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Assessing the Role of ETHYLENE RESPONSE FACTOR Transcriptional Repressors in Salicylic Acid-Mediated Suppression of Jasmonic Acid-Responsive Genes

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Salicylic acid (SA) and jasmonic acid (JA) cross-communicate in the plant immune signaling network to finely regulate induced defenses. In Arabidopsis, SA antagonizes many JA-responsive genes, partly by targeting the ETHYLENE RESPONSE FACTOR (ERF)-type transcriptional activator ORA59. Members of the ERF transcription factor family typically bind to GCC-box motifs in the promoters of JA- and ethylene-responsive genes, thereby positively or negatively regulating their expression. The GCC-box motif is sufficient for SA-mediated suppression of JA-responsive gene expression. Here, we investigated whether SA-induced ERF-type transcriptional repressors, which may compete with JA-induced ERF-type activators for binding at the GCC-box, play a role in SA/JA antagonism. We selected ERFs that are transcriptionally induced by SA and/or possess an EAR transcriptional repressor motif. Several of the 16 ERFs tested suppressed JA-dependent gene expression, as revealed by enhanced JA-induced *PDF1.2* or *VSP2* expression levels in the corresponding *erf* mutants, while others were involved in activation of these genes. However, SA could antagonize JA-induced *PDF1.2* or *VSP2* in all *erf* mutants, suggesting that the tested ERF transcriptional repressors are not required for SA/JA cross-talk. Moreover, a mutant in the co-repressor TOPLESS, that showed reduction in repression of JA signaling, still displayed SA-mediated antagonism of *PDF1.2* and *VSP2*. Collectively, these results suggest that SA-regulated ERF transcriptional repressors are not essential for antagonism of JA-responsive gene expression by SA. We further show that de novo SA-induced protein synthesis is required for suppression of JA-induced *PDF1.2*, pointing to SA-stimulated production of an as yet unknown protein that suppresses JA-induced transcription.

Keywords: *Arabidopsis thaliana* • ERF transcription factors • Hormone cross-talk • Jasmonic acid • Salicylic acid • TOPLESS.

Abbreviations: ANOVA, analysis of variance; AP2, APETALA2; BTH, benzo-(1,2,3)-thiadiazole-7-carbothioic acid; CHX, cycloheximide; COI1, CORONATINE INSENSITIVE1; ERF, ETHYLENE RESPONSE FACTOR; JA, jasmonic acid; JAZ, JASMONATE ZIM-domain; MeJA, methyl jasmonate; MS, Murashige and Skoog; ORA59, OCTADECANOID-RESPONSIVE ARABIDOPSIS59; qRT–PCR, quantitative reverse transcription–PCR; SA, salicylic acid; TPL, TOPLESS.

Introduction

Plants intimately interact with a broad range of microbial pathogens and insect herbivores. To respond to this diversity of enemies, plants possess a highly sophisticated defense system in which the plant hormones salicylic acid (SA) and jasmonic acid (JA) play important regulatory roles. Other hormones, such as ethylene, ABA, gibberellins, auxins and cytokinins, also have an effect on plant immunity, often via the modulation of the SA and JA signaling pathways (Robert-Seilaniantz et al. 2011, Pieterse et al. 2012, Broekgaarden et al. 2015). Although there are exceptions, SA-dependent defenses are generally considered to act against pathogens with a biotrophic lifestyle, whereas JA-dependent responses are often associated with defense against necrotrophic pathogens and herbivorous insects (Pieterse et al. 2012). In response to different types or combinations of attackers, the plant produces specific blends of hormones that differ in composition, quantity and timing, which is instrumental in fine-tuning the induced defense response against the invading attacker (De Vos et al. 2005).

Cross-communication between the SA and JA signaling pathways emerged as an important mechanism by which plants steer their induced defense responses and may reduce defense-associated fitness costs (Pieterse et al. 2012, Vos et al. 2013, Vos et al. 2015). Transcriptome profiling studies revealed extensive interplay between the two pathways, with

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antagonistic effects of SA on JA-responsive gene expression being most prominent (Glazebrook et al. 2003, Van Verk et al. 2011, Proietti et al. 2013, Van der Does et al. 2013). In *Arabidopsis thaliana* (Arabidopsis), activation of the SA pathway suppresses a large set of JA-responsive genes, including the JA marker genes *PLANT DEFENSIN1.2* (*PDF1.2*) and *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) (Van Wees et al. 1999, Van der Does et al. 2013). Consequently, activation of the SA pathway diminishes JA-dependent defenses against necrotrophic pathogens and insect herbivores (reviewed in Pieterse et al. 2012).

In order to study the mechanisms underlying the antagonistic effect of SA on JA-responsive gene expression (hereafter also referred to as SA/JA cross-talk), knowledge of the JA pathway that accumulated over the past recent years is highly instrumental. The F-box protein CORONATINE INSENSITIVE1 (COI1) was found to be an indispensable component of the JA signaling pathway (Devoto et al. 2002). As part of the E3 ubiquitin-ligase Skip–Cullin–F-box complex SCF^{COI1}, COI1 interacts with JASMONATE ZIM-domain (JAZ) proteins to form a complex that functions as a receptor for JA-Ile, the most bioactive derivative of JA (Fonseca et al. 2009). Binding of JA-Ile to the JAZ–COI1 receptor complex leads to degradation of JAZ via the proteasome, resulting in the onset of the JA response (Chini et al. 2007, Thines et al. 2007). In the absence of JAs, JAZ proteins act as transcriptional repressors of JA-responsive genes by binding to positive transcriptional regulators, such as MYC2, 3 and 4 (Chini et al. 2007, Fernández-Calvo et al. 2011), and ETHYLENE INSENSITIVE3 (EIN3) and EIN3-LIKE1 (EIL1) (Zhu et al. 2011). To prevent activity of their bound transcription factors, JAZ proteins recruit the general co-repressor TOPLESS (TPL) and TOPLESS-Related (TPR) proteins either directly if they contain an EAR-motif or indirectly via the adaptor protein NINJA that contains an EAR-motif (Pauwels et al. 2010, Shyu et al. 2012). In JA-stimulated cells, degradation of JAZ proteins results in the release of transcription factors, leading to activation of a large set of JA-responsive genes, including the JA marker gene *VSP2* and genes encoding APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factors, such as ERF1 and OCTADECANOID-RESPONSIVE ARABIDOPSIS59 (ORA59) that lead to activation of the JA marker gene *PDF1.2* (Lorenzo et al. 2003, Pré et al. 2008). Besides transcription factors that act positively on transcription of JA-responsive genes there are also numerous repressive transcription factors that can inhibit transcription of JA-inducible genes such as *VSP2* and *PDF1.2* (McGrath et al. 2005, Nakata et al. 2013, Caarls et al. 2015).

In Arabidopsis, significant progress has been made in the identification of targets in the JA pathway via which SA exerts its antagonistic effect (Pieterse et al. 2012, Caarls et al. 2015). SA has been shown to suppress the JA pathway downstream of JA biosynthesis and the JAZ–COI1 complex, suggesting that SA antagonizes JA signaling at the level of transcriptional regulation (Leon-Reyes et al. 2010b, Van der Does et al. 2013). Whole-genome expression profiling revealed that the GCC-box motif (AGCCGCC) is an important promoter element in JA-responsive genes that are sensitive to suppression by SA (Van der Does et al. 2013). Using a synthetic GCC-box-containing promoter fused to a reporter gene, the GCC-box was demonstrated to be sufficient for SA-mediated suppression of JA-induced expression (Van der Does et al. 2013). The GCC-box is a binding site for members of the ERF family of AP2/ERF transcription factors (Hao et al. 1998), which comprises 122 members in Arabidopsis (Nakano et al. 2006) and has been grouped into the DREB and the ERF subfamilies based on homology in DNA-binding domains (Sakuma et al. 2002). The ERF transcription factor ORA59, which binds to the GCC-box and acts as an activator of *PDF1.2* (Pré et al. 2008, Zarei et al. 2011), was then shown to be a target of SA, as both *ORA59* transcription and ORA59 protein accumulation were affected by SA (Van der Does et al. 2013, Zander et al. 2014).

Besides transcriptional activators, the ERF family of transcription factors also harbors transcriptional repressors that act at the GCC-box *cis*-regulatory element (Fujimoto et al. 2000, McGrath et al. 2005, Yang et al. 2005, Huang et al. 2015). In Arabidopsis, 14 of the 122 members of the ERF subfamily of AP2/ERF transcription factors contain an EAR-motif (Nakano et al. 2006, Ohta et al. 2001). This motif interacts with general co-repressors such as TPL, which is involved in repression of genes that are responsive to JA and auxin (Szemenyei et al. 2008, Pauwels et al. 2010, Kagale and Rozwadowski 2011). In addition, ERFs that lack an EAR-motif can also have a role in transcriptional repression, for example through interaction with a repressor such as SILENCER ELEMENT BINDING FACTOR (SEBP), as was shown for the ERF Pti4 of potato (González-Lamothe et al. 2008), or through activation of a negative regulator of the GCC-box (Caarls et al. 2015). Several *ERF* genes are SA inducible (Krishnaswamy et al. 2011). This led us to hypothesize that induction by SA of ERF repressors that act at the GCC-box could contribute to the antagonistic effect of SA on JA-responsive gene expression. To test this hypothesis, we selected and tested 16 loss-of-function *erf* mutants for their ability to display SA-mediated suppression of *PDF1.2* and *VSP2* expression. Moreover, we analyzed whether the *tpl-1* mutant is impaired in SA/JA cross-talk. By using the protein synthesis inhibitor cycloheximide (CHX), we also tested whether antagonism by SA requires de novo protein synthesis. Together, our results suggest that although de novo synthesis of an as yet unknown protein is required for SA-mediated suppression of JA-responsive gene expression, a role for SA-induced ERF repressor proteins in the antagonism between SA and JA signaling is unlikely.

Results

De novo protein synthesis is required for SA/JA cross-talk

If our hypothesis that JA-induced gene expression can be antagonized by SA-induced ERF transcriptional repressors is correct, then novel protein synthesis of these ERFs upon SA treatment is expected to be prerequisite for SA/JA cross-talk. To investigate this, the effect of the protein synthesis inhibitor CHX on the expression of JA-inducible *PDF1.2* was determined in

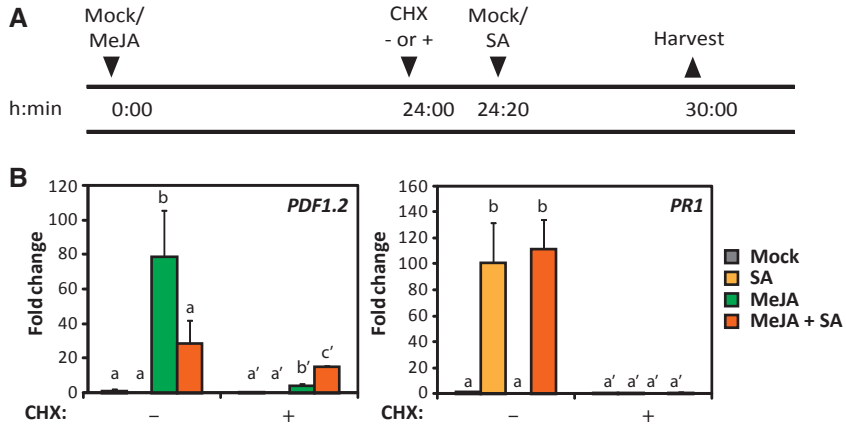

Fig. 1 SA/JA cross-talk requires de novo protein synthesis. (A) Time line of different treatments. Five-week-old wild-type Col-0 plants were treated with 0.1 mM MeJA or a mock solution; CHX was applied 24 h later and, after a subsequent 20 min, 0.5 mM SA or a mock solution were applied. At 6 h after CHX treatment, plant material was harvested for gene expression analysis. (B) qRT–PCR analysis of *PDF1.2* and *PR1* gene expression in Col-0 plants mock treated or treated with MeJA, SA or with MeJA first and then SA. All hormone treatments were combined without CHX (mock) or with CHX treatment. Fold change is relative to the expression in mock-treated plants without CHX, and normalized to the reference gene *At1g13320*. Shown are the averages of three independent biological replicates; error bars indicate the SD. Different letters indicate statistically significant differences between hormone treatments for each CHX treatment (ANOVA, Holm–Sidak post-hoc test; $P < 0.05$). –, without CHX; +, with CHX. The experiment was repeated with similar results.

Arabidopsis Col-0 wild-type plants after treatment with methyl jasmonate (MeJA), SA or a combination of both. *PDF1.2* was chosen as a read-out because its promoter region harbors two GCC-boxes that the ERF suppressors could potentially target. Plants were first dipped in a MeJA solution, treated with CHX 24 h later and treated with SA a further 20 min later (**Fig. 1A**). At 6 h after SA treatment, leaves were harvested for gene expression analysis. **Fig. 1B** shows that in the absence of CHX, *PDF1.2* expression was induced by MeJA, and subsequent treatment with SA suppressed this induction by >2-fold. This confirms previous findings that SA antagonizes JA-induced *PDF1.2*, even when SA is applied after the induction of the JA response (Koornneef et al. 2008). Application of CHX 24 h after MeJA treatment strongly reduced *PDF1.2* mRNA accumulation in comparison with plants that were not treated with CHX, showing that de novo protein synthesis is important for activation of *PDF1.2* expression by MeJA (**Fig. 1B**). However, a statistically significant 4-fold induction of *PDF1.2* expression was still detectable in MeJA/CHX-treated plants, which was probably mediated by the residual pool of transcriptional activators that was already present before the CHX treatment. Importantly, the level of MeJA-induced *PDF1.2* mRNAs that remained after the CHX treatment was no longer reduced by SA if CHX was present, and instead was even higher in the SA/JA combination treatment than in the MeJA single treatment (**Fig. 1B**). In plants that received no CHX, SA activated the expression of the SA-responsive marker gene *PR1* in both the absence and the presence of MeJA (**Fig. 1B**). CHX treatment completely abolished the induction of *PR1* by SA, confirming previous findings (Uquillas et al. 2004). Together, these results indicate that, similar to the SA-induced expression of *PR1*, de novo protein synthesis is required for the SA-mediated suppression of JA-induced *PDF1.2* expression.

SA signaling induces *ERF* transcription factor genes

Previously, we showed that the GCC-box promoter element is sufficient for SA-mediated suppression of JA-induced gene expression (Van der Does et al. 2013). The GCC-box is a binding site for ERF-type transcription factors (Hao et al. 1998), some of which can act as transcriptional repressors. Here, we tested if putative SA-inducible ERF transcriptional repressors could play a role in SA/JA cross-talk. First, we analyzed SA responsiveness of the genes encoding the 122 members of the ERF family, which belongs to the AP2/ERF superfamily of transcription factors (Sakuma et al. 2002, Nakano et al. 2006). To this end, we mined publicly available data on gene expression in Arabidopsis plants treated with SA, the SA analog benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) or the SA-inducing biotrophic pathogen *Hyaloperonospora arabidopsidis* (**Supplementary Table S1**; Atallah 2005, Wang et al. 2006, Krinke et al. 2007, Goda et al. 2008, Blanco et al. 2009, Huibers et al. 2009). Additionally, we analyzed the expression pattern of the 122 *ERF* genes by RNA sequencing of a high-density time series (14 time points) of SA-treated Arabidopsis plants (**Supplementary Table S1**, rightmost column). We selected putative SA-inducible ERF repressors in the following manner. First, the *ERF* genes whose expression was induced by one or more SA inducers [fold change > 1.5 (public data sets) or $P < 0.05$ (RNA-seq data)] in at least three different data sets were selected for further study (marked in blue in **Supplementary Table S1**). This group comprised the following 10 ERF transcription factor genes: *AtERF-1*, *CEJ1/DEAR1*, *DREB2A*, *ERF1*, *ERF2*, *ERF11*, *ERF13*, *ERF112*, *RAP2.6* and *RAP2.6L*. Then, two additional *ERF* genes were selected as interesting candidates, even though their expression was induced in only one or two of the data sets (marked in orange in **Supplementary Table S1**): *ERF5* was reported to be

Table 1 Expression of selected *ERF* genes in response to SA and/or MeJA treatment

	AGI code	ERF#[a]	Alternative name	Group[b]	Group[c]	EAR-motif[d]	Fold change expression after treatment (2 h)			Mutant line[e]
							SA	MeJA	SA + MeJA	
1	AT4G17500	AtERF#100	AtERF-1	IXa	B-3		2.11	1.24	3.77	Rioja *et al.* (2013)
2	AT5G05410	AtERF#045	DREB2A	IVa	A-2		2.26	0.72	5.30	SAIL_365_F10
3	AT3G50260	AtERF#011	CEJ1/DEAR1	IIa	A-5	CMII-2 EAR	1.84	0.76	2.23	FLAG_293H04
4	AT3G23240	AtERF#092	ERF1	IXc	B-3		2.60	0.71	2.38	See the M&M
5	AT1G50640	AtERF#082	ERF3	VIIIa	B-1	CMVIII-1 EAR	2.34	0.98	2.02	Not available
6	AT1G03800	AtERF#077	ERF10	VIIIa	B-1	CMVIII-1 EAR	1.71	1.76	2.07	SAIL_95_A08
7	AT1G28370	AtERF#076	ERF11	VIIIa	B-1	CMVIII-1 EAR	2.31	0.71	3.31	SALK_116053
8	AT1G28360	AtERF#081	ERF12	VIIIa	B-1	CMVIII-1 EAR	1.07	0.71	1.54	SAIL_873_D11
9	AT2G44840	AtERF#099	ERF13	IXa	B-3		2.69	4.47	11.50	GK_121A12
10	AT5G61600	AtERF#104	ERF104	IXb	B-3		1.63	0.57	2.05	Bethke *et al.* (2009)
11	AT2G33710	AtERF#112	ERF112	Xc	B-4		2.84	1.00	3.40	GK_604D02
12	AT1G43160	AtERF#108	RAP2.6	Xa	B-4		0.71	13.03	25.44	SAIL_1225_G09
13	AT5G13330	AtERF#113	RAP2.6L	Xa	B-4		3.15	3.10	9.88	SALK_051006
14	AT5g47220	AtERF#101	ERF2	IXa	B-3		1.30	0.65	1.13	Not selected
15	AT3G15210	AtERF#078	ERF4	VIIIa	B-1	CMVIII-1 EAR	0.87	1.17	1.29	McGrath *et al.* (2005)
16	AT5G47230	AtERF#102	ERF5	IXb	B-3		1.10	0.52	1.02	Son *et al.* (2012)
17	AT3G20310	AtERF#083	ERF7	VIIIa	B-1	CMVIII-1 EAR	1.33	0.88	1.43	Not available
18	AT1G53170	AtERF#079	ERF8	VIIIa	B-1	CMVIII-1 EAR	0.99	0.64	0.47	FLAG_157D10
19	AT5G44210	AtERF#080	ERF9	VIIIa	B-1	CMVIII-1 EAR	1.49	0.34	0.42	SALK_043407C

qRT–PCR analysis of expression of a selection of *ERF* genes in 5-week-old Col-0 plants 2 h post-treatment with 1 mM SA and/or 0.1 mM MeJA. Depicted is the fold change, which is expression in plants after treatment relative to expression in mock-treated plants. Green cells indicate that expression of the gene was induced (fold change > 1.5), while orange cells indicate reduction of gene expression (fold change < 0.67). The experiment was repeated with similar results. The 19 ERFs are divided into groups based on hierarchal clustering done by Nakano et al. (2006) and by Sakuma et al. (2002). CEJ1/DEAR1 and all proteins of group VIIIa/B-1 contain an EAR domain. The mutant lines that are selected are indicated in the rightmost column.

[a]Numbering as introduced by the phylogenetic analysis of Nakano et al. (2006).

[b]Group classification by Nakano et al. (2006).

[c]Group classification based on Sakuma et al. (2002).

[d]The presence of an EAR-domain as described by Nakano et al. (2006). CMVIII-1, (L/F)DLN(L/F)xP; CMII-2, DLNxxP.

[e]Mutants were obtained from (i) the authors of the papers; (ii) the Nottingham Arabidopsis Stock Centre (NASC) for SALK, SAIL and GABI-KAT lines; or (iii) the French National Institute for Agricultural Research (INRA) for FLAG lines.

induced by the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* in an SA-dependent and COI1-independent manner (Chen et al. 2002), and *ERF104* was described to play a role in the regulation of *PDF1.2* expression (Bethke et al. 2009).

Because of their putative function as transcriptional repressors (Ohta et al. 2001), seven additional genes encoding EAR-motif-containing ERFs of the ERF subfamily were selected (Nakano et al. 2006), namely *ERF3*, *ERF4*, *ERF7*, *ERF8*, *ERF9*, *ERF10* and *ERF12* (marked in green in **Supplementary Table S1**). They all showed SA-induced expression in at least one of the data sets. *ERF11* also encodes an EAR-motif-containing ERF, but was already selected because of its induction by SA in three different data sets. In total, out of the 122 members of the ERF family, we selected 19 genes for further study, which are listed in **Table 1**.

Selection of ERFs with a potential role in SA/JA cross-talk

To determine the time frame in which putative SA-activated transcriptional repressors need to act in order to suppress JA-induced gene expression, a time course experiment was performed with Col-0 wild-type plants in which *PDF1.2* and *VSP2* expression levels were determined in response to treatment with SA, MeJA or a combination of SA and MeJA. Even though the JA marker gene *VSP2* is considered to be regulated by basic helix–loop–helix (bHLH) transcription factors that bind to the G-box, and not by ERF transcription factors, its expression was investigated in this study because SA/JA cross-talk of this gene may be indirectly regulated by ERFs via (in)activation of other genes containing a GCC-box. *PR1* expression was taken as a control for SA inducibility. *PDF1.2* and *VSP2* transcripts accumulated at increasing levels between 1 and 6 h after treatment with MeJA (**Fig. 2**). Suppression of MeJA-induced expression of these genes by SA was evident from 2 h onwards, and was most pronounced at 4 and 6 h after treatment. Hence, if SA-induced transcriptional repressors play a role in SA/JA cross-talk, their synthesis is expected to occur within 2–4 h after treatment. Moreover, they are expected to be induced in plants treated with a combination of SA and MeJA. Therefore, we used quantitative reverse

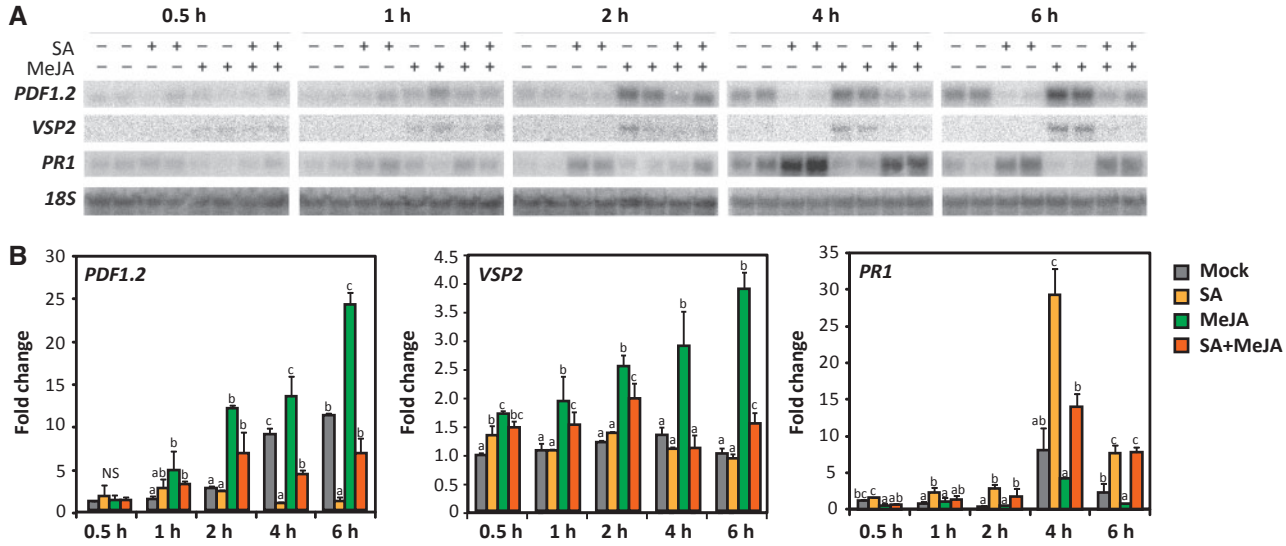

Fig. 2 Timing of SA- and JA-responsive gene expression induced by SA and/or MeJA. (A) RNA gel blot analysis of *PDF1.2*, *VSP2* and *PR1* expression in 5-week-old wild-type Col-0 plants treated with water (Mock), 1 mM SA and/or 0.1 mM MeJA, and harvested at the indicated times post-treatment. Equal loading of RNA samples was checked using a probe for *18S* rRNA. (B) Quantification of *PDF1.2*, *VSP2* and *PR1* expression as shown in (A). Fold change is relative to the expression in plants 0.5 h after mock treatment and normalized to the *18S* rRNA expression values. Shown are the means of two independent biological replicates; error bars indicate the SD. Different letters indicate statistically significant differences between hormone treatments at each time point (two-way ANOVA, Tukey post-hoc test; $P < 0.05$). NS, not significant.

transcription–PCR (qRT–PCR) to analyze the level of expression of the selected *ERF* genes at 2 h after treatment with SA, MeJA or a combination of SA and MeJA.

Table 1 shows that of these 19 *ERF* genes, 13 were induced (fold change > 1.5) within 2 h after application of a combination of SA and MeJA (i.e. *AtERF-1*, *CEJ1/DEAR1*, *DREB2A*, *ERF1*, *ERF3*, *ERF10*, *ERF11*, *ERF12*, *ERF13*, *ERF104*, *ERF112*, *RAP2.6* and *RAP2.6L*). Except for two genes, these *ERF* genes were also induced after application of SA alone. For the remaining selected *ERF* genes, transcription was not induced by any of the treatments (**Table 1**; fold change < 1.5) in this experiment. This group comprises the genes encoding EAR-motif ERFs *ERF4*, *ERF7*, *ERF8* and *ERF9*, which we decided to leave in our selection because of their great potential to act as repressors, and *ERF2* and *ERF5*, which do not code for EAR-motif ERFs. We removed *ERF2* from our selection, but *ERF5* was retained as an interesting candidate. Son et al. (2012) reported a negative effect of ERF5 on defense against the necrotrophic fungus *Alternaria brassicicola*, and a positive effect on SA-dependent gene expression and defense against *P. syringae*, suggesting a role for ERF5 in SA and JA signaling. Finally, we continued with 18 ERFs for further study: 13 *ERF* genes that are induced by treatment with a combination of SA and MeJA, four additional EAR-motif-containing ERFs, and ERF5 (**Table 1**).

Responsiveness to MeJA, SA and SA/JA cross-talk of loss-of-function *erf* mutants

To investigate whether the selected 18 ERFs are involved in SA/JA cross-talk, their respective *erf* knockout mutants were obtained (**Table 1**). No suitable mutants were available for *ERF3* and *ERF7*. The remaining 16 *erf* mutants were analyzed for their ability to display SA/JA cross-talk in comparison with wild-type Col-0 or Ws-0 (Ws-0 was included for *erf8* and *cej1/dear1*). The *erf* mutant *ora59* that is impaired in *PDF1.2* expression was included as a negative control (Zander et al. 2014). In all plants, expression of the JA marker genes *PDF1.2* and *VSP2* and the SA marker gene *PR1* was determined 5 and 24 h after treatment with MeJA, SA or a combination of MeJA with SA. In our experiments, *PDF1.2* expression was most clearly induced by MeJA and suppressed by a combination of MeJA and SA at 24 h after treatment, while for *VSP2* expression these effects were most evident at 5 h after treatment. Therefore, time point 24 h was chosen for analysis of *PDF1.2* expression and time point 5 h for *VSP2* expression.

An enhanced expression level of *PDF1.2* (>2-fold) after MeJA treatment in comparison with wild-type plants was observed in four of the selected *erf* mutants: *erf13*, *dreb2a*, *erf112* and *erf8* (**Fig. 3**; **Supplementary Fig. S1**), suggesting that these four ERFs are potential negative regulators of JA-responsive gene expression. In contrast, in three *erf* mutants, *erf9*, *erf12* and *erf104*, *PDF1.2* expression was reduced compared with Col-0. In *erf9*, *PDF1.2* induction by MeJA was completely compromised, similar to the negative control *ora59* (**Supplementary Fig. S1**). Induction of *VSP2* expression after MeJA treatment was reduced (>2-fold) in the mutants *erf12*, *erf104*, *rap2.6*, *rap2.6L*, *erf11*, *dreb2a* and *erf8* (**Fig. 3**; **Supplementary Fig. S1**), suggesting that the corresponding ERF proteins indirectly contribute to activation of the *VSP2* gene. Enhanced *VSP2* induction levels (>2-fold) upon MeJA treatment were observed in mutants *ora59* and *aterf-1* (**Fig. 3**; **Supplementary Fig. S1**). In conclusion, these results show that the majority of the ERFs we tested seem to be

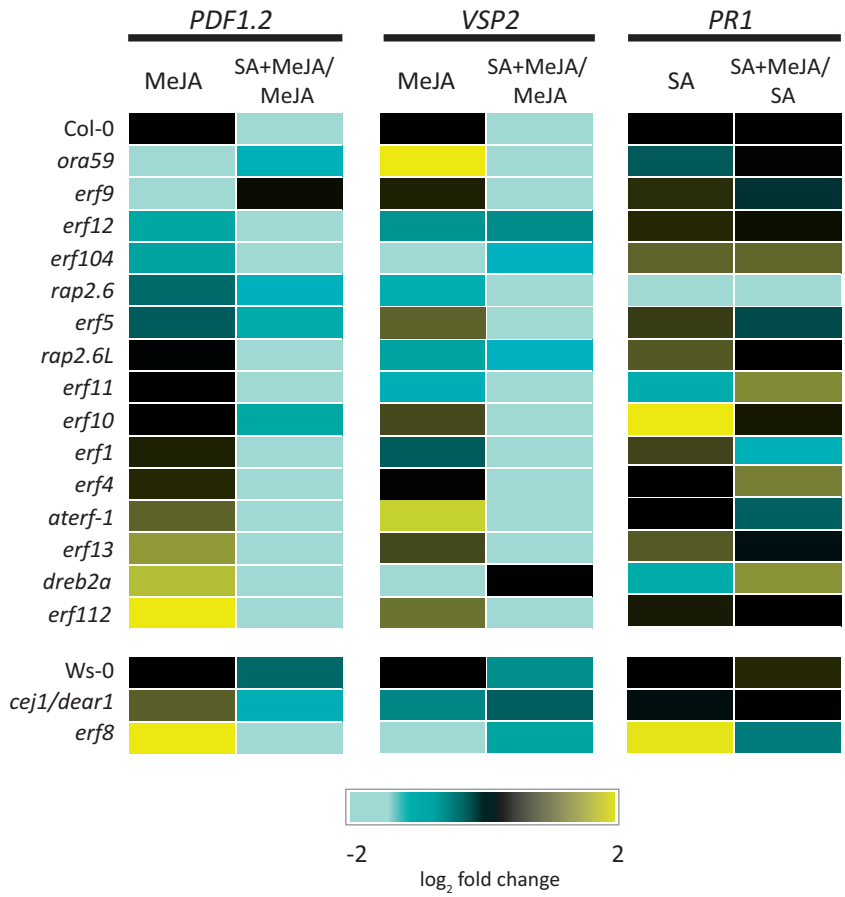

Fig. 3 Relative expression of *PDF1.2*, *VSP2* and *PR1* after MeJA, SA or SA/MeJA treatment in all tested *erf* mutants. Shown is a heat map representation of relative expression values in wild-type plants and all tested mutants at 5 h (for *VSP2*) or 24 h (for *PDF1.2* and *PR1*) after treatment, as determined by qRT–PCR in different experiments (**Supplementary Fig. S1**). Cyan and yellow represent reduced and enhanced expression, respectively, as indicated by the color bar. For each gene, fold change in expression after MeJA treatment (for *PDF1.2* and *VSP2*) or SA treatment (for *PR1*) was divided by the fold change in MeJA-treated wild-type plants of the same experiment (for *PDF1.2* and *VSP2*) or SA-treated wild-type plants of the same experiment (for *PR1*), and then $\log_2$ transformed. Fold change after the combination treatment was divided by the fold change in MeJA-treated or SA-treated plants of the same genotype, and then $\log_2$ transformed. The wild-type expression values depicted are an average of the different experiments, while the depicted values for mutants are from one experiment.

involved in regulating JA-responsive gene expression, either negatively or positively.

Induction of the *PR1* gene upon SA treatment was affected in five of the *erf* mutants (**Fig. 3**; **Supplementary Fig. S1**). In *rap2.6*, *erf11* and *dreb2a*, SA treatment led to a lower level of *PR1* expression than in wild-type Col-0 plants. In *erf8* and *erf10*, *PR1* expression was higher compared with wild-type Ws-0 or Col-0 plants, respectively, suggesting that these ERFs negatively influence SA signaling.

Importantly, in the presence of SA, MeJA-induced *PDF1.2* and *VSP2* expression was strongly suppressed in both wild types and in nearly all of the tested *erf* mutants (**Fig. 3**; **Supplementary Fig. S1**). Even when treatment with MeJA by itself induced enhanced levels of *PDF1.2* or *VSP2*, as was the case in several mutants as described above, the combination with SA evidently reduced the expression to similar levels as in combination-treated wild-type plants. Only in mutants *erf9* and *dreb2a* that already exhibited extremely low levels of *PDF1.2* (*erf9*) or *VSP2* (*dreb2a*) upon single MeJA treatment did the combination with SA not lead to further reduction (**Fig. 3**; **Supplementary Fig. S1**). SA-induced *PR1* expression was not significantly affected by the combination with MeJA, either in wild-type or in the *erf* mutant plants (**Fig. 3**; **Supplementary Fig. S1**), supporting findings of previous studies with various wild-type and mutant Arabidopsis plants (Koornneef et al. 2008, Leon-Reyes et al. 2010a, Van der Does et al. 2013). Because all the tested *erf* mutants are still highly sensitive to SA-mediated suppression of MeJA-induced *PDF1.2* and *VSP2* expression, we must conclude that none of the corresponding ERFs, that had been selected as putative SA-mediated transcriptional repressors of JA signaling, is essential for SA/JA cross-talk of the markers *PDF1.2* and *VSP2*.

SA/JA cross-talk functions independently of TOPLESS

TPL is a general co-repressor that is recruited by numerous repressors and transcription factors that contain an EAR-domain, including NINJA or JAZ, to repress JA-responsive genes in the absence of a JA stimulus. The *tpl-1* mutant exhibits enhanced sensitivity to JA in a root growth inhibition assay

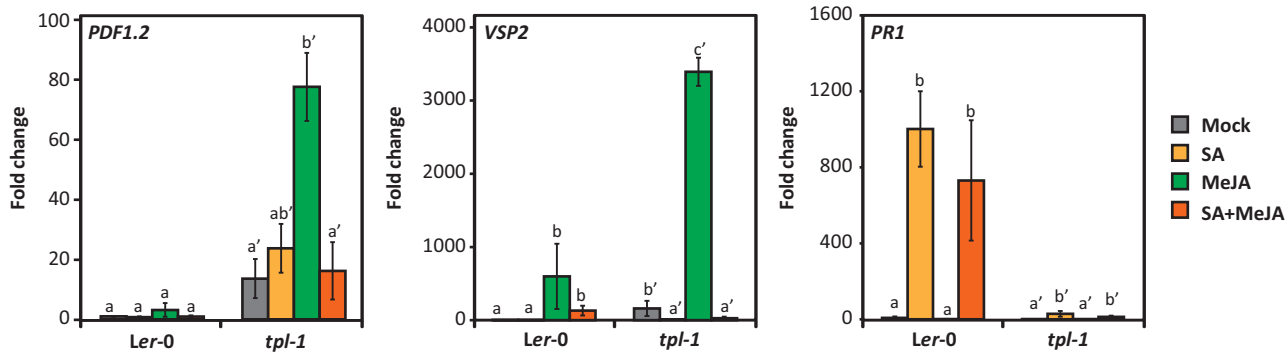

Fig. 4 The co-repressor of JA signaling TPL is not required for SA/JA cross-talk. qRT–PCR analysis of *PDF1.2*, *VSP2* and *PR1* expression in 5-week-old L*er*-0 wild-type and *tpl-1* mutant plants treated with water (Mock), 1 mM SA and/or 0.1 mM MeJA, and harvested at 24 h post-treatment. Shown are the means of three independent biological replicates; error bars indicate the SE. Fold change is relative to the expression in mock-treated wild-type plants and normalized to the reference gene *At1g13320*. Different letters indicate statistically significant differences between the treatments per genotype (two-way ANOVA on ΔCT values, Tukey post-hoc test; $P < 0.05$). The experiment was repeated twice with similar results.

(Pauwels et al. 2010). Several EAR-motif AP2/ERFs can also interact with TPL (Causier et al. 2012). Hence, we hypothesized that TPL, by interaction with different EAR-motif ERFs or with NINJA/JAZs, could play a central role in repression of JA-responsive gene expression by SA. To investigate this, we monitored the expression of *PDF1.2* and *VSP2* in *tpl-1* in response to SA, MeJA or a combination of SA and MeJA.

In the *tpl-1* mutant, basal *PDF1.2* and *VSP2* expression levels after mock treatment were 13 and 150 times higher, respectively, than in wild-type L*er*-0 adult plants (**Fig. 4**). Likewise, treatment with MeJA induced *PDF1.2* and *VSP2* expression to a higher extent in *tpl-1* than in L*er*-0 (**Fig. 4**). These effects were less prominent in seedlings, but also here MeJA induced *PDF1.2* expression to a higher level (**Supplementary Fig. S2**). These results reassert the important role of TPL in repression of JA signaling, in both basal and MeJA-induced conditions. Induction of *PR1* by SA treatment was lower in *tpl-1* compared with L*er*-0 in adult plants, but not in seedlings (**Fig. 4**; **Supplementary Fig. S2**). Importantly, SA strongly repressed the MeJA-induced *PDF1.2* and *VSP2* expression levels in *tpl-1* adult plants and seedlings (**Fig. 4**; **Supplementary Fig. S2**). These results indicate that despite its role in repression of JA signaling, TPL is not essential for SA/JA cross-talk of *PDF1.2* and *VSP2*.

Discussion

No evidence for a role of ERF repressors in SA/JA crosstalk

The antagonistic effects of SA on the JA signaling pathway have been well documented (Pieterse et al. 2012), but the mechanisms underlying this phenomenon are complex and need further exploration. Here, we show that de novo synthesis of proteins is required for suppression of JA-induced *PDF1.2* expression by SA signaling. In the presence of CHX, the induction of *PR1* by SA was completely inhibited and, while the induction of *PDF1.2* by MeJA was also strongly reduced, there was still a statistically significant 4-fold induction compared with the mock treatment (**Fig. 1**). Only if CHX was present could the *PDF1.2* expression level not be further antagonized by SA. This indicates that novel protein synthesis is required for SA to exert both its positive action on *PR1* expression and its repressive action on JA-induced *PDF1.2* expression. These SA-induced proteins could potentially interact with JA-induced transcriptional activators, and thereby reduce JA-dependent transcription. Alternatively, the SA-induced proteins could act as transcriptional repressors by occupying *cis*-regulatory elements in the promoters of JA-responsive genes and hence compete with JA-regulated transcriptional activators, leading to repression of transcription of JA-inducible genes (Caarls et al. 2015).

In this study, we investigated the potential role of SA-inducible ERF transcriptional repressors in the SA-mediated attenuation of JA-responsive gene expression. We focused on the ERF family of the AP2/ERF superfamily of transcription factors, because the 122 members of this family share a common DNA-binding domain with affinity for the GCC-box promoter element, which was previously shown to be a central target site of SA/JA antagonism (Nakano et al. 2006, Van der Does et al. 2013). Moreover, the ERF transcriptional activator ORA59 has been reported to be targeted by SA to suppress JA/ethylene signaling, indicating that ERFs can have a significant role in SA/JA cross-talk (Van der Does et al. 2013, Zander et al. 2014). Finally, several ERFs were described before as repressors of *PDF1.2* expression (Huang et al. 2015). Based on the SA-inducible expression pattern of *ERF* genes and/or the presence of an EAR-domain in their protein sequence, we selected and tested 16 ERF transcription factors as potential SA-induced repressors of JA signaling. To our knowledge, this is the first study in which a large set of ERF transcription factors is systematically screened for their potential role in SA/JA antagonism and in SA and JA responsiveness.

Induction of *PDF1.2* and *VSP2* by single MeJA treatment was affected in 12 *erf* mutants, while *PR1* induction by SA was affected in five *erf* mutants. Strikingly, all tested *erf* mutants,

except the two (*erf9* and *dreb2a*) that are fully compromised in MeJA-induced expression of *PDF1.2* or *VSP2*, respectively, displayed wild-type levels of SA-mediated suppression of JA-induced *PDF1.2* and *VSP2* expression, suggesting that the corresponding ERFs do not play an essential role as repressors in SA/JA cross-talk (**Fig. 3**; **Supplementary Fig. S1**). There may be functional redundancy among different ERF proteins affecting SA/JA cross-talk, in which case mutation of single genes does not result in a significant effect on SA-mediated suppression of JA-induced gene expression. However, the fact that several single *erf* mutants already displayed an effect on induction of *PDF1.2* or *VSP2* expression by MeJA treatment shows that even mutations in single *ERF* genes can result in measurable effects on transcription of these two JA-responsive genes. Moreover, a loss-of-function mutation in TPL, which is a general repressor of JA signaling and interacts with different EAR-motif AP2/ERFs (Pauwels et al. 2010, Causier et al. 2012), also did not affect SA-mediated suppression of JA-induced *PDF1.2* and *VSP2* expression (**Fig. 4**; **Supplementary Fig. S2**). Together, these results make it unlikely that the tested ERF transcription factors or TPL play a major role as repressor in the antagonistic effect of SA on JA-responsive gene expression. The transcription factors ERF9 and DREB2A may, however, still be involved in SA/JA cross-talk, not as repressors but as activators of JA signaling, that could be subjected to post-translational regulation by SA, as shown previously for ORA59 (Van der Does et al. 2012).

However, a role for ERF repressors in SA/JA cross-talk cannot be fully excluded. First, we were not able to assess the role of two of the selected ERFs, ERF3 and ERF7, as we did not obtain homozygous loss-of-function mutants after screening several T-DNA insertion lines. Overexpression of *ERF7* has been shown to result in suppression of *PDF1.2* expression in stable transgenic lines, indicating that it functions as a repressor (Song et al. 2005). Secondly, we selected only those ERFs whose gene expression was induced after treatment with SA or BTH, infection by *H. arabidopsidis* or that contain an EAR-domain. SA might, however, activate other repressive ERFs at the protein level, as post-translational regulation has previously been demonstrated for several ERFs (Koyama et al. 2013, Van der Does et al. 2013). Therefore, we cannot exclude a role in SA/JA cross-talk for other ERF repressors.

ERF transcriptional repressors of JA-inducible genes

It has been demonstrated that all the transcription factors of the ERF subfamily that contain an EAR-motif are capable of suppressing gene transcription in protoplast transactivation assays (Fujimoto et al. 2000, Song et al. 2005, Yang et al. 2005, Wehner et al. 2011). In accordance with this, we found a role for the EAR-motif-ERF ERF8 as a negative regulator of *PDF1.2* expression, evidenced by increased *PDF1.2* expression after MeJA treatment in the *erf8* mutant (**Fig. 3**; **Supplementary Fig. S1**). ERF8 was previously described to be able to suppress induced luciferase activity in transgenic GCC:LUC lines (Wehner et al. 2011); here, its relevance as a suppressor of the GCC-box containing *PDF1.2* gene is confirmed. Moreover, we found that *erf8* also displayed enhanced SA-induced *PR1* levels, indicating a role for ERF8 in repression of multiple defense responses. Overexpression of *ERF4* was previously shown to result in suppression of MeJA-induced *PDF1.2* expression and reduction of resistance against *Fusarium oxysporum* (McGrath et al. 2005). However, the *erf4* mutant did not show higher *PDF1.2* expression in our experiments. Loss-of-function mutants of three ERF transcription factors without an EAR-domain did, however, display enhanced *PDF1.2* expression levels after MeJA treatment, namely *erf13*, *erf112* and *dreb2a* (**Fig. 3**; **Supplementary Fig. S1**). This indicates that the corresponding ERFs can repress MeJA-induced *PDF1.2* expression through means other than through interaction with EAR-binding co-repressors.

In contrast to the increased *PDF1.2* levels in the *erf8* and *dreb2a* mutants, the MeJA-induced *VSP2* levels were very low (**Fig. 3**; **Supplementary Fig. S1**), suggesting that ERF8 and DREB2A can activate parts of the MYC branch of JA signaling. In accordance with this, overexpression of DREB2A was demonstrated to increase expression of *VSP1* (Sakuma et al. 2006). The mutual antagonism between the classical ERF and MYC branch of JA signaling (Lorenzo et al. 2004, Verhage et al. 2011) could possibly explain the association between high *PDF1.2* and low *VSP2* levels in *erf8* and *dreb2a* by a reduction of the antagonism on the ERF branch. In contrast, in mutant *ora59*, enhanced expression levels of *VSP2* upon MeJA treatment are associated with reduced *PDF1.2* levels (**Supplementary Fig. S1**), which is also in line with a mutually antagonistic interaction between the ERF and MYC branch of JA signaling.

ERF transcriptional activators of JA-induced genes

Previously, the ERF transcription factor ORA59 was shown to be a dominant positive regulator of JA-induced *PDF1.2* (Pré et al. 2008). We find that *PDF1.2* expression in the *ora59* mutant was virtually absent, independent of the treatment applied (**Fig. 3**; **Supplementary Fig. S1**), confirming previous findings (Zander et al. 2014). The *erf9* mutant was as affected in *PDF1.2* expression as *ora59* (**Fig. 3**; **Supplementary Fig. S1**). This was unexpected as ERF9 contains an EAR-motif and was previously described to have higher *PDF1.2* expression in response to *B. cinerea* infection than wild-type plants (Maruyama et al. 2009). These differences could possibly be due to differences in treatment or sampling times. Further research should elucidate the role of this ERF in *PDF1.2* expression and SA/JA cross-talk. Interestingly, although overexpression of the ERF gene *ERF1* was previously shown to activate *PDF1.2* strongly (Lorenzo et al. 2003), we did not observe an effect on the level of *PDF1.2* transcription in the *erf1* mutant (**Supplementary Fig. S1**). This supports the idea that ORA59 is the central ERF transcription factor positively regulating *PDF1.2* expression (Pré et al. 2008). Contrasting reports on ERF5 have been published (Moffat et al. 2012, Son et al. 2012). In our experiments, induction of *PDF1.2* and *VSP2* by MeJA in *erf5* was nearly 2-fold reduced or enhanced, respectively, but these effects were not statistically significant.

Other *erf* mutants, namely *erf12* and *erf104*, also showed reduced *PDF1.2* expression levels after induction by MeJA

(Fig. 3; Supplementary Fig. S1). Moreover, *VSP2* induction levels by MeJA were reduced in seven *erf* mutants (*erf8*, *dreb2a*, *erf104*, *erf12*, *erf11*, *rap2.6* and *rap2.6L*). In *erf8* and *dreb2a*, this reduced *VSP2* expression correlated with enhanced *PDF1.2* expression, as discussed above. In *erf104* and *erf12*, induction of both *VSP2* and *PDF1.2* was affected, suggesting that the corresponding ERFs have a role in activation of the ERF as well as the MYC branch of JA signaling. The ERF104 protein has previously been shown to bind to the *PDF1.2* promoter to regulate its expression positively (Bethke et al. 2009). ERF12 and ERF11 are EAR-containing proteins, so their effect on activation of JA-responsive gene expression could be indirect.

Together, these results indicate that different ERFs can have redundant roles in the activation of JA-responsive genes. However, the fact that effects on *PDF1.2* and *VSP2* expression are detectable in the respective single *erf* mutants suggests that different ERFs might act additively or be active in different tissues, developmental stages or at different times following induced signaling.

The general co-repressor TOPLESS

The co-repressor TPL is recruited to repress gene expression in several hormonal signaling pathways. It interacts with EAR-domain-containing suppressors NINJA or JAZ in the JA signaling pathway, but also with the auxin repressors AUX/IAA via their EAR-motifs (Szemenyei et al. 2008, Pauwels et al. 2010, Shyu et al. 2012). The AP2/ERF transcription factor APETALA2 (AP2) was shown to interact with TPL, and the recruitment of TPL and its interactor HDA19 represses downstream targets (Krogan et al. 2012). Several EAR-motif ERF transcriptional repressors, including ERF4 and ERF11, were shown to interact with TPL (Causier et al. 2012). We investigated if suppression via TPL could play a role in SA/JA cross-talk. *PDF1.2* and *VSP2* expression in basal and MeJA-induced conditions is significantly higher in the *tpl-1* mutant compared with wild-type L*er*-0 (Fig. 4), thus demonstrating convincingly the repressive effect of TPL on JA-inducible gene expression. SA-inducible *PR1* expression is reduced in the *tpl-1* mutant, which may be due to the increase in JA signaling that could suppress SA-responsive gene expression. The effects of TPL on adult plants are in general greater than on seedlings (Supplementary Fig. S2). Importantly, suppression of MeJA-induced *PDF1.2* and *VSP2* expression by SA is still intact in *tpl-1* adults and seedlings (Fig. 4). Hence, TPL is not required for SA-mediated suppression of *PDF1.2* and *VSP2* expression. Indirectly, this also suggests that EAR-motif ERF transcriptional repressors are unlikely to play a role in SA/JA cross-talk.

Potential SA-inducible players in suppression of JA signaling

In summary, we demonstrate that SA-mediated suppression of JA-dependent *PDF1.2* gene expression requires de novo SA-induced protein synthesis and that it is not likely that ERF-repressive transcription factors or the transcriptional co-repressor TPL play a major role in this process. So, which SA signaling components are de novo synthesized upon induction by SA and may act in the suppression of the JA pathway? Previously, the transcriptional co-activator NPR1, the TGA transcription factors TGA2, TGA5 and TGA6, glutaredoxin GRX480, and the WRKY transcription factors WRKY50, WRKY51 and WRKY70 were reported to play important roles in SA/JA cross-talk (reviewed by Caarls et al. 2015). Regulation of *ORA59* expression by TGA transcription factors, possibly in an interaction with SA-induced GRX480, is probably essential in suppression of the GCC-box (Leon-Reyes et al. 2010a, Zander et al. 2014). A future challenge for research on SA/JA cross-talk will be to identify if these, or as yet unknown, SA-induced proteins can interact with JA signaling components to suppress JA-dependent gene transcription.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana seeds were sown on river sand. Two weeks after germination, seedlings were transferred to 60 ml pots containing a sand/potting soil mixture (5 : 12, v/v) that had been autoclaved twice for 20 min with a 24 h interval. For plate assays, seeds were sown on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) including vitamins, at pH 6.0, supplemented with 1% (w/v) sucrose and 0.85% (w/v) plant agar. Plants were cultivated in a growth chamber with a 10 h day (75 µmol m^{-2} s^{-1}) and 14 h night cycle at 70°C.

For several of the studied genes, knockout mutants were previously published and available, namely: *aterf-1* (At4g17500) (Rioja et al. 2013), *erf4* (At3g15210) (McGrath et al. 2005), *erf5* (At5g47230) (Son et al. 2012), *erf104* (At5g61600) (Bethke et al. 2009), *ora59* (At1g06160) (Zander et al. 2014) (all in the Col-0 background) and *tpl-1* (At1g15750) (L*er*-0 background) (Long et al. 2002).

Knockouts of other genes were obtained by acquiring T-DNA insertion lines from the Nottingham Arabidopsis Stock Centre (NASC): *dreb2a* (SAIL_365_F10; At5g05410), *erf9* (SALK_043407C; At5g44210), *erf10* (SAIL_95_A08; At1g03800), *erf11* (SALK_166053; At1g28370), *erf12* (SAIL_873_D11; At1g28360), *erf13* (GK_121A12; At2g44840), *erf112* (GK_604D02; At2g33710), *rap2.6* (SAIL_1225_G09; At1g43160) and *rap2.6L* (SALK_051006; At5g13330), all in the Col-0 background (Sessions et al. 2002, Alonso et al. 2003, Kleinboelting et al. 2011). Other knockout lines were obtained from the French National Institute for Agricultural Research (INRA): *cej1*/*dear1* (FLAG_293H04; At3g50260) and *erf8* (FLAG_157D10; At1g53170), both in the Ws-0 background (Samson et al. 2002). Lines that were homozygous for the T-DNA insert were selected with PCR using the primers listed in Supplementary Table S2. Mutants were used to analyze SA/JA cross-talk only when the T-DNA insertion was located in an exon, or when the expression of the mutated gene was proven absent by qRT–PCR in mutants carrying the T-DNA up- or downstream of the coding sequence. The primers used in the qRT–PCR for analysis of expression of the *ERF* target gene in the mutants were as described by Czechowski et al. (2004) or Supplementary Table S2. For details on the qRT–PCR analysis see 'RNA extraction, RNA gel blotting, qRT–PCR and gene expression analysis'.

For *ERF1* (At3g23240), a T-DNA insertion line was obtained through screening of 80,000 lines of the SALK collection (Alonso et al. 2003), which was based on a four-dimensional pooling strategy. In order to identify a T-DNA insertion in the *ERF1* gene, a PCR-based approach was taken, using two T-DNA-specific primers for the LB (JMLB1) and RB (JMRB) of the pROK2 vector and the corresponding *ERF1* Forward and Reverse primer (Supplementary Table S2). In each screening, at least four primer combinations were tested (JMLB1 and *ERF1* Forward; JMLB1 and *ERF1* Reverse; JMRB and *ERF1* Forward; and JMRB and *ERF1* Reverse). A PCR product was considered valid when it hybridized with an *ERF1*-specific probe and was present in each of the four DNA pools. PCR products were run in a 1% agarose gel (1 × TBE) containing ethidium bromide and

transferred to a Hybond-N^+ membrane using 0.4 N NaOH. For pre-hybridization and hybridization, Church and Gilbert solution was used (7% SDS, 0.3 M NaPi pH 7.0 and 1 mM EDTA). In the *erf1* T-DNA insertion line, specific amplification with the primer combination JMLB1 and *ERF1* Reverse was obtained.

Chemical treatments

Five-week-old plants were treated with SA and/or MeJA by dipping the leaves into a solution containing 0.015% (v/v) Silwet L77 (Van Meeuwen Chemicals BV) and either 1 mM SA (Mallinckrodt Baker), 0.1 mM MeJA (Serva, Brunschwig Chemie) or a combination of these chemicals. For mock treatments, plants were dipped into a solution containing 0.015% (v/v) Silwet L77. MeJA was added to the solutions from a 1,000-fold concentrated stock in 96% ethanol. To the solutions without MeJA, a similar volume of 96% ethanol was added.

For the CHX experiments, 5-week-old plants were dipped in mock or MeJA solution. Twenty-four hours later, leaf discs (19.6 mm^2) were cut from the fourth to sixth leaf and placed in 6-well plates containing 3 ml of MES buffer (5 mM MES, 1 mM KCl, pH 5.7) per well. Fifteen leaf discs were used per sample; three independent biological replicates were included per treatment. CHX (Sigma-Aldrich) was added from a 100-fold concentrated stock in 10% ethanol/MES buffer, resulting in a final concentration of 0.1 mM CHX, after which leaf discs were kept under vacuum for 20 min. Subsequently, SA was added from a 4-fold concentrated stock in MES buffer, resulting in a final concentration of 0.5 mM SA. Similar volumes of ethanol or MES buffer were added to solutions without CHX or SA. After 6 h, leaf discs were snap-frozen in liquid nitrogen.

For treatment of L*er*-0 and *tpl-1* seedlings, 14-day-old plate-grown plants were transferred to fresh MS agar plates with or without 0.5 mM SA, 20 μM MeJA or a combination of both. As described above, MeJA was added from a 1,000-fold concentrated stock in 96% ethanol; media without MeJA received a similar volume of 96% ethanol. Seedlings were harvested 48 h after transfer.

RNA extraction, RNA gel blotting, qRT–PCR and gene expression analysis

RNA was extracted from leaf material from five plants per treatment per sample. For gene expression analysis with RNA gel blotting (**Fig. 2**), the protocol as described by Van Wees et al. (1999) was followed for RNA extraction, RNA gel blotting and blot hybridization with gene-specific probes for *PR1* (At2g14610), *PDF1.2* (At5g44420), *VSP2* (At5g24770) and *18S* rRNA. Probes were synthesized by PCR amplification on cDNA using previously described primers (Van der Does et al. 2013). After hybridization with [α-^{32}P]dCTP-labeled probes, blots were exposed for autoradiography. Signal intensities of probes were quantified using a Bio-Rad Molecular Imager FX with Quantity One software. Fold changes of *PR1*, *PDF1.2* or *VSP2* expression levels as determined by RNA gel blotting were calculated by normalizing the expression value of the different samples to the *18S* rRNA expression value and subsequently calculating the relative level of expression compared with that of the mock treatment.

For gene expression analysis with a two-step qRT–PCR, RNA was extracted as described for vegetative tissues by Oñate-Sánchez and Vicente-Carbajosa (2008). RNA that was used for qRT–PCR was pre-treated with DNase I (Fermentas) to remove genomic DNA. RevertAid H minus Reverse Transcriptase (Fermentas) was used to convert DNA-free total RNA into cDNA using oligo(dT) primers. PCRs were performed in optical 384-well plates with a ViiA 7 realtime PCR system (Applied Biosystems), using SYBR® Green to monitor the synthesis of double-stranded DNA. A standard thermal profile was used: 50°C for 2 min, 95°C for 1 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplicon dissociation curves were recorded after cycle 40 by heating from 60 to 95°C with a ramp speed of 1.0°C min^{-1}. Expression levels were normalized to the reference gene *At1g13320*, which encodes PROTEIN PHOSPHATASE 2A SUBUNIT A3 (PP2A-A3) (Czechowski et al. 2005) using the $2^{-\Delta\Delta}$Ct method described previously (Livak and Schmittgen 2001, Schmittgen and Livak 2008). The primers used to analyze gene expression with qRT–PCR were as described by Czechowski et al. (2004), except the primers for expression of *ERF5*, *PDF1.2*, *VSP2*, *PR1* and *At1g13320* (**Supplementary Table S2**; Van der Does et al. 2013). Fold change was calculated relative to the mock treatment in wild-type plants, as indicated in the figure legends. A two-way analysis of variance (ANOVA) was performed on fold changes to determine the statistical significance of differences in expression levels. For data that did not fulfill the requirement of equal variance, the ANOVA was performed on ΔCt values.

To construct the heat map depicted in **Fig. 3**, the fold change in expression level of *PDF1.2* and *VSP2* after MeJA treatment was divided by the fold change in MeJA-treated wild-type plants of the same experiment, while the fold change in *PR1* expression level after SA treatment was divided by the fold change in SA-treated wild-type plants of the same experiment. Fold change after the combination treatment was divided by the fold change in MeJA-treated (*PDF1.2* and *VSP2*) or SA-treated (*PR1*) plants of the same genotype. Subsequently, all values were $\log_2$ transformed. As these data are from a large number of independent experiments in which *erf* mutants were tested, the wild-type expression values depicted are an average of the different experiments, while the depicted values for mutants are from one experiment and are relative to the expression of the single MeJA- or single SA-treated wild-type plants in that specific experiment or, in the case of the combination treatment, relative to its own single treatment value. All mutants have been tested in minimally two independent experiments with similar results.

RNA sequencing of SA-treated plants

For RNA sequencing, the sixth leaf (counted from the oldest to the youngest) was harvested from four individual SA- or mock-treated wild-type Col-0 plants at each of the following time points post-treatment: 15 min, 30 min and 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 16 h. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), including a DNase treatment step in accordance with the manufacturer's instructions. RNA-seq library preparation and sequencing was performed by UCLA Neuroscience Genomics Core (Los Angeles, CA, USA). Sequencing libraries were prepared using the Illumina TruSeq RNA Sample Prep Kit, and sequenced on the Illumina HiSeq 2000 platform with single read lengths of 50 bases. Basecalling was performed using the Casava v1.8.2. pipeline with default settings except for the additional argument '–use-bases-mask y50,y6n', to provide an additional fastq file containing the barcodes for each read in each sample. Sample demultiplexing was performed by uniquely assigning each barcode to sample references, allowing for a maximum of two mismatches and only considering barcode nucleotides with a quality score of ≥28. Reads were mapped to the Arabidopsis genome (TAIR version 10) using TopHat version 2.0.4 (Trapnell et al. 2009) with parameter settings: 'max-intron-length 2000', 'transcriptome-mismatches 3', 'N 3', 'bowtie1', 'no-novel-juncs', 'genome-read-mismatches 3', 'p 6', 'read-mismatches 3', 'G', 'min-intron-length 40'. Aligned reads were summarized over annotated gene models (TAIR version 10) using HTseq-count version 0.5.3p9 (http://www-huber.embl.de/users/anders/HTSeq/) with parameters: 'stranded no', '-i gene_id'. Sample counts were depth-adjusted using the median-count-ratio method available in the DESeq package (Anders and Huber 2010). Genes that were significantly altered over time in response to SA when compared with the mock treatment were identified using a generalized linear model (GLM) with a log link function and negative binomial distribution. Within this model, we considered both the time after treatment (time) and the treatment itself (treat) as a factor. To assess the effect of SA application on the total read count for each gene, a saturated model (total counts ~ treatment + time + treatment:time) was compared with a reduced model considering time alone (total counts ~ time) using an ANOVA χ^2 test. The obtained *P*-values for all genes were corrected for multiple testing using a Bonferroni correction. All statistics associated with testing for differential gene expression were performed with R (www.r-project.org). All raw RNA-Seq read data are deposited in the Short Read Archive (http://www.ncbi.nlm.nih.gov) with BioProject ID PRJNA224133.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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