytis cinerea. Four-week-old soil-grown plants were drop-inoculated with a B. cinerea spore solution (5 ï 10^4 spores ml$^{)1}$) or with quarter-strength potato dextrose broth (mock). The diameters of at least 40 lesions per experiment were measured and grouped according to their size into the three indicated classes. The mean percentage distribution (SE) of the lesion sizes of three independent experiments is shown. Different letters denote significant differences (Student's t-test, P < 0.05) between the relative abundances of lesion size classes in both genotypes.

(b) Quantitative real-time RT-PCR analysis of relative PDF1.2 transcript levels in wild-type and tga256 mutant plants after 4 days of spray inoculation with B. cinerea. Four-week-old soil-grown plants were sprayed with a B. cinerea spore solution (2 ï 10^5 spores ml$^{)1}$) or with quarter-strength potato dextrose broth (mock). The average of the relative PDF1.2 transcript levels in 12 infected wild-type plants was set to 100%. The mean values (SE) obtained from 12 individual wild-type and 12 individual tga256 plants are shown. Asterisks represent significant differences between wild-type and tga256 plants within a treatment (two-way ANOVA, ***P < 0.001).

(c) Quantitative real-time RT-PCR analysis of relative PDF1.2 transcript levels in wild-tpye and tga256 mutant plants at 1 day after dip inoculation with Pseudomonas syringae pv. maculicola. Five-week-old soil-grown plants were infected by immersing whole rosettes into bacterial suspensions of approximately 0.2 OD_{600}, containing 0.02% (v/v) Silwet and 10 mM $MgCl_2$. The average of the relative PDF1.2 transcript levels of six infected wild-type plants was set to 100%. The mean values (SE) obtained from six individual wild-type and six individual tga256 plants are shown. Asterisks represent significant differences between wild-type and tga256 plants within a treatment (two-way ANOVA, ***P < 0.001).

damage on the mutant than on the wild-type plants. As activation of defense responses against B. cinerea requires functional JA and ET signaling cascades (AbuQamar et al., 2006), we tested whether the enhanced susceptibility correlates with a defect in these pathways using the defensin gene PDF1.2 as a marker. As shown in Figure 1b, PDF1.2 induction was impaired in the tga256 mutant as compared with wild-type plants after infection with B. cinerea, indicating that class-II TGA factors play an important role in the JA/ET-activated network. Lower inducibility of PDF1.2 as a result of deleted TGA alleles was also observed in plants infected with P. syringae maculicola ES4326 at 1 day post-infection (1 dpi) (Figure 1c).

As PDF1.2 expression is not affected in JA-treated tga256 mutant plants (Ndamukong et al., 2007), we deduced that ET, which is generated upon infection with necrotrophic fungi and P. syringae (De Vos et al., 2005), might introduce the requirement for class-II TGA factors. Synergistic effects of JA and ET on PDF1.2 expression are predominantly observed in seedlings grown on agar (Penninckx et al., 1998). Therefore, wild-type and tga256 mutant plants were grown for 12 days on MS plates, and then treated for 48 h with either the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC; 0.5 mM), methyl jasmonate (MeJA; 50 lM) or a combination of both chemicals. As observed before (Penninckx et al., 1998), ACC or JA only slightly induced PDF1.2 expression, whereas a more than 1000-fold induction was observed after simultaneous application of both chemicals (Figure 2a). This induction was severely compromised in the tga256 knock-out mutant. In contrast, LOX2, which is a marker gene for the ET-independent JA pathway (Lorenzo and Solano, 2005), was unaffected (Figure 2b). Similar results were obtained with the JA/ET-inducible gene b-CHI and the JA-inducible gene VSP2 (Figure S1).

To corroborate our conclusion that TGA factors play a crucial role in mediating PDF1.2 expression only in the presence of ET, we investigated the effect of JA and ET in 4-week-old soil-grown plants. JA treatment (8 h; 4.5 lM gaseous MeJA) caused the induction of PDF1.2 independently of TGA factors (Figure 2c). In contrast, ACC treatment (48 h; 0.5 mM ACC) failed to stimulate PDF1.2 expression in the tga256 mutant (Figure 2d).

JA/ET induction of the PDF1.2 promoter does not depend on the TGA binding site

TGA factors bind to the TGACG motif at bp position –397 to –393 relative to the predicted transcriptional start site within the PDF1.2 promoter in vitro (Spoel et al., 2003). In order to analyze whether this motif is important for JA/ET-induced expression, the sequence TGACG was mutated to a stretch of five Ts, and the wild-type and the mutant promoters (from bps)1 to)931) were fused to the reporter gene GUS. The chimeric genes were transformed into the Arabidopsis genome by Agrobacterium tumefaciens-mediated gene transfer. Seedlings of 14 independent transformants of each construct were germinated on agar plates and subjected to either mock or JA/ACC treatment. As shown in Figure 3, the

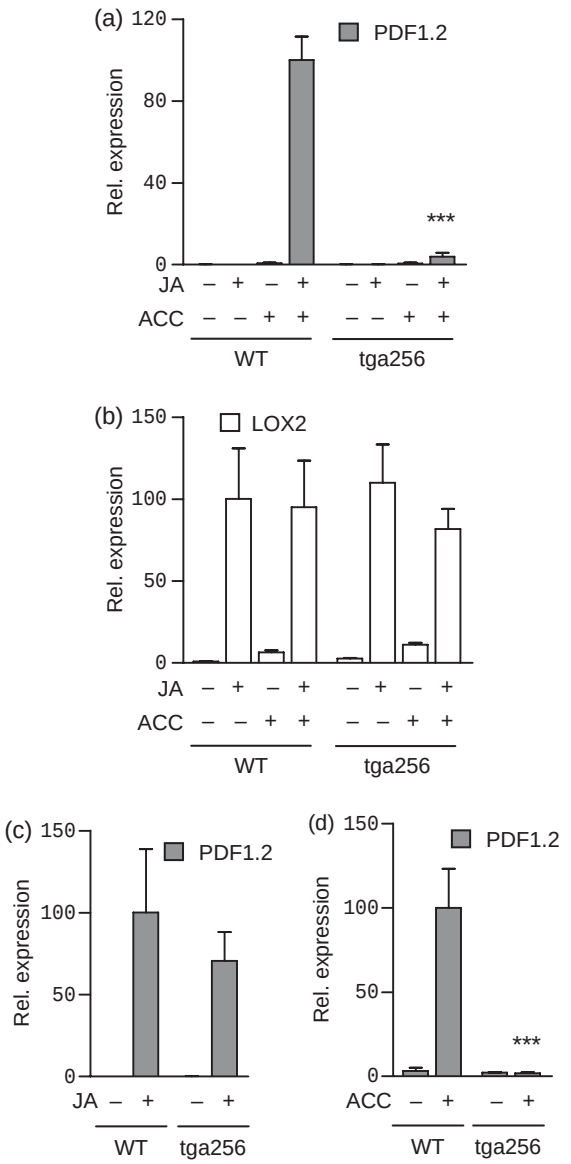

Figure 2. Expression of PDF1.2 and LOX2 in wild-type and tga256 mutant plants after treatment with jasmonic acid (JA), 1-aminocyclopropane-1-carboxylic acid (ACC), or JA and ACC.
(a, b) Twelve-day-old wild-type and tga256 mutant seedlings grown on MS medium were transferred to medium supplemented with 0.01% ethanol (mock) or 50 l M MeJA/0.01% ethanol (JA). Transferred plants were sprayed with 0.5 m M of the ethylene (ET) precursor ACC. After 48 h of treatment, approximately 50 seedlings were harvested for RNA extraction. PDF1.2 (a) and LOX2 (b) transcript levels were determined by quantitative real-time RT-PCR analysis. Values from JA/ACC- (a) or JA-treated (b) wild-type plants were set to 100%. The mean values (SE) from three independent experiments (one plate with 50 seedlings per experiment) are shown. Asterisks represent significant differences between wild-type and tga256 plants within a treatment (two-way ANOVA, ***P < 0.001).
(c, d) Four-week-old wild-type and tga256 mutant plants grown on soil were treated for 8 h with 4.5 l M MeJA or for 48 h with 0.5 m M ACC. Relative PDF1.2 transcript levels were determined by quantitative real-time RT-PCR analysis. Values from JA- (c) or ACC-treated (d) wild-type plants were set to 100%. The mean values (SE) obtained from six individual wild-type and six individual tga256 plants are shown. Asterisks represent significant differences between wild-type and tga256 plants within a treatment (two-way ANOVA, ***P < 0.001).

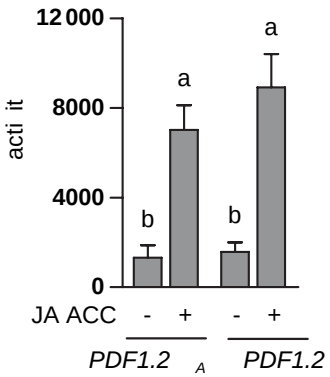

Figure 3. Expression of PDF1.2$_{(TGACG)}$:GUS and PDF1.2$_{(TTTTT)}$:GUS after treatment of plants with jasmonic acid (JA) and 1-aminocyclopropane-1-carboxylic acid (ACC).
A 959-bp fragment of the PDF1.2 promoter was cloned upstream of the GUS reporter gene. In construct PDF1.2$_{(TTTTT)}$, the TGACG motif at bp position)397 to)393 was replaced by TTTTT. Col-0 plants were transformed with these constructs and approximately 50 F_2 plants from 14 independent transformants of each construct were grown on MS medium for 12 days, transferred to MS plates containing 0.01% ethanol (mock) or 50 l M MeJA/0.01% ethanol (JA), sprayed with 0.5 m M ACC, and then harvested for quantitative GUS expression analysis after 48 h. Values indicate the mean GUS activities (pmol methylumbelliferyl glucuronide per mg protein) of 14 independent F_2 lines (SE) of each construct (see Figure S2 for values of the individual lines). Different letters denote significant differences between treatments within a PDF1.2:GUS construct (Student's t-test, P < 0.05).

TGACG motif does not play an important role for the JA/ACC inducibility of the PDF1.2 promoter. GUS values of the independent transgenic lines are shown in Figure S2.

JA/ET-induced expression of ORA59 or ERF1 is not severely affected in the tga256 knock-out mutant

Next, we tested whether expression of the two known transcription factors ERF1 and ORA59, which act upstream of PDF1.2 (Lorenzo et al., 2004; Pre et al., 2008), was affected in the tga256 mutant. As transcriptional activation of regulatory factors might precede the regulation of their target genes, a time-course experiment was performed with JA/ACC-treated seedlings. Under these conditions, the synergistic effect of JA and ACC on the expression of PDF1.2 was observed after 48 h (Figure 4a). In contrast, transcription of ERF1 and ORA59 was already induced after 2 h (Figures 4b,c). As judged from two biological replicates, expression of ORA59 was not affected at any of the analyzed time points in the tga256 knock-out mutant. ERF1 expression was significantly enhanced in mock- and JA/ACC-treated samples of the tga256 mutant at 4, 8 and 12 h. However, this enhancement did not lead to increased PDF1.2 expression. We therefore assume that TGA factors activate PDF1.2 expression by influencing the expression of other regulatory factors.

In B. cinerea-infected tga256 plants (4 dpi), expression of ORA59 was reduced by a factor of two when compared with

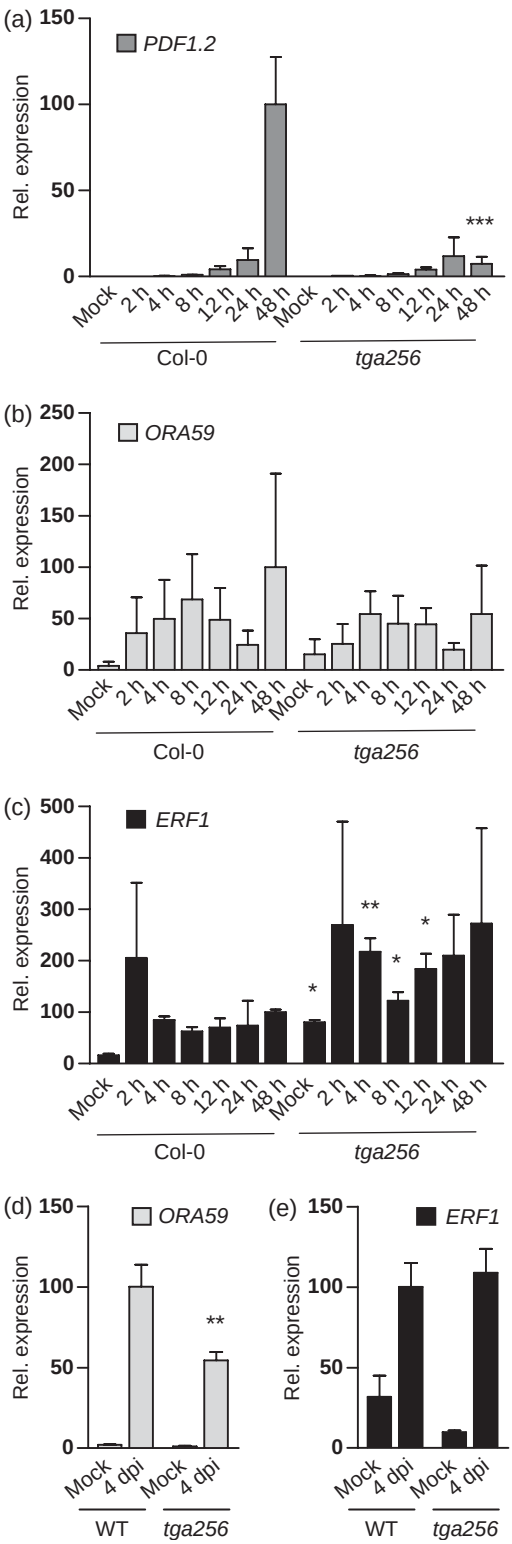

Figure 4. Time course of PDF1.2, ORA59 and ERF1 expression after treatment with jasmonic acid (JA) and 1-aminocyclopropane-1-carboxylic acid (ACC) in wild-type and tga256 mutant plants.

Twelve-day-old wild-type and tga256 mutant seedlings grown on MS medium were transferred to fresh MS medium containing 50 μM methyl jasmonate (MeJA)/0.01% ethanol (JA) and subsequently sprayed with 0.5 mM ACC. Approximately 50 seedlings were harvested for RNA extraction after the indicated number of hours. The mock value is from plants transferred for 48 h to MS plates containing 0.01% ethanol. PDF1.2 (a), ORA59 (b) and ERF1 (c) transcript levels were determined by quantitative real-time RT-PCR analysis. The relative expression in wild-type plants after 48 h of JA/ACC treatment was set to 100%. The mean values (SE) of two samples from two independent experiments are shown. To demonstrate the effect of the tga256 mutations on ORA59 (d) and ERF1 (e) transcript levels after Botrytis cinerea infections, the same cDNAs were used as in Figure 1b. Asterisks represent significant differences between wild-type and tga256 plants (two-way ANOVA, *P < 0.05, **P < 0.01, ***P < 0.001).

wild-type plants (Figure 4d). Under these conditions, expression levels were approximately 20-fold higher than in the pharmacological experiments (Figure S3). Thus, TGA factors might be needed for a high level of ORA59 expression. Expression of ERF1 was unaffected (Figure 4e).

The tga256 jin1 mutant shows induced PDF1.2 transcript levels that cannot be suppressed by SA

Another regulatory gene that influences PDF1.2 expression is JASMONATE-INSENSITIVE1 (JIN1) that encodes the transcription factor AtMYC2 (Lorenzo et al., 2004). AtMYC2 activates JA-responsive genes like VSP2 and LOX2, but has a negative effect on JA/ET-inducible genes like PDF1.2 and b-Chi. In order to define the genetic interaction between TGA factors and AtMYC2, the tga256 mutant was crossed with the jin1-1 mutant, which encodes a truncated AtMYC2 protein (Nickstadt, 2005). Seedlings of the homozygous tga256 jin1 quadruple mutant and the parental lines were grown on MS medium and treated with JA and ACC. As displayed in Figure 5a, PDF1.2 expression was enhanced by about 40-fold in the jin1-1 mutant as compared with the induced PDF1.2 transcript levels in wild-type plants, confirming the previously described strong negative effect of AtMYC2 on the JA/ET pathway (Lorenzo et al., 2004). Simultaneous inactivation of class-II TGA factors and AtMYC2 resulted in plants that induce PDF1.2 expression to wild-type levels (Figure 5a). Thus, with respect to PDF1.2 expression, the jin1-1 allele is a strong suppressor of the tga256-mediated phenotype, and vice versa.

As TGA factors have been described as regulatory components of the SA signaling network (Zhang et al., 2003), we speculated that the positive effect of TGA factors on JA/ET-induced PDF1.2 expression might be regulated by SA, thus establishing a molecular link between the two competing defense programs. In order to challenge this hypothesis, we analyzed the effect of exogenously applied SA on JA/ACC-induced PDF1.2 expression in the tga256 jin1 quadruple mutant. As described before (Leon-Reyes et al., 2009), application of SA impaired PDF1.2 expression in wild-type plants after JA/ACC treatment (Figure 5b). Likewise, the JA/ACC-treated jin1-1 mutant showed 20-fold lower activation

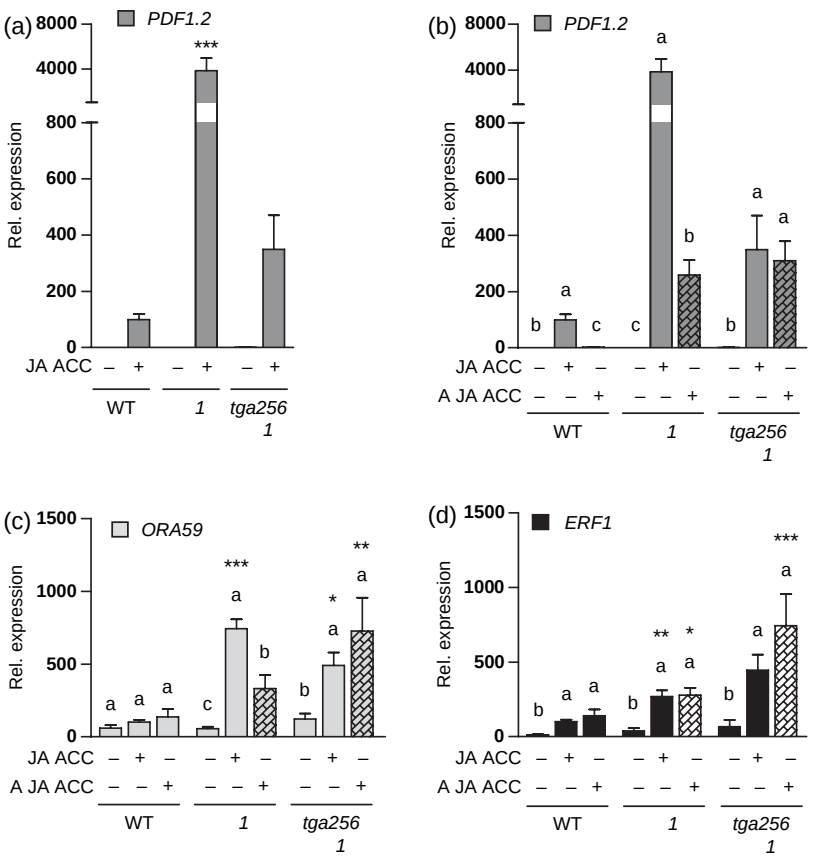

Figure 5. Analysis of PDF1.2, ORA59 and ERF1 transcript accumulation in wild-type, jin1-1 and tga256 jin1 mutant plants after treatment with jasmonic acid (JA)/1-aminocyclopropane-1-carboxylic acid (ACC) and salicylic acid (SA).
Quantitative real-time RT-PCR analysis of relative PDF1.2 (a, b), ORA59 (b) and ERF1 (c) transcript levels in wild-type, jin1-1 and tga256 jin1 mutant plants. Plantlets were germinated on MS medium, transferred after 12 days to MS plates containing 50 μM methyl jasmonate (MeJA)/0.01% ethanol (JA), which were supplemented with 200 μM SA when indicated, sprayed with 0.5 mM ACC and then incubated for 48 h. Mock control plants were transferred to MS plates containing 0.01% ethanol. Approximately 50 plantlets per sample were harvested for RNA extraction after 48 h. Transcript levels were determined by quantitative real-time RT-PCR analysis. Values of wild-type plants after JA/ACC induction were set to 100%. The mean values (SE) of three independent experiments with between one and five independent replicates each are shown. Different letters in (b, c, d) indicate significant differences among treatments within a genotype (Student's t-test, $P < 0.05$). Asterisks in (a, c, d) represent significant differences between the genotypes within a treatment compared with the wild type (two-way ANOVA, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

of PDF1.2 after treatment with SA as compared with the JA/ACC-induced levels. The remaining PDF1.2 transcript levels were still in the same range as in the JA/ACC-treated wild-type and tga256 jin1 plants. Thus, SA treatment of the jin1-1 mutant diminished PDF1.2 expression to the same extent as deletion of the TGA genes. In the JA/ACC-treated tga256 jin1 quadruple mutant, expression was not affected by SA in six out of eight samples (Figure 5), and was enhanced by 10-fold in two samples (Figure S4). Despite these fluctuations, this analysis indicates that – at least in the jin1-1 mutant – TGA factors are necessary for mediating the negative effect of exogenous SA on PDF1.2 expression.

In order to investigate whether AtMYC2 represses PDF1.2 expression indirectly by negatively regulating the expression of the corresponding upstream factors, ORA59 and ERF1 transcription was analyzed using the same cDNAs as in Figure 5b. This analysis indicates that JA/ACC-induced ORA59 transcript levels increase in the absence of AtMYC2, and that this increase is reduced twofold by SA (Figure 5c). Likewise, mutations of the TGA alleles in the jin1-1 mutant background significantly reduced ORA59 expression (Student's t-test, $P < 0.05$). Thus, SA and deletion of the TGA genes had the same effect, as observed above for PDF1.2 expression. However, the effects on ORA59 transcript levels were only twofold as compared with 20-fold in the case of PDF1.2. Again, no crosstalk was observed in the tga256 jin1 quadruple mutant. ERF1 transcript levels were not as strongly affected by the jin1-1 allele (Figure 5d). SA had no effect. Variable ERF1 expression was observed in the tga256 jin1 quadruple mutant after JA/ACC/SA treatment, which most likely accounts for the highly induced PDF1.2 expression levels in the two samples that were not considered for calculating the means (Figure S4).

PDF1.2 transcription activated by ectopic expression of ORA59 is subject to SA crosstalk

Our data demonstrate that AtMYC2 negatively affects ORA59 transcription (Figure 5c), suggesting that enhanced PDF1.2 levels in jin1-1 mutant plants are to the result of increased ORA59 levels. Although SA and TGA factors had some influence on ORA59 transcription, at least in the jin1-1 mutant, their effect on PDF1.2 was much stronger. In order to obtain independent supportive evidence for the idea that SA can affect PDF1.2 expression without affecting ORA59 expression, we made use of transgenic plants expressing ORA59 under the control of an estradiol-inducible promoter (Pre et al., 2008). SA treatment reduced the ORA59-induced expression of PDF1.2, when we applied 10 nM estradiol, which induces PDF1.2 expression to approximately the same levels as JA and ACC (Figure 6). Although the crosstalk was not as stringent as in JA/ACC/SA-treated wild-type plants, this experiment provides evidence that SA can work

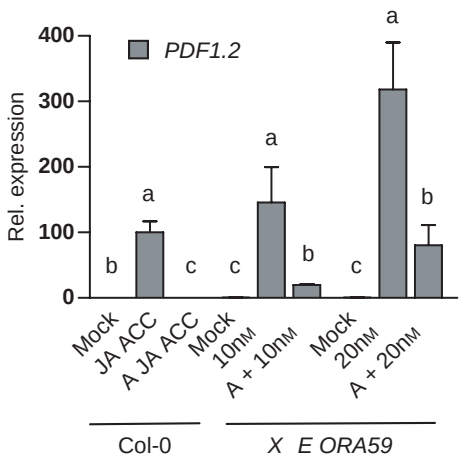

Figure 6. Effect of salicylic acid (SA) on PDF1.2 expression in plants ectopically expressing ORA59.
Seedlings of wild-type and transgenic plants expressing ORA59 under the control of an b-estradiol-inducible promoter (XVE:ORA59) were grown for 12 days on MS medium. Transgenic XVE:ORA59 plants were transferred to MS plates containing 10 or 20 nM b-estradiol with or without 200 lM SA. Mock control plants were transferred to MS plates containing 0.01% ethanol. Wild-type plants were treated as described in Figure 2. Approximately 50 plantlets were harvested for RNA extraction after 48 h of incubation. Transcript levels were determined by quantitative real-time RT-PCR analysis. The mean value of three independent samples of JA/ACC-treated wild-type plants was set to 100%. The mean values (SE) of three independent samples are shown. Different letters indicate significant differences among treatments within wild-type plants: XVE:ORA59 plants treated with 10 nM b-estradiol (E) and XVE:ORA59 plants treated with 20 nM b-estradiol (Student's t-test, P < 0.05).

through a mechanism that does not involve the suppression of ORA59 transcription.

Increased expression of TGA5 leads to hyperinduction of PDF1.2

In order to test, whether TGA2, TGA5 and TGA6 act redundantly with respect to their ability to promote PDF1.2 expression, the tga25 and tga6 mutants were analyzed. As displayed in Figure 7a, PDF1.2 expression was reduced in the tga25 mutant, indicating that endogenous levels of TGA6 cannot promote transcription. The tga6 mutant behaved like the wild type, revealing that TGA6 is not required. Next, the

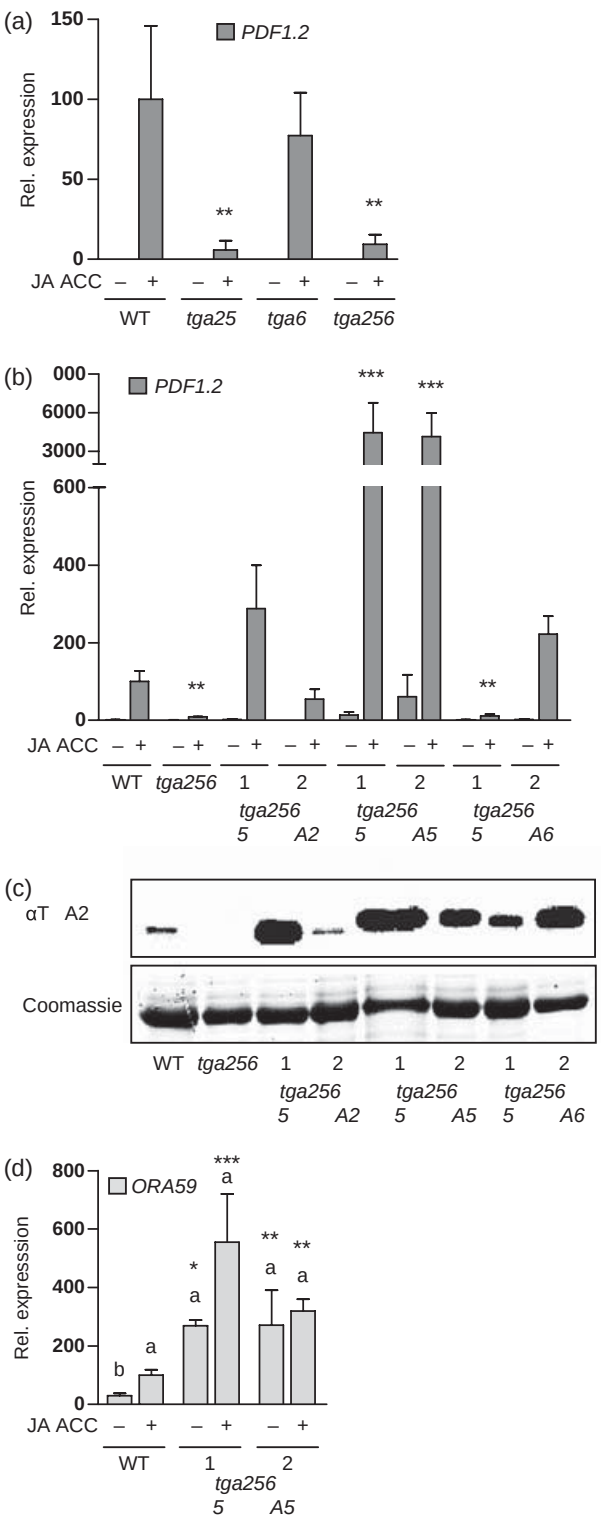

Figure 7. Analysis of PDF1.2 and ORA59 expression in tga25 and tga6 mutants, and in plants ectopically expressing TGA2, TGA5 or TGA6.
Twelve-day-old wild-type, tga256, tga25 and tga6 mutant seedlings, or transgenic lines ectopically expressing TGA2, TGA5 or TGA6 in the tga256 mutant background, were grown on MS medium and transferred to medium supplemented with 0.01% ethanol (mock) or 50 lM methyl jasmonate (MeJA)/0.01% ethanol (JA). Transferred plants were sprayed with 0.5 mM of the ethylene (ET) precursor 1-aminocyclopropane-1-carboxylic acid (ACC). After 48 h of treatment, approximately 50 seedlings were harvested for RNA or protein extraction. PDF1.2 (a, b) and ORA59 (d) transcript levels were determined by quantitative real-time RT-PCR analysis. Values of JA/ACC-treated wild-type plants were set to 100%. The mean values (SE) of three independent biological replicates are shown. (c) Western blot analysis of the transgenic lines analyzed in (b) using the antibody generated against the C termini of TGA2 and TGA5 (Fode et al., 2008). The samples for protein extraction were taken from untreated seedlings grown in the same experiment as for RNA extraction. Asterisks represent significant differences between the genotypes within a treatment compared with the wild type (two-way ANOVA, *P < 0.05, **P < 0.01, ***P < 0.001). Different letters in (d) indicate significant differences between treatments within a genotype (Student's t-test, P < 0.05).

cDNAs of TGA2, TGA5 and TGA6 were placed under the control of the Cauliflower mosaic virus (CaMV) 35S promoter and transformed into the tga256 mutant. All three factors were able to support the activation of PDF1.2 expression in the presence of JA and ACC (Figure 7b). Western blot analysis showed that lines with similar levels of different TGA proteins yielded different levels of PDF1.2 transcript (Figure 7c). TGA5 revealed the highest capacity to promote transcription. In these lines, ORA59 expression was constitutively enhanced (Figure 7d). However, this did not lead to significantly elevated PDF1.2 transcript levels in the absence of JA and ACC, suggesting that TGA2 or TGA6 might be necessary to support the activating capacity of ORA59. In the presence of JA/ACC, a hyperinduction of PDF1.2, which was similar to the hyperinduction in the jin1-1 mutant, was observed. Apparently, TGA5 is sufficient to support this effect.

DISCUSSION

TGA transcription factors TGA2, TGA5 and TGA6 are essential regulators of the SA-dependent defense response SAR (Zhang et al., 2003). Moreover, they are crucial for the activation of a detoxification pathway upon chemical stress (Fode et al., 2008; Mueller et al., 2008). This study assigns another function to these factors: they are indispensable for the induction of JA-inducible genes like PDF1.2 and b-Chi under conditions of increased ET levels, and contribute to the defense against the necrotrophic pathogen B. cinerea. Moreover, genetic evidence suggests that this activity is negatively modulated by SA.

TGA factors are required to antagonize the strong negative effect of AtMYC2

Up to now, the two transcriptional activators ERF1 and ORA59 have been described as integrators of the signaling events elicited by JA and ET. Both factors belong to the family of AP2/ERF domain proteins, and their transcript levels are synergistically activated by JA and ET (Lorenzo et al., 2003; Pre et al., 2008). The role of TGA factors as essential activators of PDF1.2 expression has not been described before. However, TGA factors are only necessary for PDF1.2 expression when increased ET levels come into play, e.g. under conditions of infection with B. cinerea and P. syringae, simultaneous application of JA and ACC to MS-grown plantlets, and ACC treatment of soil-grown plants (Figures 1 and 2). A similar modulating effect of signaling cascades by ET has been recently reported with respect to the NPR1 dependency of the SA–JA crosstalk (Leon-Reyes et al., 2009). Whereas the suppression of JA-induced PDF1.2 expression by SA depends on NPR1, NPR1 is not required when ET levels are elevated.

As revealed by the tga256 jin1 quadruple mutant, TGA factors are dispensable for PDF1.2 induction when the negative regulator AtMYC2 is mutated (Figure 5a). In this mutant, transcript levels of ORA59 are elevated, apparently compensating for the lack of TGA factors (Figure 5c). We propose that TGA factors and AtMYC2 act as antagonistic modulators of the JA/ET pathway, with ERF1 and ORA59 functioning as JA/ET-induced core regulators (Figure 8). TGA factors and AtMYC2 act at different levels (Figure 5b,c). AtMYC2 has an as yet unexplored negative effect on the expression of the ORA59 gene that acts upstream of PDF1.2. In contrast, TGA factors do not influence ORA59 transcript levels after JA/ACC treatment (Figure 4b). Still, they do not function directly at the PDF1.2 promoter, as the only TGACG binding site within this promoter can be deleted without affecting promoter activity (Figure 3). Thus, it seems more likely that TGA factors act indirectly by regulating transcription of a yet unknown protein that controls PDF1.2 promoter activity. Given that more than 24 h are needed until the synergistic effect of JA and ACC can be observed (Figure 4a), it seems plausible that TGA-dependent

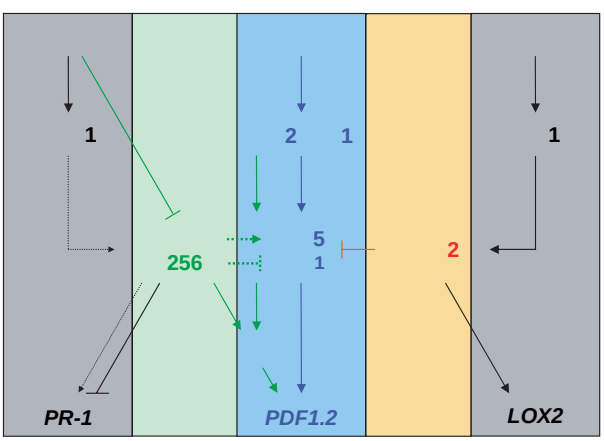

Figure 8. Schematic representation of the role of AtMYC2 and TGA factors for the regulation of PDF1.2 promoter activity.
Under conditions of elevated levels of jasmonic acid (JA) and ethylene (ET), the expression of the two AP2 transcription factors ORA59 and ERF1, which are activators of PDF1.2 expression (Lorenzo et al., 2004; Pre et al., 2008), is induced through an COI1/EIN2-dependent process (blue box; Penninckx et al., 1998). ORA59 and, to a lesser extent, ERF1 expression is repressed by AtMYC2, which is transcriptionally induced by elevated JA levels, and positively regulates genes like LOX2 (grey box on the right; Lorenzo et al., 2004). TGA factors counteract the negative effect of AtMYC2 by promoting PDF1.2 expression (green arrows) through regulation of an unknown target gene (represented by X). TGA factors might synergistically interact with signaling components activated by the ET-signaling cascade. Their positive influence on ORA59 expression (dashed arrow) and their negative influence on ERF1 expression (dashed line) is of lesser importance. As deduced from the salicylic acid (SA) insensitivity of JA/ET-induced PDF1.2 expression in the absence of TGA256 and AtMYC2, we speculate that the positive function of TGA factors with respect to the JA/ET pathway is abolished in the presence of SA. As the SA–JA/ET antagonism is independent from NPR1 (Leon-Reyes et al., 2009), we assume that SA regulates the activity of TGA factors in this context through a yet unexplored mechanism. At the same time, TGA2 represses basal levels of PR-1 in the absence of SA (Rochon et al., 2006; Kesarwani et al., 2007). This inhibitory effect is turned into a positive effect in the presence of SA through NPR1 (Cao et al., 1997) (left grey box). As the activating role of the three TGA factors is not observed under all conditions (Blanco et al., 2009), we used dashed lines for this pathway.

ET-induced transcriptional changes have to occur to ensure the efficient induction of PDF1.2. None of the three TGA factors was transcriptionally induced by JA/ACC treatment (Figure S5), and it might well be that they act synergistically with transcriptional regulators connected to the ET pathway. A major goal of the future is to find the direct target genes of class-II TGA factors that are necessary for PDF1.2 expression in the presence of elevated ET levels.

In addition to controlling this yet unknown regulator of PDF1.2 expression, TGA factors can contribute to the activation of the ORA59 promoter. This becomes evident after infection with B. cinerea, when ORA59 transcript levels are activated to a higher degree than after a single application of the hormones (Figure S3). TGA factors are involved in this high level of expression, as revealed by twofold lower PDF1.2 transcript levels in the tga256 mutant (Figure 4d). In addition, high ORA59 transcription in the jin1-1 mutant partially depends on class-II TGA factors (Figure 5c). Consistently, plants expressing high levels of TGA5 show an increased activation of ORA59 (Figure 7d). As the ORA59 promoter contains a TGACG motif, it might well be that it represents one of the direct target promoters of TGA5.

TGA factors are required to mediate the SA–JA/ET crosstalk in the jin1-1 mutant background

In wild-type Arabidopsis plants, SA has a strong negative effect on JA/ET-induced PDF1.2 expression. As deletion of the TGA256 genes also has a negative effect, one might speculate that SA modulates the activity of TGA factors in the context of the JA/ET network. Evidence in favor of this hypothesis was obtained by analysis of the impact of either SA treatment or deletion of the TGA256 alleles in the jin1-1 mutant background (Figure 5b). High induction of PDF1.2 in the jin1-1 mutant was reduced to the same level, irrespective of whether SA was applied or whether the jin1-1 allele was crossed into the tga256 mutant background. Consistently, the SA–JA/ET crosstalk was abolished in the tga256 jin1 mutant. Although we cannot exclude that TGA factors are only important for the SA–JA/ET crosstalk in the jin1-1 mutant background, we propose that the modulation of the JA/ET pathway by TGA factors serves to install the SA sensitivity in wild-type plants. As ORA59 transcript levels are only slightly affected by either SA or the tga256 alleles (Figure 5c), the SA–JA/ET crosstalk most likely targets a yet unknown direct target of the TGA factors. One possible scenario is that TGA factors repress transcription of ERFs with an ERF-associated amphiphilic repression (EAR) domain that negatively regulate transcription. These EAR-ERFs might displace ORA59 of the PDF1.2 promoter. Previously, AtERF4 has been suggested as a mediator of SA–JA/ET crosstalk (McGrath et al., 2005). Either mutation or SA-mediated inhibition of TGA factors would lead to the constitutive expression of these EAR-ERFs, which would block PDF1.2 expression. Results obtained with transgenic plants expressing ORA59 under the control of an estradiol-inducible promoter are consistent with this idea. In these plants, PDF1.2 expression can be triggered in the absence of the hormones by estradiol-induced elevation of ORA59 transcript levels, and this activation can be suppressed by SA (Figure 6).

How the activity of TGA factors in the context of the JA/ET pathway is influenced by SA is not known. NPR1, which confers the SA sensitivity on the PR-1 promoter, is not involved in the crosstalk (Leon-Reyes et al., 2009). We have previously presented evidence that the SA-induced glutaredoxin GRX480, which interacts with TGA factors, might be involved in the suppression of PDF1.2 expression by SA (Ndamukong et al., 2007). However, as GRX480 is induced efficiently by SA only in the presence of NPR1, we consider it unlikely that it plays a major role in the NPR1-independent SA–JA/ET crosstalk described here.

In conclusion, we have shown that TGA factors, which are essential for the SA-dependent establishment of SAR, play a pivotal role in the activation of the JA/ET pathway, both after pathogen infection and hormone treatment. Under these conditions, they serve to counteract the repressing activity of AtMYC2 in an SA-dependent manner.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions and chemical treatments

Arabidopsis thaliana (accession Columbia, Col-0) was used. Mutants [tga25, tga6, tga256 (Zhang et al., 2003) and jin1-1 (Berger et al., 1996)] were obtained from Y. Zhang (University of British Columbia, Vancouver, Canada) and from S. Berger (Julius-Maximilians University, Würzburg, Germany), respectively. Line XVE-ORA59 (Pre et al., 2008) was obtained from J. Memelink (University of Leiden, the Netherlands). Plants were vertically grown under controlled environmental conditions (21/19 C, 16-h light/8-h dark cycle, 60% relative humidity) on agar plates containing MS medium. After 12 days, plants were transferred to MS plates containing 0.01% ethanol or 50 μM MeJA (Sigma-Aldrich, http://www.sigmaaldrich.com) with or without 200 μM SA (Merck, http://www.merck.com). For ET induction, plants were sprayed after transfer with 0.5 mM of the ethylene precursor ACC (Sigma-Aldrich). Plants grown for 4 weeks in soil (21/19 C, 16-h light/8-h dark cycle, 60% relative humidity) were sprayed with 0.5 mM ACC. JA induction of 4-week-old soil-grown plants was performed in closed glass boxes, where 4.5 μM MeJA was applied via the gaseous phase. Plant material was harvested after 48 and 8 h, respectively. XVE-ORA59 plants were grown as described above and transferred to MS plates containing 10 or 20 nM b-estradiol (Sigma-Aldrich) with or without 200 μM SA. Unless indicated otherwise, plant material was harvested after 48 h.

Pathogen infections

For B. cinerea infection experiments, wild-type and tga256 mutant plants were grown on a pasteurized soil mix of humus/perlite (3 : 1) under controlled environmental conditions (20–22/16–18 C, 12-h light/12-h dark cycle, 60–70% relative humidity). The B. cinerea strain BMM, provided by Brigitte Mauch-Mani (University of Neuchâtel, Switzerland), was grown on potato dextrose agar (Merck). After

harvesting the spores and subsequent filtration through glass wool to remove hyphae, the spores were diluted in quarter-strength potato dextrose broth. For assessment of symptom development, droplets of 5 µl of spore suspension (5×10^4 spores ml^{-1}) were deposited on leaves of 4-week-old plants. The diameter of the lesions was measured after 3 days. PDF1.2 expression analysis was performed with plants that had been spray-inoculated for 4 days (2×10^5 spores ml^{-1}). Quarter-strength potato dextrose broth served as the mock for drop and spray inoculations of control plants.

For infection of wild-type and tga256 mutant plants with P. syringae pv. maculicola ES4326, plants were grown on soil for 5 weeks under controlled environmental conditions (20–22/16–18 C, 8-h light/16-h dark cycle, 60–70% relative humidity). Infections were achieved using dip inoculation with a bacterial culture diluted to 0.02 OD_{600} in 10 mM $MgCl_2$, 0.02% (v/v) Silwet L-77 (OSi Specialties, Crompton, http://www.crompton-instruments.com) (Laurie-Berry et al., 2006). PDF1.2 expression analysis was carried out at 1 dpi.

Genetic analysis

The tga256 mutant was crossed into the jin1-1 background. The F_1 progeny was allowed to self-fertilize and the resulting F_2 generation was screened for homozygosity. The tga2-1 tga5-1 allele results from a deletion induced by fast-neutron bombardment (Zhang et al., 2003), and was detected using primers P1, P2 and P3 (for primer sequences see Table S1). The tga6-1 allele was confirmed by PCR with primers P4 and P5. In addition, quantitative real-time RT-PCR analysis was performed to confirm the lack of expression of TGA2, TGA5 and TGA6 in the respective mutants. Primers P6, P7 and P8 were used to detect the mutant jin1-1 allele. Homozygosity was further confirmed by northern blot analysis, which allows detection of the truncated jin1-1 transcript.

Binary vectors and plant transformation

Gateway technology (Invitrogen, http://www.invitrogen.com) was used to generate binary vectors for analysis of the PDF1.2 promoter. The promoter region from bp positions)959 to)1 relative to the predicted transcriptional start site of the PDF1.2 (At5g44420) gene was amplified using primers P9 and P10 (for primer sequences see Table S1), which add GATEWAY recombination sites to the promoter fragment. Genomic DNA extracted from Col-0 plants using the DNeasy Plant Mini Kit (Qiagen, http://www.qiagen.com) was used as a template. The fragment was inserted into pDONR223 (Invitrogen), resulting in pDONR223/PDF1.2-Pro. Mutation of the TGACG motif at position bps –397 to –393 to TTTTT was achieved by PCR using primer pairs P11/P12 and P13/14, and pDONR223/PDF1.2-Pro as a template, resulting in two fragments that subsequently served as templates for overlapping PCR with primers P11 and P13. Both promoter fragments were recombined upstream of the GUS gene in the binary vector pBGWFS7 (http://www.psb.ugent.be/gateway).

The coding region of TGA5 and TGA6 was amplified from cDNA fragments with primer pairs P15/P16 and P17/P18, and was cloned into the pDONR223 vectors (Invitrogen). The resulting pDONR223/TGA5, pDONR223/TGA6 and pDONR201/TGA2 were incubated with the binary destination vector pB2GW7 (http://www.psb.ugent.be/gateway), thus placing them under the control of the CaMV 35S promoter.

To generate transgenic plants the binary plasmids were electroporated (GenePulser II; Bio-Rad, http://www.bio-rad.com) into A. tumefaciens strain GV3101 (pMP90). These agrobacteria were used to transform Col-0 plants with pGBWFS7 derivatives for the promoter analysis and to transform tga256 mutant plants for ectopic expression of TGA2, TGA5 and TGA6 (Clough and Bent, 1998). Transgenic lines expressing TGA2, TGA5 or TGA6 were identified by protein gel blot analysis using the aTGA25 antiserum (Fode et al., 2008).

Quantitative GUS assay

Crude protein extracts were prepared from at least 50 seedlings from 14 individual T_2 transformants. Growth conditions and hormone treatments were as described above. Quantitative GUS assays using 4-methyl-umbelliferyl-b-D-glucuronide (Sigma-Aldrich) as a substrate was performed in microtitre plates (Jefferson et al., 1987). The released fluorescence was measured with a Cyto Fluor Series 4000 plate reader (Perspektive Biosystems, Hertford, Hertfordshire, UK). The total quantity of protein was determined using a commercial Bradford assay solution (Carl Roth, http://www.carlroth.com).

Quantitative real-time RT-PCR analysis

RNA extraction and real-time RT-PCR analysis was performed as described by Fode et al. (2008). Calculations were made according to the 2_T^{DC} method (Livak and Schmittgen, 2001). UBQ5 served as a reference (Kesarwani et al., 2007). Primers used to amplify and quantify the cDNA are indicated in Table S1 [PDF1.2 (At5g44420), VSP2 (At5g24770), UBQ5 (At3g62250)]. QuantiTect Primers to amplify mRNA for TGA2 (At5g06950), TGA5 (At5g06960), TGA6 (At3g12250), b-CHI (At3g12500), LOX2 (At3g45140), ORA59 (At1g06160) and ERF1 (At3g23240) were obtained from Qiagen.

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SUPPORTING INFORMATION

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

Figure S1. Expression of b-CHI and VSP2 in wild-type and tga256 mutant plants after treatment with jasmonic acid (JA), 1-aminocyclopropane-1-carboxylic acid (ACC), or JA and ACC.

Figure S2. Expression of $PDF1.2_{(TGACG)}$:GUS and $PDF1.2_{(TTTTT)}$:GUS in independent plant lines after treatment with jasmonic acid (JA) and 1-aminocyclopropane-1-carboxylic acid (ACC).

Figure S3. Comparison between the relative ORA59 transcript levels in wild-type plants after infection with Botrytis cinerea or treatment with jasmonic acid (JA) and 1-aminocyclopropane-1-carboxylic acid (ACC).

Figure S4. Expression of PDF1.2, ORA59 and ERF1 in wild-type and tga256 jin1 mutant plants after treatment with jasmonic acid (JA)/1-aminocyclopropane-1-carboxylic acid (ACC) and salicylic acid (SA)/JA/ACC.

Figure S5. Time course of TGA2, TGA5 and TGA6 expression after treatment with jasmonic acid (JA) and 1-aminocyclopropane-1-carboxylic acid (ACC).

Table S1. List of primers used for genotyping, cloning and real-time RT-PCR analysis.

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