PCP
PLANT & CELL PHYSIOLOGY

Special Focus Issue – Regular Paper

Chemical Activation of EDS1/PAD4 Signaling Leading to Pathogen Resistance in Arabidopsis

Shachi Joglekar[1], Mohamed Suliman[1], Michael Bartsch[2], Vivek Halder[1], Jens Maintz[1,4], Jaqueline Bautor[2], Jürgen Zeier[3], Jane E. Parker[2,*] and Erich Kombrink[1,*]

[1]Chemical Biology Laboratory, Max Planck Institute for Plant Breeding Research, D-50829 Köln, Germany
[2]Department of Plant-Microbe Interactions, Max Planck Institute for Plant Breeding Research, D-50829 Köln, Germany
[3]Department of Biology, Heinrich Heine University, D-40225 Düsseldorf, Germany
[4]Present address: Department of Crop Genetics, John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK
*Corresponding authors: Jane E. Parker, E-mail, parker@mpipz.mpg.de; Fax, +49 221 5062 352; Erich Kombrink, E-mail, kombrink@mpipz.mpg.de; Fax, +49 221 5062 353.
(Received October 26, 2017; Revised May 29, 2018)

In a chemical screen we identified thaxtomin A (TXA), a phytotoxin from plant pathogenic *Streptomyces scabies*, as a selective and potent activator of *FLAVIN-DEPENDENT MONOOXYGENASE1* (*FMO1*) expression in Arabidopsis (*Arabidopsis thaliana*). TXA induction of *FMO1* was unrelated to the production of reactive oxygen species (ROS), plant cell death or its known inhibition of cellulose synthesis. TXA-stimulated *FMO1* expression was strictly dependent on *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) and *PHYTOALEXIN DEFICIENT4* (*PAD4*) but independent of salicylic acid (SA) synthesis via *ISOCHORISMATE SYNTHASE1* (*ICS1*). TXA induced the expression of several *EDS1/PAD4*-regulated genes, including *EDS1*, *PAD4*, *SENESCENCE ASSOCIATED GENE101* (*SAG101*), *ICS1*, *AGD2-LIKE DEFENSE RESPONSE PROTEIN1* (*ALD1*) and *PATHOGENESIS-RELATED PROTEIN1* (*PR1*), and accumulation of SA. Notably, enhanced *ALD1* expression did not result in accumulation of the product pipecolic acid (PIP), which promotes *FMO1* expression during biologically induced systemic acquired resistance. TXA treatment preferentially stimulated expression of *PAD4* compared with *EDS1*, which was mirrored by PAD4 protein accumulation, suggesting that TXA leads to increased PAD4 availability to form EDS1–PAD4 signaling complexes. Also, TXA treatment of Arabidopsis plants led to enhanced disease resistance to bacterial and oomycete infection, which was dependent on *EDS1* and *PAD4*, as well as on *FMO1* and *ICS1*. Collectively, the data identify TXA as a potentially useful chemical tool to conditionally activate and interrogate EDS1- and PAD4-controlled pathways in plant immunity.

Keywords: *Arabidopsis thaliana* • Bioactive small molecules • Chemical genetics • Defense gene activation • Plant immunity • Plant–pathogen interaction.

Abbreviations: AAD, α-amino adipic acid; ALD1, AGD2-like defense response protein1; DMSO, dimethylsulfoxide; EDS1, enhanced disease susceptibility1; FMO1, flavin-dependent monooxygenase1; GFP, green fluorescent protein; GUS, β-glucuronidase, HR, hypersensitive response; ICS1, isochorismate synthase1; JA, jasmonic acid; MAPK, mitogen-activated protein kinase; MER, merbromin; MON, monensin; PAD4, phytoalexin deficient4; NHP, *N*-hydroxypipecolic acid; PIP, pipecolic acid; PR1, pathogenesis-related protein1; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; SA, salicylic acid; SAG101, senescence associated gene101; SAR, systemic acquired resistance; SARD4, SAR-deficient4, TXA, thaxtomin A; YFP, yellow fluorescent protein.

Introduction

Plants utilize multilayered defense strategies consisting of preformed and inducible mechanisms to resist pathogen infection (Jones and Dangl 2006, Spoel and Dong 2012, Cui et al. 2015). The Arabidopsis (*Arabidopsis thaliana*) protein ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1), together with its direct signaling partners, PHYTOALEXIN DEFICIENT4 (PAD4) and SENESCENCE ASSOCIATED GENE101 (SAG101), regulates plant basal immunity against virulent biotrophic pathogens and effector-triggered immunity (ETI), mediated by intracellular TOLL/INTERLEUKIN-1 RECEPTOR–NUCLEOTIDE BINDING–LEUCINE RICH REPEAT (TNL) receptors, against avirulent pathogenic strains (Wiermer et al. 2005, Bhattacharjee et al. 2011, Heidrich et al. 2011, Rietz et al. 2011, Wagner et al. 2013). Defense responses activated by EDS1 heteromeric complexes with PAD4 or SAG101 include transcriptional reprogramming of infected cells and the production of salicylic acid (SA) and other stress signals, which limit pathogen growth (Zhou et al. 1998, Feys et al. 2001, Zhang et al. 2003, Glazebrook 2005, Wirthmueller et al. 2007, García et al. 2010, Rietz et al. 2011, Wagner et al. 2013). Analysis of Arabidopsis mutants showed that *EDS1* and *PAD4* are of crucial importance for disease resistance. The role of *SAG101* is less clear because its loss is frequently compensated by *PAD4* (Feys et al. 2005, Rietz et al. 2011). However, *SAG101* contributes non-redundantly to certain Arabidopsis immune responses (Zhu et al. 2011, Xu et al. 2015). Also, a unique *PAD4* defense activity independent of *EDS1* was reported in resistance to aphid feeding (Pegadaraju et al. 2007, Zhu et al. 2011, Louis et al. 2012).

Plant Cell Physiol. 59(8): 1592–1607 (2018) doi:10.1093/pcp/pcy106, Advance Access publication on 21 June 2018, available online at www.pcp.oxfordjournals.org

EDS1 with PAD4 promotes two immunity pathways: (i) an SA-dependent branch in which pathogen-induced SA accumulation amplifies resistance; and (ii) an SA-independent branch which conditions resistance in the absence of *ISOCHORISMATE SYNTHASE1* (*ICS1*)-generated SA (Bartsch et al. 2006, Cui et al. 2017). *FLAVIN-DEPENDENT MONOOXYGENASE1* (*FMO1*) is a marker gene of the *EDS1*- and *PAD4*-controlled, SA-independent signaling branch, its expression being locally and systemically activated in Arabidopsis plants upon inoculation with virulent or avirulent *Pseudomonas syringae* bacteria (Bartsch et al. 2006, Mishina and Zeier 2006). Arabidopsis plants constitutively overexpressing *FMO1* displayed enhanced resistance to bacterial (*P. syringae*) and oomycete (*Hyaloperonospora arabidopsidis*) pathogens (Bartsch et al. 2006, Koch et al. 2006), whereas Arabidopsis *fmo1* loss-of-function mutants were compromised in local resistance to *P. syringae* or *H. arabidopsidis*, and defective in the establishment of systemic acquired resistance (SAR) triggered by virulent or avirulent bacteria (Bartsch et al. 2006, Mishina and Zeier 2006).

Biochemical profiling and genetic studies established a tight functional link between inducible plant immunity, in particular the establishment of SAR, and systemic accumulation of the lysine metabolite pipecolic acid (PIP), which is produced via the aminotransferase AGD2-LIKE DEFENSE RESPONSE PROTEIN1 (ALD1) (Song et al. 2004, Návarová et al. 2012, Bernsdorff et al. 2016). PIP is a critical regulator of induced plant defense responses including *FMO1* and *ALD1* expression and FMO1-mediated SA biosynthesis via ICS1 in a positive amplification loop (Návarová et al. 2012, Hartmann et al. 2018). Because pathogen-induced expression of *FMO1* and *ALD1* also occurred in the Arabidopsis *sid2/ics1* mutant, which is defective in SA production via ICS1 (Nawrath and Métraux 1999, Wildermuth et al. 2001), it was proposed that FMO1 and ALD1 are involved in SA-independent processes upstream of SA biosynthesis and SAR induction (Song et al. 2004, Bartsch et al. 2006, Mishina and Zeier 2006, Návarová et al. 2012). Recently, the biochemical functions of both ALD1 and FMO1 were identified. ALD1 catalyzes the first step in the biosynthesis of PIP by an α-transamination of L-lysine leading to the formation of 2,3-hydroxypipecolic acid (Hartmann et al. 2017). FMO1 hydroxylates PIP to *N*-hydroxypipecolic acid (NHP), which acts as a potent inducer of SAR (Hartmann et al. 2018).

Here we chose a chemical biology approach to dissect signaling pathways controlling *FMO1* expression. Searches for bioactive small molecules via phenotypic screening of chemical libraries have recently found broader application in plants (Kaschani and van der Hoorn 2007, McCourt and Desveaux 2010, Tóth and van der Hoorn 2010, Hicks and Raikhel 2012, Serrano et al. 2015). Many aspects of plant biology have been subjected to chemical interrogation, including hormone signaling (De Rybel et al. 2009, Park et al. 2009, Meesters et al. 2014, Rigal et al. 2014), endomembrane trafficking (Hicks and Raikhel 2010), cell wall formation (Desprez et al. 2002, Park et al. 2014) and host–pathogen interactions (Serrano et al. 2007, Schreiber et al. 2008, Knoth et al. 2009, Serrano et al. 2010, Noutoshi et al. 2012). Groundbreaking studies using a novel small molecule ABA agonist, pyrabactin, led to the identification of long sought after ABA receptors (Park et al. 2009, Cutler et al. 2010, Mosquna et al. 2011).

We applied a marker-based chemical biology approach to identify bioactive small molecules that induce *FMO1* expression using a transgenic Arabidopsis line expressing a fluorescent FMO1–yellow fluorescent protein (YFP) fusion protein under control of the native *FMO1* promoter. Screening of a collection of 1,488 natural and synthetic chemicals yielded five candidate activators of *FMO1* expression, of which one compound, the fungal phytotoxin thaxtomin A (TXA), induced *EDS1*- and *PAD4*-dependent defense responses without affecting other stress-related outputs. We found that TXA-induced expression of *FMO1* is strictly dependent on *EDS1* and *PAD4* but independent of SA production, indicating that TXA selectively promotes SA-independent EDS1/PAD4-controlled immunity. Moreover, TXA treatment resulted in enhanced resistance against virulent pathogenic strains of *P. syringae* and *H. arabidopsidis*. Our analysis shows that TXA is a potent chemical activator of *EDS1*- and *PAD4*-regulated defense outputs. Thus, TXA is a promising new tool to conditionally activate and dissect further EDS1/PAD4-regulated plant immune responses.

Results

Identification of TXA, a potent and selective activator of Arabidopsis *FMO1* expression

We developed a chemical screening assay to identify small molecules that activate expression of the defense marker gene *FMO1*, representing an *EDS1*- and *PAD4*-controlled, SA-independent resistance pathway (Bartsch et al. 2006). For this, we generated transgenic Arabidopsis lines in the *fmo101* (Col-0) mutant expressing FMO1 with a C-terminal YFP tag under the control of the native *FMO1* promoter (*FMO1p::FMO1-YFP*). Functionality of the transgene was established by performing infection assays with *H. arabidopsidis*, which showed restored disease resistance (Supplementary Fig. S1). To establish appropriate screening conditions, we initially monitored induced YFP fluorescence in seedlings treated with fumonisin B1 (Supplementary Fig. S2), which was shown previously to activate *FMO1* expression (Olszak et al. 2006). Transgenic seedlings were grown in liquid medium in 96-well plates and incubated with 1,488 diverse chemicals (comprising natural, semi-synthetic and recognized bioactive compounds) followed by quantifying enhanced reporter gene expression at 3–6 d after treatment. This primary screening, performed at various consecutive time points and with two or three replications per chemical, showed that several compounds increased FMO1–YFP fluorescence in seedlings, whereas most chemicals had little or no effect (**Fig. 1A**). To not miss bioactive compounds with low activity, we initially used a low cut-off value of 1.5-fold enhanced fluorescence in sample vs. control [dimethylsulfoxide (DMSO)] treatments and selected approximately 200 compounds for further characterization. Rigorous rescreening ($n \geq 2$) by quantifying YFP fluorescence reduced the number of confirmed hit compounds, which were then used to monitor their impact on FMO1–YFP accumulation at 24 h by protein blot analysis (Supplementary Fig. S3). This process

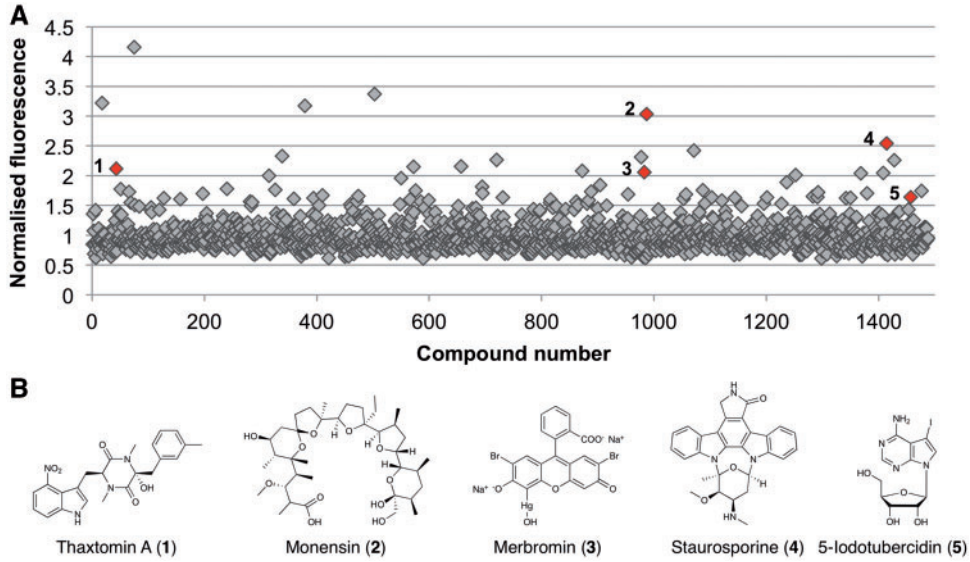

Fig. 1 Chemical screen for activators of defense-related reporter gene expression. (A) Arabidopsis seedlings harboring the *FMO1p::FMO1-YFP* reporter, grown hydroponically in 96-well microplates for 14 d, were treated with 1,488 diverse compounds (10–20 μM). YFP fluorescence was determined after 6 d in two or three replicates and the mean values were normalized to the averaged activity of the whole plate (96 samples). Compounds with normalized fluorescence values >1.5 (~200 compounds) were subjected to extensive rescreening ($n \geq 3$), which identified five activators (red diamonds). (B) Structures of the confirmed activators of *FMO1* expression: thaxtomin A (1), monensin (2) merbromin (3), staurosporine (4), 5-iodotubercidin (5).

eliminated false-positive hits and provided a short list of five robust activators of *FMO1p::FMO1-YFP* expression (hit rate 0.34%) (**Fig. 1B**).

Since our aim was to identify chemicals that selectively activate *EDS1/PAD4* defense pathways represented by *FMO1*, two of the confirmed hit compounds, staurosporine and 5-iodotubercidin (**Fig. 1B**), were excluded from further analysis because they are general protein kinase inhibitors, which perturb numerous processes in plants, including signaling mediated by jasmonic acid (JA), SA and calcium (Massillon et al. 1994, Meggio et al. 1995, Conrath et al. 1997, Allen et al. 1999, Asai et al. 2002, Jensen et al. 2002, Lecourieux et al. 2002). The remaining three candidate compounds, the phytotoxin TXA, the polyether antibiotic monensin (MON) and the topical antiseptic merbromin (MER), were subjected to several orthogonal assays and counter assays to validate their bioactivity and selectivity in *FMO1* induction. First, we determined the compound's impact on other plant stress responses. We found that treatment with TXA, MON or MER did not induce production of reactive oxygen species (ROS), which is a common response to infection or microbial elicitors [microbe-associated molecular patterns (MAMPs)] such as the flagellin-derived peptide, flg22 (**Table 1**; Supplementary Fig. S4A). However, we observed different effects when monitoring mitogen-acivated protein kinase (MAPK) signaling or intracellular calcium spiking. TXA and MER did not influence MAPK phosphorylation status, whereas MON caused phosphorylation of MPK6, and to a lower extent MPK3, compared with MPK6 and MPK3 activation by flg22 (**Table 1**; Supplementary Fig. S4B). Also, TXA and MER did not affect the flg22-induced transient increase in cytoplasmic calcium concentration ($[Ca^{2+}]_{cyt}$), whereas MON resulted in elevated $[Ca^{2+}]_{cyt}$ and partial inhibition of the flg22-induced calcium response (**Table 1**; Supplementary Fig. S5). Secondly, we determined the compound's impact on expression of the SA-responsive defense marker *PATHOGENESIS-RELATED PROTEIN1* (*PR1*) by monitoring β-glucuronidase (GUS) activity in a transgenic Arabidopsis line expressing the GUS reporter under control of the *PR1* promoter (*PR1p::GUS*). While TXA strongly activated *PR1p::GUS* expression, MON and MER did not (**Table 1**; Supplementary Fig. S6). Conversely, SA-induced *PR1p::GUS* expression remained unaffected by TXA but was strongly impaired by MON and MER (**Table 1**; Supplementary Fig. S6). Thirdly, we determined the effect of TXA, MON and MER on plant hormone responses, such as JA-activated expression of *VSP1* and *LOX2* (Ellis and Turner 2001, Jensen et al. 2002, Meesters et al. 2014), ABA-induced *RAB18* expression (Fujii et al. 2007, Gao et al. 2007) and auxin-activated expression of *PIN1* (Vieten et al. 2005). Of the three candidate compounds, only TXA did not alter these plant hormone responses (**Table 1**; Supplementary Fig. S7).

Based on this survey, TXA was chosen for further analysis as a potent and selective activator of *FMO1* expression. Chemically, TXA is a piperazinedione derivative and the major phytotoxin produced by the plant-pathogenic soil bacterium, *Streptomyces scabies* (King et al. 1989, King et al. 1991). In addition to acting as a virulence factor causing scab-like lesions on potato tubers, TXA exhibits herbicidal and fungicidal activities (King et al. 1991, Goyer et al. 1998, Kinkel et al. 1998, Zhang et al. 2013).

TXA-induced *FMO1* expression is not a consequence of ROS production

To gain insight into the TXA mode of action, we characterized its effect on endogenous *FMO1* expression in Arabidopsis wild-type

Table 1 Summary of biological activity of primary hit compounds on diverse biological readouts

Responsive to:	Infection or flg22 (plant defense responses)					SA			Jasmonate					ABA	Auxin
Compound	FMO1p::FMO1-YFP	FMO1 mRNA	ROS accumulation	MPK3/6	Ca^{2+} spiking	PR1p::GUS activation [1]	PR1p::GUS inhibition [2]	PR1 mRNA	VSP1p::GUS activation [1]	VSP1p::GUS inhibition [2]	LOX2p::GUS activation [1]	LOX2p::GUS Inhibition [2]	VSP1 mRNA	RAB18 mRNA	PIN1 mRNA
Thaxtomin A (**1**)															
Monensin (**2**)															
Merbromin (**3**)								NT							

The activity of compounds **1**, **2**, and **3** (for structures see **Fig. 1B)** was evaluated in various quantitative and qualitative bioassays that are selectively activated by infection (or flg22), salicylic acid (SA), jasmonate (JA), abscisic acid (ABA), or auxin as presented in **Fig. 1** and **Fig. 2** and **Supplemental Figs. S4-S7**. Activity is presented in five categories: strong inhibition (**red**; 70-100%), weak inhibition (**pink**; 40-70%), no effect (**yellow**), weak activation (**light green**; < 2-fold), strong activation (**green**; > 2-fold). NT, not tested.

[1] *Arabidopsis* seedlings were treated only with specified compounds and monitored for reporter gene activation.

[2] Reporter gene expression was activated by specific inducer (flg22, SA, or JA) and the interference by specified compounds recorded as inhibition.

Activity scale:
- strong inhibition (70-100%)
- weak inhibition (40-70%)
- neutral (no effect)
- weak activation (< 2-fold)
- strong activation (> 2-fold)

(Col-0) seedlings by monitoring *FMO1* mRNA accumulation by quantitative real-time PCR (qRT-PCR). Increasing TXA concentrations (up to 1,000 nM) resulted in progressively increased *FMO1* expression, determined at 24 h after treatment (**Fig. 2A**). A concentration of 100 nM TXA was chosen for all subsequent experiments because it yielded reproducible and consistently high *FMO1* induction (≥30-fold). Higher TXA concentrations caused seedling growth retardation and necrosis. This detrimental effect of TXA on seedlings, recorded as increased electrolyte leakage, became apparent at concentrations >100 nM and extended incubation times (>24 h), and was independent of the Arabidopsis genotype tested (Supplementary Fig. S8). In a time course study, we found that *FMO1* mRNA amounts increased up to 24 h after treatment with 100 nM TXA and subsequently declined (**Fig. 2B;** Supplementary Fig. S9A). These results show that TXA is a potent and robust chemical activator of Arabidopsis endogenous *FMO1* expression.

In view of the reported activity of TXA as a phytotoxin, we tested the relationship between TXA-induced *FMO1* expression and ROS production as a proxy of cellular damage (programmed cell death). We found that TXA-induced *FMO1* expression was not impaired in the Arabidopsis *rbohd/f* mutant, which is defective in ROS production (Torres et al. 2002, Morales et al. 2016). Enhanced and temporally extended *FMO1* expression in *rbohd/f* mutant plants compared with wild-type plants rather suggested a negative effect of RboHD/F-generated ROS on TXA-induced *FMO1* expression (Supplementary Fig. S9B). Continuous monitoring of ROS production over an extended time period (up to 24 h) revealed no significant difference between TXA- and DMSO-treated seedlings (Supplementary Fig. S10), although flg22-induced ROS production was enhanced by TXA. These data suggest that TXA-induced *FMO1* expression over 24 h is not a consequence of rapid or extended ROS production. However, a contribution of induced cell death caused by TXA toxicity cannot be excluded (cf Supplementary Fig. S8).

FMO1 induction by TXA is not coupled to its activity as a cellulose synthesis inhibitor

TXA was shown previously to inhibit cellulose biosynthesis in plants (Scheible et al. 2003). We explored whether *FMO1* induction is a consequence of this cellulose biosynthesis inhibition by comparing the effect of TXA on *FMO1* expression with that of isoxaben, another known inhibitor of cellulose synthesis (Bischoff et al. 2009, Duval and Beaudoin 2009). For this, we treated Arabidopsis (Col-0) seedlings with 100 nM TXA or 10 nM isoxaben, to achieve approximately equal efficacy in repression of genes encoding enzymes involved in cell wall biosynthesis, such as cellulose synthases *CESA5*, *CESA6*, *CESA7* and *CESA8*, as previously reported (Bischoff et al. 2009). Treatment with solvent (DMSO) served as a negative control. Under conditions of equal inhibition (**Fig. 3A**), the induction of *FMO1* expression by TXA was significantly greater (≥40-fold) than by isoxaben (≤10-fold) (**Fig. 3B**). This result suggests that TXA-induced *FMO1* expression is not tightly coupled to TXA inhibition of cellulose synthesis. We conclude that TXA most probably has at least one additional cellular target, which influences *FMO1* expression.

TXA treatment promotes SA accumulation but not AAD or PIP synthesis

Activation of *FMO1* expression upon plant infection by necrotizing pathogens is accompanied by the induction of defense responses including up-regulation of the SA biosynthesis gene *ICS1* and accumulation of the defense hormone SA (Mishina and Zeier 2006). We quantified SA levels in Arabidopsis (Col-0) seedlings after treatment with TXA and found elevated levels of total and free SA (4- to 5-fold increase) compared with DMSO-treated controls (**Fig. 4A**). Therefore, TXA treatment stimulates SA production, in line with the observed activation of *ICS1* and *PR1* expression (**Fig. 4C;** Supplementary Fig. S6).

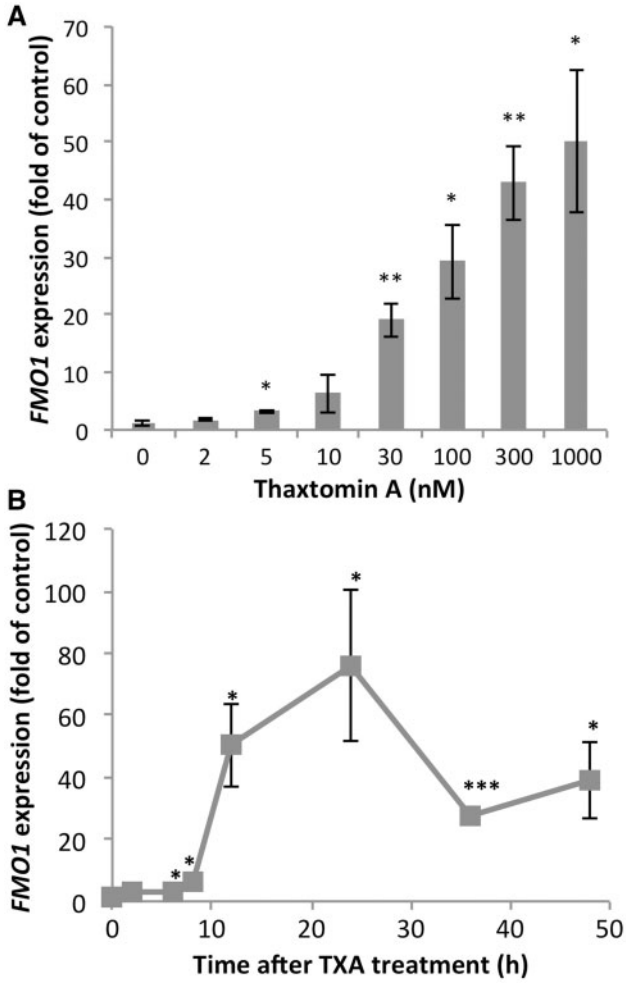

Fig. 2 Dose response and time course of TXA-induced *FMO1* expression. Arabidopsis wild-type (Col-0) seedlings, grown for 14 d in liquid culture, were treated (A) for 24 h with different thaxtomin A (TXA) concentrations (or 1% DMSO as control) or (B) 100 nM TXA (or 1% DMSO as control) for the indicated time periods. *FMO1* expression was quantified by qRT-PCR and normalized to the controls. Values represent the mean (±SD) of three biological replicates. The experiment was repeated twice with similar results. Asterisks indicate significant differences from control values (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t*-test). Values at 12 and 24 h differ significantly from values at 36 and 48 h ($P < 0.024$), demonstrating a decline of *FMO1* expression after 24 h (cf. Supplementary Fig. S9A).

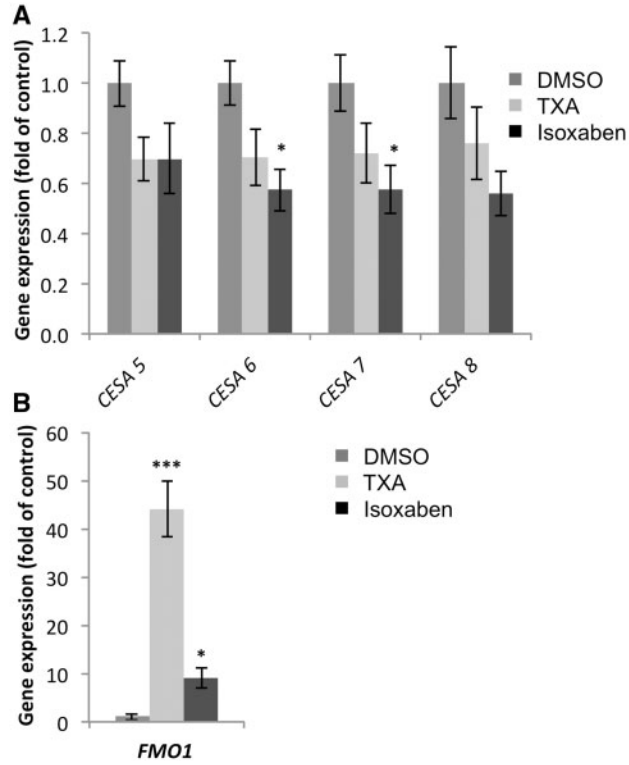

Fig. 3 Differential effect of cellulose synthesis inhibitors TXA and isoxaben on gene expression. Arabidopsis wild-type (Col-0) seedlings, grown for 14 d in liquid medium, were treated for 24 h with TXA (100 nM) or isoxaben (10 nM); DMSO (1%) served as control. Expression of (A) cellulose synthase genes, *CESA5*, *CESA6*, *CESA7* and *CESA8*, and (B) *FMO1* was quantified by qRT-PCR and normalized to control treatment (1% DMSO). Values represent the mean (±SD) of three biological replicates. Asterisks indicate significant differences from control values (*$P < 0.05$, ***$P < 0.001$, Student's *t*-test).

FMO1 is also an essential component of SAR, a resistance phenomenon in leaves distal from the site of primary pathogen attack (Mishina and Zeier 2006). Establishment of SAR in plants, accompanied by induced *FMO1* expression, was associated with massive local (>50-fold) and systemic (>10-fold) accumulation of the lysine metabolite PIP, whereas the related α-amino adipic acid (AAD) also accumulated to high levels (>50-fold) in local but to only moderate levels (>2-fold) in systemic tissue (Návarová et al. 2012). We tested whether TXA treatment affects the amino acid profile of Arabidopsis seedlings, but did not observe an impact of TXA on PIP or AAD levels (**Fig. 4B**). Other free amino acid levels were similarly unaffected (not shown). Therefore, TXA-induced *FMO1* expression in seedlings is apparently independent of amino acid signaling mediated by PIP or AAD.

TXA-induced *FMO1* expression requires *EDS1* and *PAD4*

During pathogen infection, *FMO1* expression was fully dependent on *EDS1* and *PAD4*, but independent of SA (Bartsch et al. 2006, Mishina and Zeier 2006). Also, *ALD1*, encoding an amino acid transferase participating in PIP (but not AAD) biosynthesis, was essential for systemic *FMO1* expression during SAR induction (Návarová et al. 2012). We tested various Arabidopsis mutant lines (in accession Col-0) for effects on TXA-induced *FMO1* expression. For this, we quantified *FMO1* expression in each line by qRT-PCR in relation to a constitutive reference gene, At4g26410 (Czechowski et al. 2005), because the different genotypes differed in basal *FMO1* transcript accumulation. This analysis revealed that TXA-induced *FMO1* expression was strongly reduced in the *eds1-2* and *pad4-1* single mutants as well as in the *pad4-1 sag101-3* or *eds1-2 sid2-2/ics1* double mutants (**Fig. 5**). In contrast, TXA-induced *FMO1* expression was unimpaired in the *sag101-3*, *ald1* and *sid2-2/ics1* single mutant lines. Therefore, the genetic requirements for TXA action on *FMO1* appear to be similar to those in pathogen-induced *FMO1*

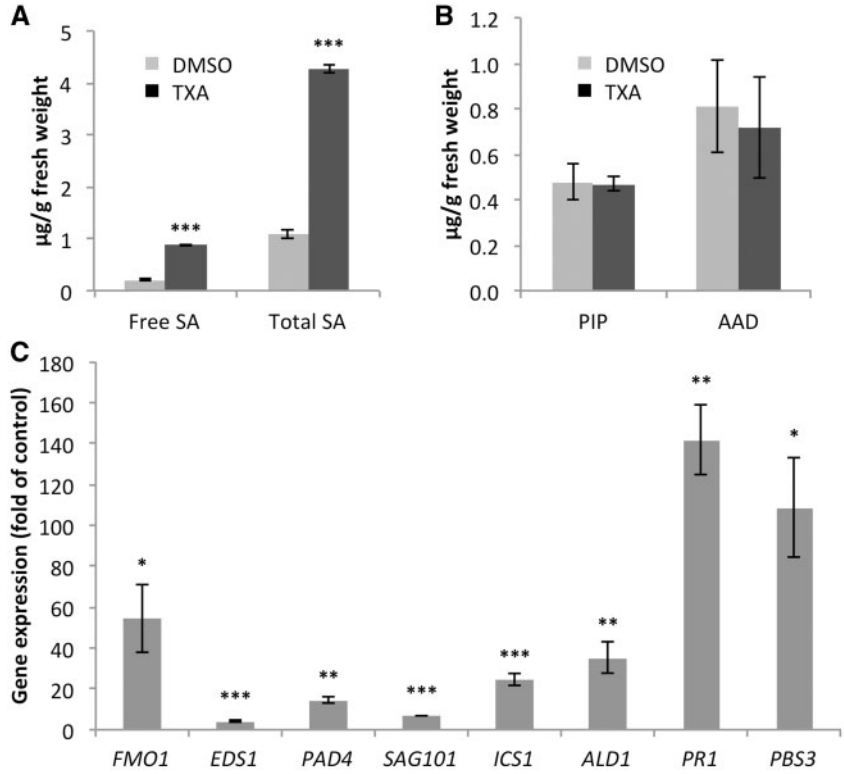

Fig. 4 Effect of TXA on salicylic acid (SA) and amino acid (PIP and AAD) levels and defense gene expression. Arabidopsis wild-type (Col-0) seedlings, grown for 14 d in liquid medium, were treated for 24 h with TXA (100 nM) or DMSO (1%) as control. The content of (A) free and total SA and (B) the amino acids PIP and AAD was quantified by GC-MS analysis. (C) Expression of the indicated genes was quantified by qRT-PCR and normalized to the controls. Values represent the mean (±SD) of three biological replicates. Experiments were repeated with similar results. Asterisks indicate significant differences from control values (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t*-test).

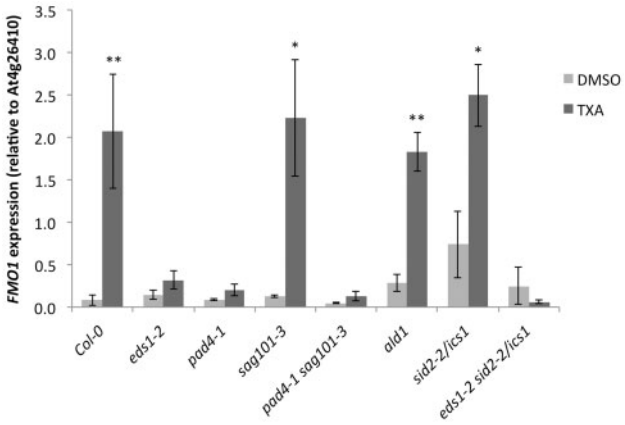

Fig. 5 TXA-induced *FMO1* expression in Arabidopsis mutants. Seedlings of the indicated Arabidopsis genotypes, grown for 14 d in liquid medium, were treated for 24 h with TXA (100 nM) or DMSO (1%) as control. Expression of *FMO1* was quantified by qRT-PCR relative to expression of the reference gene At4g26410. Values represent the mean (±SD) of three biological replicates. Asterisks indicate significant differences from expression values after control treatment (*$P < 0.05$, **$P < 0.01$, Student's *t*-test). Two independent repetitions of the experiment gave similar results.

gene expression (Bartsch et al. 2006). These results underscore the importance of both *EDS1* and *PAD4*, but not ICS1-generated SA, in TXA-induced *FMO1* expression. They also support the notion that TXA-induced *FMO1* expression does not require *ALD1*. This fits with a lack of increased PIP upon TXA treatment (**Fig. 4B**) because *ALD1* expression and function are tightly linked to PIP biosynthesis (Návarová et al. 2012).

TXA activates expression of *EDS1*- and *PAD4*-dependent defense genes

Next we tested whether TXA affects the expression of known *EDS1*- and *PAD4*-controlled defense genes besides *FMO1*, by monitoring their mRNA levels in Arabidopsis wild-type (Col-0) seedlings at 24 h after TXA treatment. Expression of *EDS1*, *PAD4* and *SAG101* was activated by TXA, with *PAD4* responding most strongly (>12-fold) compared with control treatment with DMSO (**Fig. 4C**). There were major increases in expression of the SA marker gene *PR1* (140-fold) and genes affecting SA production, *ICS1* (24-fold) and *AVRPPHB SUSCEPTIBLE3* (*PBS3*; 100-fold) (**Fig. 4C**), all of which are *EDS1* dependent in pathogen resistance (García et al. 2010). The induced expression of *ICS1* corresponds to SA accumulation in seedlings upon TXA

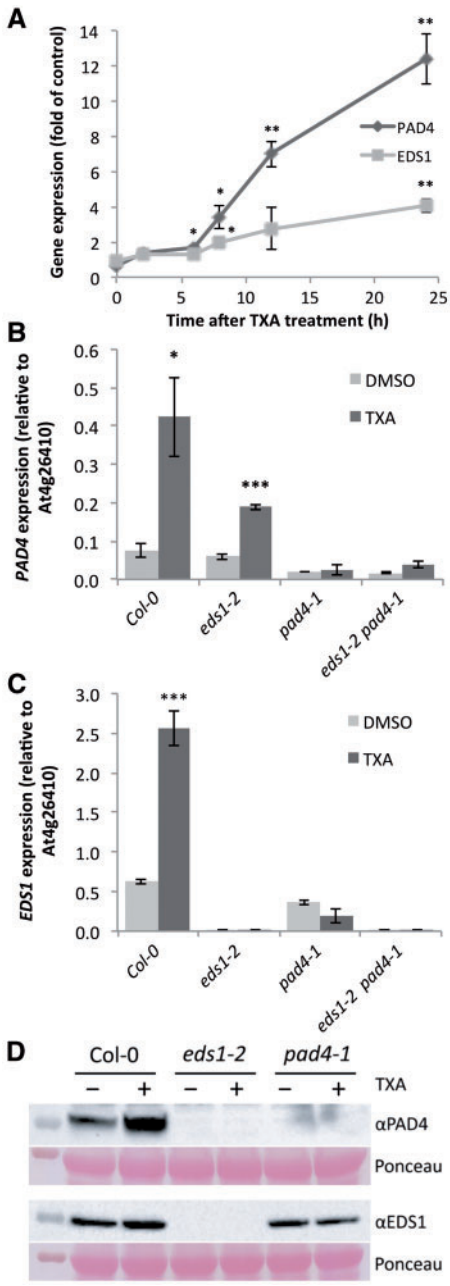

Fig. 6 Effect of TXA on *EDS1* and *PAD4* gene expression and protein accumulation. Arabidopsis wild-type (Col-0) or mutant seedlings, grown for 14 d in liquid medium, were treated for 24 h (or the indicated times) with TXA (100 nM) or DMSO (1%) as control. (A) Time course of *EDS1* and *PAD4* expression in wild-type (Col-0) seedlings. (B) *EDS1* expression and (C) *PAD4* expression in the indicated Arabidopsis genotypes. Gene expression was quantified by qRT-PCR and normalized to control (DMSO) treatment (A) or expression of the reference gene At4g26410 (B, C). Values represent the mean (±SD) of three biological replicates. Asterisks indicate significant differences from control values (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t*-test). (D) Accumulation of PAD4 and EDS1 proteins in response to TXA treatment of different Arabidopsis genotypes was visualized by protein blot analysis with anti-PAD4 or anti-EDS1 serum after SDS–PAGE. Staining with Ponceau S shows equal protein loading. All experiments were repeated two or three times with similar results.

treatment (Fig. 4A). Interestingly, *ALD1* expression, which was reported to be partially dependent on *PAD4* (Song et al. 2004), also increased (35-fold) in response to TXA, but this did not result in a corresponding accumulation of PIP (Fig. 4B). Together, the results suggest that TXA treatment leads to increased expression of various *EDS1*- and *PAD4*-dependent genes.

TXA treatment preferentially stimulates *PAD4* expression

Given the importance of EDS1 and PAD4 as defense regulators, we followed their expression upon treatment with TXA over a 24 h time course. The relative increase in *PAD4* mRNA was higher compared with that of *EDS1* mRNA (>12-fold vs. 4-fold at 24 h) (Fig. 6A). We then tested whether TXA-induced *PAD4* mRNA accumulation depends on *EDS1*, and vice versa. *PAD4* expression in the *eds1-2* null mutant increased upon TXA treatment to a lower extent (~40%) compared with the response of Arabidopsis wild-type (Col-0) plants (Fig. 6B). In contrast, TXA failed to activate *EDS1* expression in the *pad4-1* null mutant (Fig. 6C). As expected, *PAD4* and *EDS1* transcripts were not detectable in the corresponding *pad4-1* and *eds1-2* mutants, or an *eds1-2 pad4-1* double mutant with or without TXA (Fig. 6B, C). Therefore, TXA-induced *PAD4* expression is partly independent of *EDS1*, whereas TXA-induced *EDS1* expression absolutely requires *PAD4*. This suggests that PAD4 is the key limiting factor in determining TXA stimulation of *EDS1*.

In pathogen resistance responses, EDS1 operates with PAD4 in a heterodimeric complex, which mutually stabilizes each partner and up-regulates the expression of both genes as part of a transcriptional feed-forward loop (Feys et al. 2001, Feys et al. 2005, Rietz et al. 2011, Wagner et al. 2013). We therefore tested the effect of TXA treatment on EDS1 and PAD4 protein levels in Arabidopsis wild-type (Col-0), *eds1-2* and *pad4-1* mutant plants at 24 h after treatment with TXA (or DMSO as control). On protein blots of total leaf extracts, probed with anti-EDS1 or anti-PAD4 serum, we found that TXA treatment markedly stimulated PAD4 accumulation, whereas EDS1 levels did not change in wild-type (Col-0) plants (Fig. 6D). EDS1 amounts also remained unchanged in the *pad4-1* mutant in response to TXA (Fig. 6D). PAD4 protein was not detectable in the *eds1-2* mutant (Fig. 6D), suggesting that the residual TXA-induced *PAD4* mRNA accumulation seen in *eds1-2* (Fig. 6C) does not compensate for reduced PAD4 protein stabilization in an EDS1–PAD4 complex. Collectively, these data suggest that TXA principally stimulates *PAD4* expression and hence increases availability of PAD4 to form active EDS1–PAD4 complexes, which mediate the enhanced expression of their own and other *EDS1*- and *PAD4*-dependent defense genes, such as *FMO1*. The fact that overexpression of PAD4 neither initiated *FMO1* expression (in the absence of TXA) nor enhanced its TXA-induced expression (Supplementary Fig. S11) further supports that co-ordinated actions of PAD4 together with EDS1, presumably as EDS1–PAD4 complexes (Wagner et al. 2013, Cui et al. 2017), are important for TXA-triggered defense pathway activation.

TXA treatment enhances resistance to bacterial and oomycyte pathogens

Because TXA treatment of Arabidopsis plants induced expression of several genes within the *EDS1–PAD4* pathway, we tested whether TXA also leads to enhanced pathogen resistance. Leaves of 5-week-old wild-type (Col-0) and mutant plants were infiltrated with TXA (100 nM) and inoculated 24 h later with the virulent bacterial pathogen *P. syringae* pv *tomato* strain DC3000. Quantification of bacterial growth at 3 d post-inoculation showed that TXA caused an approximately 10-fold reduction in bacterial titer compared with control (DMSO) treatment in wild-type (Col-0) plants (**Fig. 7A**). In contrast, there was no effect of TXA treatment on *P. syringae* pv *tomato* DC3000 growth in the *pad4-1*, *eds1-2*, *sid2-2/ics1* and *fmo1-1* mutant lines (**Fig. 7A**), indicating that these genes are necessary for increased bacterial resistance by TXA. Notably, infiltration of TXA (100 nM, 24 h) did not lead to visible necrosis in leaves, further suggesting that TXA-induced resistance is not related to cell death propagation. Also, the TXA response was not associated with increased SA or PIP, since both compounds were only slightly higher in TXA-treated tissues compared with DMSO treatment (SA from 0.33 ± 0.07 to 0.40 ± 0.08 μg g FW^{-1}; PIP from 0.13 ± 0.03 to 0.37 ± 0.08 μg g FW^{-1}). By comparison, *Pseudomonas*-inoculated leaves accumulated 20- to 100-fold higher SA and PIP levels (~10 and 42 μg g FW^{-1}, respectively) (Bartsch et al. 2006, Návarová et al. 2012). Thus, with respect to SA accumulation, sterile seedlings grown in liquid culture were more responsive to TXA (100 nM) treatment than 5-week-old plants grown on soil. The different cultivation conditions, mode of TXA application, as well as developmental stages and tissues (young seedlings vs. older leaves), of plants used for chemical screening or infection assays, respectively, probably contribute to altered TXA-induced SA accumulation.

Leaves of 5-week-old Arabidopsis plants treated for 24 h with TXA (100 nM) were also spray-inoculated with conidiospores of *Hyaloperonospora arabidopsidis* isolate Noco2, which is virulent on the wild type (Col-0). Leaves were then stained with trypan blue at 4 d post-inoculation to visualize pathogen structures and dead or dying host cells under a light microscope (Aarts et al. 1998). In wild-type (Col-0) plants treated with DMSO as control, *H. arabidopsidis* hyphae had spread throughout leaves (**Fig. 7B**). In contrast, there was no hyphal spread observed in TXA-treated Col-0 leaves, which instead displayed localized host reactions indicative of a hypersensitive response (HR) at pathogen infection sites (**Fig. 7B**). Similar to the bacterial infection assays, TXA pre-treatment did not induce resistance against *H. arabidopsidis* isolate Noco2 in the *pad4-1*, *eds1-2*, *sid2-1/ics1* and *fmo1-1* mutant lines, which supported equivalent pathogen hyphal growth to that in the DMSO-treated samples (**Fig. 7B**). These phenotypes were consistent with *H. arabidopsidis* conidiospore counts on inoculated leaves (Supplementary Fig. S12). The data show that TXA treatment promotes Arabidopsis resistance to a virulent bacterial and oomycete pathogen and that TXA-induced resistance requires the defense regulatory genes, *EDS1* and *PAD4*. The failure of

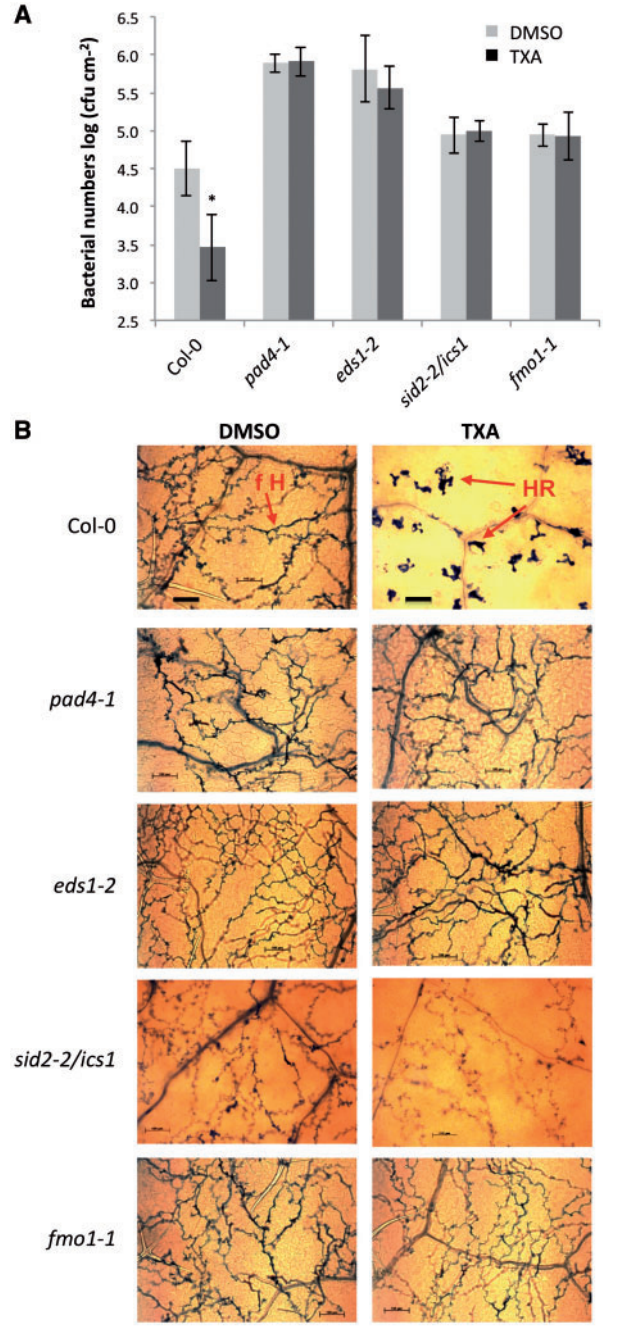

Fig. 7 TXA treatment leads to enhanced resistance of Arabidopsis wild-type plants to *Pseudomonas syringae* pv. tomato and *Hyaloperonospora arabidopsidis*. Leaves of the indicated 5-week-old Arabidopsis genotypes were infiltrated with TXA (100 nM) or DMSO (0.001%) as control and 24 h after treatment spray-inoculated with (A) virulent *P. syringae* pv tomato DC3000 or (B) virulent *H. arabidopsidis* isolate Noco2. Bacterial titers were quantified at 3 d post-inoculation; values represent the mean (±SD) of six replicates; the asterisk indicates a significant difference from the control (*$P < 0.05$, Student's *t*-test). To monitor the plant's hypersensitive response (HR) and hyphal growth (free hyphae, fH), ≥20 leaves of each line were stained at 4 d post-inoculation with trypan blue and examined by light microscopy, and representative pictures are shown. An HR was observed in TXA-treated wild-type (Col-0) plants, whereas all other panels show growth of free hyphae (fH). Scale bar = 100 μM. Experiments were repeated four times with similar results.

TXA to induce resistance in *sid2-2/ics1* and *fmo1-1* genotypes suggests that EDS1- and PAD4-controlled SA-dependent and SA-independent pathways contribute to this immunity phenotype.

Discussion

Intricate signaling networks mediate plant responses to attacking pathogens and other environmental stresses. Genetic approaches have identified key components and actions of these pathways (Glazebrook 2005, Jones and Dangl 2006, Tsuda et al. 2009, Spoel and Dong 2012, Cui et al. 2015), but many mechanistic details remain unclear. Here, we used a chemical biology approach to interrogate an SA-independent signaling branch in Arabidopsis that is controlled by the central immunity regulators EDS1 and PAD4 (**Fig. 8**). Previous studies showed that EDS1–PAD4 complexes transduce signals generated by plant pathogen infection to reprogram host cells transcriptionally (inducing expression of genes such as *FMO1*, *ICS1* and *ALD1* representing different defense outputs), leading to immunity and often to localized cell death (**Fig. 8**) (Gruner et al. 2013, Cui et al. 2015). How EDS1–PAD4 complexes molecularly activate these defense pathways is unclear.

Primary chemical screening and hit selection

We identified TXA as a selective activator of the *EDS1*- and *PAD4*-dependent but SA-independent defense pathway represented by *FMO1* (Bartsch et al. 2006, Mishina and Zeier 2006) by screening a library of diverse natural and synthetic compounds for activators of reporter gene expression. TXA was selected from a large number of primary hit compounds by rigorous filtering using multiple biological readouts (**Table 1**). This was an essential step because of the relatively low fluorescence emitted from accumulated FMO1–YFP (~3- to 4-fold compared with the control) in the primary screen. Endogenous *FMO1* transcripts accumulated to maximum levels at 24 h after infection or chemical stimulation (**Fig. 2B;** Supplementary Fig. S9A) (Bartsch et al. 2006, Mishina and Zeier 2006), whereas FMO1–YFP fluorescence was detectable at 3–6 d after TXA treatment (Supplementary Fig. S2) (Olszak et al. 2006). Accordingly, this relatively late time frame was used for consecutive monitoring of stimulated YFP fluorescence in Arabidopsis seedlings expressing the *FMO1p::FMO1-YFP* reporter. For a critical discussion of many issues related to designing chemical screening experiments, we refer readers to our recent methodology article (Serrano et al. 2015).

We analyzed the chemical screening data by applying stringent statistical methods to avoid missing hit compounds with intermediate activity. Hence, we calculated the so-called Z-score, which relates the mean fluorescence value obtained with each compound (determined with three replications) to the mean of a collective control value and corresponding standard deviation (Malo et al. 2006, Serrano et al. 2015), and selected all compounds yielding values $\geq$1.5 for re-screening. These verified candidate compounds were subjected to further validation by monitoring accumulation of FMO1–YFP on protein blots and endogenous *FMO1* mRNA by qRT-PCR after chemical treatment (**Fig. 2**; Supplementary Fig. S3). This ultimately yielded five robust activators of *FMO1* expression including TXA, MON and MER.

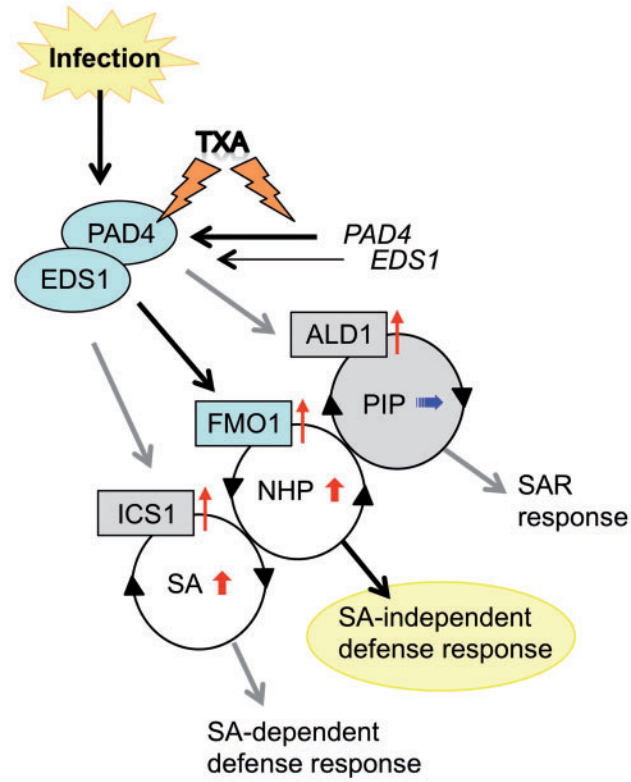

Fig. 8 Model for TXA action in *EDS1*- and *PAD4*-mediated defense signaling involving *FMO1* expression. Upon infection, EDS1–PAD4 complexes activate defense pathways represented by the enzymes ICS1, FMO1 and ALD1, mediating (primarily) SA-dependent, SA-independent and systemic defense responses (SAR), respectively. Thaxtomin A (TXA) preferentially activates expression of *PAD4*, thereby increasing the abundance, stability and/or activity of the EDS1–PAD4 complex, leading to activation of downstream defense responses, including *FMO1*, *ICS1* and *ALD1* gene expression. Enhanced FMO1 activity, via conversion of pipecolic acid (PIP) to *N*-hydroxy-PIP (NHP), mediates SA-independent defense responses. Increased ICS1 activity leads to SA accumulation and induction of SA-dependent defense genes such as *PR1*, but these responses are not necessary for TXA-induced *FMO1* expression. TXA-activated *ALD1* expression is not accompanied by accumulation of the corresponding product PIP (gray shaded area), presumably because another necessary enzyme, SARD4, is not co-expressed. Consequently, TXA-induced *FMO1* expression is uncoupled from PIP and ALD1. However, TXA's precise mode of action remains to be clarified.

TXA activates a subset of defense-related responses

From the identified compounds, TXA was the only selective activator of *FMO1* expression. Other early defense responses such as ROS production, MAPK activation and intracellular calcium spiking were not affected (**Table 1**). Thus, TXA appears to act on a particular sector of the defense signaling network,

which also leads to induction of *PR1* gene expression (140-fold at 100 nM TXA; **Fig. 4C**) mediated by enhanced SA production (**Fig. 4A**). This observation is in accordance with previous reports showing that TXA treatment activates defense responses in Arabidopsis (Duval et al. 2005, Errakhi et al. 2008, Bischoff et al. 2009, Duval and Beaudoin 2009). However, some of these reports are conflicting with respect to *PR1* expression, which was up-regulated (Bischoff et al. 2009) or not affected by TXA treatment (Duval et al. 2005, Errakhi et al. 2008, Duval and Beaudoin 2009). TXA is a phytotoxin causing cell necrosis in a range of plants (including Arabidopsis), but it also has fungicidal and antiviral activity (Zhang et al. 2013). It is conceivable that application of TXA at high concentrations (5–10 μM) prevents *PR1* expression as a result of extensive tissue damage (Duval et al. 2005, Errakhi et al. 2008, Duval and Beaudoin 2009), in contrast to localized cell death occurring upon pathogen infection. Moreover, TXA is thought to be responsible for disease symptoms on host plants infected by *S. scabies* as the pathogenicity of strains correlated with TXA amounts produced (King et al. 1991, Goyer et al. 1998, King and Calhoun 2009). Inoculation of potato plants with TXA-producing *S. scabies* strains increased expression of various SA-dependent defense genes including *PR-1b*, *PR-2* and *PR-5* (Arseneault et al. 2014). Notably, TXA-activated responses did not include ROS production, proton uptake (alkalization of growth medium) or activation of the ethylene/JA pathways (Duval et al. 2005), which our observations confirm and extend to calcium spiking, MAPK activation and the JA, ABA and auxin signaling pathways (**Table 1**). Under our assay conditions, TXA treatment (100 nM, 24 h) did not initiate visible necrosis or ion leakage (Supplementary Fig. S8). We therefore excluded TXA-induced cell death as a major driver of *FMO1* expression and activator of defense responses.

Positioning TXA action in the defense network

Several studies have established that *FMO1* expression is activated independently of SA production in pathogen-infected leaves (Bartsch et al. 2006, Koch et al. 2006, Mishina and Zeier 2006, Olszak et al. 2006, Gruner et al. 2013). Here, we found that TXA-induced *FMO1* expression also did not require ICS1-generated SA (**Fig. 5**). Nevertheless, TXA did lead to accumulation of free and conjugated SA and stimulation of *PR1* gene expression (**Fig. 4**). SA or SA-related molecules also stimulate *EDS1* and *PAD4* expression as part of a positive amplification loop that enhances pathogen resistance locally and systemically (Zhou et al. 1998, Jirage et al. 1999, Feys et al. 2001), thereby probably contributing to *EDS1*- and *PAD4*-dependent increases in SA and induction of *ALD1* and *FMO1* in response to TXA (**Fig. 4**) (Song et al. 2004) [data accessible at the NCBI Gene Expression Omnibus (GEO), accession GSE14961; Expression data from Arabidopsis seedlings treated with salicylic acid]. The correlation between pathogen-induced *FMO1* expression and SA accumulation is not restricted to infected leaves (local response) but extends to distal tissues (SAR). Indeed, establishment of SAR in Arabidopsis was associated with systemic *FMO1* expression and SA accumulation (Bernsdorff et al. 2016). In the Arabidopsis *fmo1* mutant, systemic SA accumulation and SAR were defective, whereas the local SA accumulation, defense activation and disease resistance were not impaired (Bartsch et al. 2006, Mishina and Zeier 2006). Thus, activation of *ICS1* and *FMO1* expression and accumulation of SA occurs through independent pathways, with TXA probably affecting a common regulatory hub, the EDS1–PAD4 complex (**Fig. 8**).

The non-proteinogenic amino acid PIP is an essential regulator of inducible plant immunity (SAR) and systemic *FMO1* and *PR1* expression (Návarová et al. 2012, Bernsdorff et al. 2016). Based on previous results (Gupta and Spenser 1969, Song et al. 2004, Návarová et al. 2012), it was recently shown that PIP biosynthesis from lysine proceeds via two enzymatic steps, catalyzed by the aminotransferase ALD1 and the reductase SAR-DEFICIENT4 (SARD4) (Hartmann et al. 2017). However, when quantifying PIP (and other amino acids), we did not detect significant differences in PIP levels in response to TXA treatment, although *ALD1* expression was strongly up-regulated (**Fig. 4**). Possibly, *ALD1* mRNA is not effectively translated or SARD4 might not be co-ordinately expressed with ALD1 in response to TXA treatment. Notably, TXA-induced *FMO1* expression was unimpaired in the Arabidopsis *ald1* mutant, which is defective in PIP production, suggesting that PIP is not involved in TXA-mediated *FMO1* expression (**Fig. 5**). This corroborates earlier findings that *ald1* plants activate *FMO1* expression similar to wild-type plants upon inoculation with *P. syringae* (Bernsdorff et al. 2016). A complex role for PIP in *FMO1* expression was previously reported. Mere elevation of PIP levels (e.g. by exogenous PIP application) only moderately activated *FMO1* expression, but, when combined with pathogen infection, *FMO1* expression was strongly enhanced, suggesting that PIP amplifies another signal (or signals) rather than being an exclusive trigger (Návarová et al. 2012, Hartmann et al. 2018). TXA treatment caused strongly increased *PAD4* expression (**Figs. 4, 6A**), and pathogen-induced *ALD1* expression and PIP production in systemic tissues were strongly dependent on *PAD4* (Návarová et al. 2012). Thus, the observed increase in *ALD1* expression is likely to be a consequence of increased PAD4 abundance in TXA-treated plants (**Fig. 8**). The recently discovered activity of FMO1 as PIP *N*-hydroxylase suggests that TXA treatment induces resistance via production of the SAR-inducer NHP (Hartmann et al. 2018)(**Fig. 8**). As in induction of resistance by TXA, NHP accumulation upon *P. syringae* inoculation also depended on functional *PAD4* and *EDS1* signaling (Hartmann et al. 2018).

PAD4 is highly responsive to TXA

Pathogen-induced *FMO1* expression is controlled by *EDS1* and *PAD4* (Bartsch et al. 2006). In our experiments, TXA-induced *FMO1* expression was also fully dependent on *EDS1* and *PAD4*, and genetically independent of *SAG101* (**Fig. 5**). EDS1 forms structurally related heterodimers with PAD4 or SAG101, which activate downstream defense responses (Wagner et al.

2013). With respect to TXA treatment, our data suggest that EDS1 operates principally with PAD4 in *FMO1* activation (**Fig. 6**). Whereas EDS1–PAD4 co-operation defines many pathogen-triggered TNL receptor-mediated and basal immune responses, EDS1 shows a preference for interaction with SAG101 in at least one TNL receptor-mediated autoimmune response (Xu et al. 2015), suggesting that EDS1–PAD4 and EDS1–SAG101 complexes can be engaged selectively in different immunity pathways.

Expression of *EDS1* and *PAD4* was induced by TXA treatment (**Fig. 6**) and therefore the EDS1–PAD4 complex or components mediating its function, such as TNL receptors, are potential targets of TXA. Analysis of *eds1-2* and *pad4-1* mutants revealed that TXA-induced *PAD4* expression was partly independent of *EDS1*, whereas TXA-induced *EDS1* expression was strictly dependent on *PAD4* (**Fig. 6**). Increased *PAD4* expression was mirrored by PAD4 protein accumulation in TXA-treated wild-type plants (**Fig. 6D**). In *pad4-1* seedlings, *EDS1* mRNA and EDS1 protein accumulated before and after TXA treatment (**Fig. 6**), possibly due to EDS1 stabilization by SAG101 (Feys et al. 2005, Wagner et al. 2013). In contrast, PAD4 protein was undetectable in the *eds1-2* mutant before and after TXA treatment, despite appreciable *PAD4* mRNA accumulation (**Fig. 6**). This underscores the importance of EDS1 in stabilizing PAD4 in heterodimeric complexes (Feys et al. 2005, Rietz et al. 2011, Cui et al. 2017). Based on these results, we propose that TXA targets a component (or components) acting upstream of *EDS1* and *PAD4* expression (**Fig. 8**). TXA's preferential stimulation of *PAD4* expression (**Fig. 6A**) might reflect higher turnover of *PAD4* mRNA compared with *EDS1* mRNA, or a unique role for PAD4 in responding to TXA treatment. In this regard, PAD4-controlled resistance against green peach aphids in Arabidopsis was found to work independently of EDS1 or SA accumulation (Pegadaraju et al. 2007, Louis et al. 2012), indicating a unique PAD4 function in this stress response.

TXA activates EDS1- and PAD4-dependent disease resistance

Small bioactive molecules of natural or synthetic origin that induce plant defense responses and thus mimic infection by pathogens have been applied in basic research and natural field conditions to protect crops from disease (Bektas and Eulgem 2015). Because TXA treatment selectively activated EDS1–PAD4-controlled defense outputs, we asked whether TXA also provides protection against pathogen infection.

TXA treatment of Arabidopsis leaves, followed by inoculation with virulent *P. syringae* pv. *tomato* strain DC3000, led to reduced bacterial titers compared with mock-treated control leaves (**Fig. 7A**). No chemically induced resistance was observed in *pad4-1*, *eds1-2*, *sid2-2/ics1* or *fmo1-1* mutants, in accordance with TXA-induced expression of the corresponding genes in wild-type plants (**Fig. 4C**). Treatment with a low dose of TXA (100 nM, used throughout our work) did not cause macroscopic necrotic lesioning or measurable ion leakage, which were apparent at higher concentrations (e.g. 1 mM) or extended exposure times (Supplementary Fig. S8). We conclude that TXA does not mediate its effects on defense gene expression through general toxicity or stimulation of plant cell death.

TXA-induced resistance in Arabidopsis was also effective against the virulent oomycete, *H. arabidopsidis* (**Fig. 7B**). Formation of HR-like lesions restricting hyphal growth in TXA-treated leaves marked a clear phenotypic difference from inoculated control leaves (**Fig. 7B;** Supplementary Fig. S12). In accordance with TXA-activated expression of *EDS1* and *PAD4*, TXA-induced resistance was ineffective in *eds1-2* and *pad4-1* mutant leaves. The failure also of *fmo1-1* and *sid2-2/ics1* mutants to mount TXA-induced resistance towards *H. arabidopsidis* is consistent with the importance of these components, respectively, in EDS1/PAD4-driven SA-independent and SA-dependent signaling branches (Nawrath and Métraux 1999, Bartsch et al. 2006, Mishina and Zeier 2006). Notably, equivalent growth of bacteria and oomycete pathogens in the various mutant backgrounds with or without TXA treatment indicates that the compound does not exert a direct antimicrobial effect under the conditions applied here.

In conclusion, activation of *EDS1*/*PAD4* signaling by TXA leading to mobilization of SA-dependent and SA-independent defense pathways is associated with strong up-regulation of *PAD4* expression and PAD4 accumulation, and co-ordinated actions of PAD4 with EDS1, probably as heterodimeric complexes (**Fig. 8**). This suggests that one or several components controlling promoter activity (e.g. transcription factors or regulators) are potential targets of TXA in Arabidopsis. TXA might also have an effect on the distribution or activity of the EDS1–PAD4 complex. The consequence of TXA targeting this regulatory node is mobilization of defense pathways represented by *FMO1*, *ICS1* and *ALD1* (**Fig. 8**). TXA does not activate other common defense responses, such as ROS production, MAPK signaling and calcium spiking (**Table 1**). Although the precise mode of action of TXA remains to be clarified, its selective and potent activity suggest it might be a powerful chemical tool to interrogate EDS1/PAD4 signaling in plant immunity further.

Materials and Methods

Plant material and growth conditions

We used *Arabidopsis thaliana* accession Col-0 (wild-type) and the previously described mutants *fmo1-1*, *eds1-2* (Bartsch et al. 2006), *pad4-1* (Jirage et al. 1999), *sag101-3* (Feys et al. 2005), *sid2-2/ics1* (Nawrath and Métraux 1999), *ald1* (Song et al. 2004) and the double mutant *eds1-2 pad4-1* (Wagner et al. 2013). A new *sag101* mutation in accession Col-0, named *sag101-3*, was selected from the GABI-KAT T-DNA collection (https://www.gabi-kat.de) and verified as having a T-DNA disruption of *SAG101* using PCR-based mutant detection and sequencing. A homozygous *sag101-3* mutant was crossed with *pad4-1* to generate a *pad4-1 sag101-3* double mutant line, which was verified by PCR-based mutant detection and pathogen phenotyping. An *eds1-2 sid2-1* double mutant line was kindly provided by Haitao Cui (Max Planck Institute for Plant Breeding Research). Primers used for mutant detection are available on request. Transgenic Arabidopsis lines expressing genomic *FMO1* fused to *YFP* under control of 5′-regulatory *FMO1* gene sequences (*FMO1p::FMO1-YFP*) were generated in the *fmo1-1* mutant by *Agrobacterium*-mediated transformation (Clough and Bent 1998). *FMO1* genomic DNA was cloned to generate

pENTR/D FMO1p::FMO1, which was then recombined into a pAMPAT::YFP destination binary vector pXCG-YFP (Witte et al. 2004) producing pXC::FMO1p::FMO1-YFP. Three selected homozygous transgenic lines expressing *FMO1p::FMO1-YFP* (lines 36-4, 60-3 and 65-5) fully complemented *fmo1-1* in resistance to *H. arabidopsidis* isolate Cala2 (Supplementary Fig. S1). One line (internal number 60-3) was selected for library screening and subsequent experiments. In addition, we used *Arabidopsis* (Col-0) lines expressing the GUS reporter under the control of different promoters, SA-responsive *PR1p::GUS* (Shapiro and Zhang 2001), JA-responsive *VSP1p::GUS* (Zheng et al. 2006) and *LOX2p::GUS* (Jensen et al. 2002), or the cytosolic calcium sensor apoequorin, *pMAQ2* (Knight et al. 1991, Maintz et al. 2014).

For chemical treatment and screening, Arabidopsis seedlings were grown hydroponically in transparent 96-well microplates (PerkinElmer) containing 160 µl of half-strength Murashige and Skoog (MS) basal salt medium (Murashige and Skoog 1962) supplemented with 0.5% sucrose (one surface-sterilized seed per well). Unless otherwise stated, plates were placed for 12–16 d in a growth chamber with a cycle of 16 h light (21°C) and 8 h dark (19°C). For maintenance and infection experiments, plants were cultivated in peat-based soil (Stender AG) with a cycle of 10 h light (22°C, 200 µE m^{-2} s^{-1}) and 14 h dark (20°C), and 65% relative humidity.

Screening for activators of FMO1–YFP expression

For chemical screening, Arabidopsis seedlings expressing *FMO1p::FMO1-YFP* were grown in liquid culture (96-well microplates) for 14 d. Growth medium was removed and replaced by fresh medium with added chemicals (10–20 µM, depending on the library) and a final solvent (DMSO) concentration not exceeding 1%. YFP fluorescence was recorded in seedlings after 3–6 d of incubation in various consecutive readings using a FloroCount BF10000 (Packard Bioscience) or a Centro *XS*^{3} LB 960 Microplate Reader (Berthold Technologies) with an excitation/emission wavelength of 485/535 nm. Treatment with the fungal toxin fumonisin B1 (10 µM), which was previously shown to activate expression of *FMO1* in Arabidopsis (Olszak et al. 2006), served as positive control. For initial library screening, two or three biological replicates per chemical were averaged and normalized to appropriate control samples, e.g. averaged fluorescence values of the whole screening plate or samples without added chemicals that were contained on the same plate. For confirmation of selected primary hits and other assay conditions, 2–6 biological replicates were analyzed.

Quantification of GUS activity in reporter lines

Arabidopsis lines harboring different GUS reporters were grown in liquid medium (96-well microplates) for 12–16 d and gene expression activated by treatment with the appropriate inducer as previously reported to yield maximum activity, i.e. *PR1p::GUS* was treated with 200 µM SA for 24 h, and *VSP1p::GUS* and *LOX2p::GUS* with 100 µM jasmonic acid methyl ester (JAMe) for 24 h (Halder and Kombrink 2015). For combined treatment with TXA or other compounds, these chemicals were added 1 h prior to induction of gene expression to determine their potential as inhibitors; alternatively, the inducer was omitted to determine the chemicals' potential as activators. Following this treatment, the medium was removed by aspiration and seedlings were used immediately (or stored at −80°C) for quantification of GUS activity in situ using a microplate reader (FluoroCount, Packard Bioscience) with an excitation/emission wavelength of 365/455 nm as previously described (Halder and Kombrink 2015). All reported values are the mean (±SD) of at least four biological replicates.

Quantitative Ca^{2+} measurement

Arabidopsis line pMAQ2 (Knight et al. 1991), expressing cytosolic apoaequorin, was used to monitor rapid, elicitor-induced changes in cellular Ca^{2+} concentrations as previously reported (Maintz et al. 2014). In brief, 14-day-old seedlings, grown hydroponically in microplates, were charged with coelenterazine (10 µM, 100 µl) by overnight treatment, then the liquid was replaced by water or solutions of chemicals (20 µM TXA, 20 µM MON or 20 µM MER) and seedlings incubated for 30 min in the dark, before the Ca^{2+} response was initiated by addition of 1 µM peptide epitope of bacterial flagellin (flg22). The Ca^{2+}-dependent bioluminescence was recorded for 30 min in a Centro *XS*^{3} LB 960 or TriStar LB 942 Microplate Reader (Berthold Technologies) as described (Maintz et al. 2014). For each treatment, at least three replicates were recorded and the resulting Ca^{2+} traces averaged to provide the result of one experiment, which was repeated at least two or three times.

Oxidative burst assay

Production of ROS was determined by H_2O_2-dependent luminescence of luminol as previously described, with minor modifications (Serrano et al. 2007, Meesters et al. 2014). Arabidopsis seedlings, grown for 14 d in liquid medium, were washed in 300 µl of water and transferred to 96-well microplates containing 100 µl of water. Following incubation for 2 h, ROS production was triggered by adding chemicals (100 nM TXA, 10 µM MON or 10 µM MER), 1 µM flg22 as positive control or 1% DMSO as negative control, followed by immediate injection of 100 µl of assay solution containing 400 µM luminol and 0.02 mg ml^{-1} horseradish peroxidase (HRP; Sigma-Aldrich, P6782). Luminescence was recorded for 2 s per well and the cycle was repeated every 2 min for a total time period of 72 min (in exceptional cases, this period was extended up to 24 h), using a TriStar LB 942 Microplate Luminometer (Berthold Technologies). Each measurement was carried out in 12 replicates.

MAPK assays

MAPK activation was detected by immunoblot analysis of soluble proteins extracted from the seedlings in a lysis buffer as described earlier (Saijo et al. 2009, Ranf et al. 2011), using anti-phospho p44/p42 MAPK (Erk1/2) (Tyr202/Tyr204) antibody (Cell Signaling Technology, New England Biolabs).

Protein blot analysis

Total soluble proteins were separated by SDS–PAGE and blotted onto a Hybond ECL nitrocellulose membrane (GE Healthcare Life Sciences) as previously described (Serrano et al. 2010). Equal protein loading and transfer onto the membrane was monitored by staining with 0.1% Ponceau S (Sigma-Aldrich), followed by extensive washing. For detection of EDS1 or PAD4, polyclonal antisera were used as described (Feys et al. 2005, Rietz et al. 2011). For FMO1–YFP detection, an antiserum directed against green fluorescent protein (GFP; Thermo Fisher Scientific) was used. All primary antisera were used at dilutions of 1:1,000 and incubated with the membrane overnight at 4°C. Specific protein detection was achieved using HRP-conjugated secondary antibodies (rabbit) and Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific); the results were documented on a ChemicDoc MP system (Bio-Rad Laboratories).

Measurement of electrolyte leakage

The quantification of electrolyte leakage for evaluation of tissue damage during infection and abiotic stress has previously been described (Mur et al. 2006, Chen et al. 2015). Arabidopsis seedlings, grown for 14 d in liquid medium, were placed in 2 ml of water (24-well microplate) and treated with chemicals (100 nM TXA) or DMSO (1%). The conductivity of the medium was determined at selected time intervals (2–48 h) in a 60 µl sample using a HORIBA B-173 Compact Twin Conductivity Meter (Horiba Ltd.). The experiment was carried out in six replicates per time point and genotype, and repeated three times with similar results.

Quantification of SA and amino acids

SA measurements were obtained from seedlings (100–200 mg) as previously described (Straus et al. 2010, Schlicht and Kombrink 2013). Amino acid levels were determined according to Návarová *et al.* (2012) using 50–100 mg of seedlings that were frozen after treatment with TXA (or DMSO as control).

Disease resistance assays

Leaves of 5-week-old Arabidopsis plants were infiltrated with TXA (100 nM) or DMSO (0.001%) as control and 24 h later spray-inoculated with *P. syringae* pv. *tomato* strain DC3000 (10^5 c.f.u. ml^{-1}), which is virulent on Arabidopsis wild type (Col-0), as previously reported (Birker et al. 2009). Bacterial titers were quantified at 3 d after inoculation in 18 leaf disks that were taken from six plants per genotype. Infection assays were performed four times independently.

Conidiospores of *H. arabidopsidis* isolate Noco2 (4×10^4 spores ml^{-1}) were spray-inoculated onto 5-week-old plants after 24 h pre-treatment with TXA

(100 nM) or DMSO (0.001%) as previously described (Stuttmann et al. 2011). Four days after inoculation, development of a host HR and growth of *H. arabidopsidis* hyphae were visualized by trypan blue staining of leaves as described previously (Aarts et al. 1998, Stuttmann et al. 2011). A minimum of 20 leaves per genotype were analyzed with an AxioImager.A2 microscope equipped with an AxioCam HRc camera system (Carl Zeiss). For quantitative conidiospore assays, 3–4 pots of each genotype were pre-treated and inoculated as above. After 6–7 d, the leaves of each pot (sampled as one replicate) were used for counting conidiospores in a hemocytometer as done previously (Stuttmann et al. 2011). Spore counting assays were performed at least three times independently.

qRT-PCR analysis

Relative transcript levels were determined by qRT-PCR according to established protocols (Schmittgen and Livak 2008, Weßling and Panstruga 2012). Total RNA was extracted from 2-week-old seedlings (approximately 20 mg) using the RNeasy Plant Mini Kit (QIAGEN) or the my-Budget Plant RNA Kit (Bio-Budget Technologies GmbH) following the manufacturer's instructions. cDNA was synthesized from 1 µg of RNA using SuperScript™II Reverse Transcriptase (Invitrogen) and random hexamer primers following the supplier's instructions. All qPCR assays were performed with cDNA (diluted 1:20) corresponding to 25 ng of RNA using the iQTM SYBR Green Supermix kit (Bio-Rad Laboratories) on the iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories) as previously described. Gene-specific primers were used for target gene amplification at final concentrations of 0.1 µM, and gene At4g26410 served as control (FMO1-forward: 5′-GTTCGTGGTTGTGTGTACCG-3′, FMO1-reverse: 5′-TGTGCAAGCTTTTCCTCCTT-3′; EDS1-forward: 5′-CGAAGACACA GGGCCGTA-3′, EDS1-reverse: 5′-AAGCATGATCCGCACTCG-3′; PAD4-forward: 5′-G GTTCTGTTCGTCTGATGTTT-3′, PAD4-reverse: 5′-GTTCCTCGGTGTTTTGAGTT-3′; SAG101-forward: 5′-CATTCCTCTGCTCCGAGAAC-3′, SAG101-reverse: 5′-CGTTTT AACGTCGGTTCGAT-3′; PR1-forward: 5′-TTCTTCCCTCGAAAGCTCAA-3′, PR1-reverse: 5′-AAGGCCCACCAGAGTGTATG-3′; ALD1-forward: 5′-ACTTGGTGGCAGCA CAAAAC-3′, ALD1-reverse: 5′-ATCACCAGTCCCAAGGCTTATC-3′; ICS1-forward: 5′-TTCTGGGCTCAAACACTAAAAC-3′, ICS1-reverse: 5′-GGCGTCTTGAAATCTCCATC-3′; PBS3-forward: 5′-ACACCAGCCCTGATGAAGTC-3′, PBS3-reverse: 5′-CCCAAGTCT GTGACCCAGTT-3′; VSP1-forward: 5′-TCATACTCAAGCCAAACGG-3′, VSP1-reverse: 5′-ATCCTCAACCAAATCAGC-3′; CESA5-forward: 5′-CATCGTCCCTGAGATTAGCAA CT-3′, CESA5-reverse: 5′-AGCAATCGACCCGAAAAGTG-3′; CESA6-forward: 5′-GGAA AATTCATCGTTCCCGAG-3′, CESA6-reverse: 5′- AGAAGAGCGCCATGAAGAGG-3′; CESA7-forward: 5′-GAGCTGGGTTGGATCTATGGC-3′, CESA7-reverse: 5′- AATGCAT CTTGAATCCCGTCA-3′; CESA8-forward: 5′-AGCTATTTGCCTTCTTACCGGC-3′, CESA8-reverse: 5′- CATGCTTGCTAGGTTTGATAGCG-3′; At4g26410-forward: 5′-GA GCTGAAGTGGCTTCCATGAC-3′, At4g26410-reverse: 5′-GGTCCGACATACCCATGA TCC-3′). qPCR assays were carried out in three biological samples per treatment and two technical replicates per sample according to the following conditions: denaturation at 95°C for 2 min, 40 repeats at 95°C for 20 s, 56°C for 30 s and 72°C for 25 s. Relative expression levels were calculated using the ΔΔCT method (Schmittgen and Livak 2008) and normalized to the expression in control plants.

Statistical analyses

Quantitative data analyses were performed in Excel spreadsheets with the embedded basic statistical functions (mean, SD, Student's *t*-test, root-mean-square linear regression). For statistical evaluation of the screening data, the *Z*-score was calculated as previously described (Malo et al. 2006; Serrano et al. 2015) and compounds producing values exceeding 1.5 were selected for further analysis.

Supplementary Data

Supplementary data are available at PCP online.

Funding

This work was supported by the Max Planck Society and the Deutsche Forschungsgemeinschaft [Priority Programm 1212, grant PA 917/2-2 to J.E.P] and the Max Planck Institute for Plant Breeding Research, Köln [an International Max Planck Research School doctoral fellowship to S.J.].

Disclosures

The authors have no conflicts of interest to declare.

Acknowledgments

We thank Dr. Thomas Griebel (Max Planck Institute for Plant Breeding Research, Köln) for help with salicylic acid quantification, and Drs. Marc R. Knight, Chuanyou Li, John Mundy, Dierk Scheel and Allan D. Shapiro for kindly providing seeds of Arabidopsis mutants and reporter lines.

References

Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J. and Parker, J.E. (1998) Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two *R* gene-mediated signaling pathways in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 95: 10306–10311.

Allen, G.J., Kuchitsu, K., Chu, S.P., Murata, Y. and Schroeder, J.I. (1999) Arabidopsis *abi1-1* and *abi2-1* phosphatase mutations reduce abscisic acid-induced cytoplasmic calcium rises in guard cells. *Plant Cell* 11: 1785–1798.

Arseneault, T., Pieterse, C.M.J., Gérin-Ouellet, M., Goyer, C. and Filion, M. (2014) Long-term induction of defense gene expression in potato by *Pseudomonas* sp. LBUM223 and *Streptomyces scabies*. *Phytopathology* 104: 926–932.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.-L., Gomez-Gomez, L., et al. (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature* 415: 977–983.

Bartsch, M., Gobbato, E., Bednarek, P., Debey, S., Schultze, J.L., Bautor, J., et al. (2006) Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in *Arabidopsis* immunity and cell death is regulated by the monooxygenase *FMO1* and the nudix hydrolase *NUDT7*. *Plant Cell* 18: 1038–1051.

Bektas, Y. and Eulgem, T. (2015) Synthetic plant defense elicitors. *Front. Plant Sci.* 5: 804.

Bernsdorff, F., Döring, A.-C., Gruner, K., Schuck, S., Bräutigam, A. and Zeier, J. (2016) Pipecolic acid orchestrates plant systemic acquired resistance and defense priming via salicylic acid-dependent and -independent pathways. *Plant Cell* 28: 102–129.

Bhattacharjee, S., Halane, M.K., Kim, S.H. and Gassmann, W. (2011) Pathogen effectors target *Arabidopsis* EDS1 and alter its interactions with immune regulators. *Science* 334: 1405–1408.

Birker, D., Heidrich, K., Takahara, H., Narusaka, M., Deslandes, L., Narusaka, Y., et al. (2009) A locus conferring resistance to *Colletotrichum higginsianum* is shared by four geographically distinct Arabidopsis accessions. *Plant J.* 60: 602–613.

Bischoff, V., Cookson, S.J., Wu, S. and Scheible, W.-R. (2009) Thaxtomin A affects CESA-complex density, expression of cell wall genes, cell wall composition, and causes ectopic lignification in *Arabidopsis thaliana* seedlings. *J. Exp. Bot.* 60: 955–965.

Chen, Q.-F., Xu, L., Tan, W.-J., Chen, L., Qi, H., Xie, L.-J., et al. (2015) Disruption of the *Arabidopsis* defense regulator genes *SAG101*, *EDS1*, and *PAD4* confers enhanced freezing tolerance. *Mol. Plant* 8: 1536–1549.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16: 735–743.

Conrath, U., Silva, H. and Klessig, D.F. (1997) Protein dephosphorylation mediates salicylic acid-induced expression of *PR-1* genes in tobacco. *Plant J.* 11: 747–757.

Cui, H., Gobbato, E., Kracher, B., Qiu, J., Bautor, J. and Parker, J.E. (2017) A core function of EDS1 with PAD4 is to protect the salicylic acid defense sector in Arabidopsis immunity. *New Phytol.* 213: 1802–1817.

Cui, H., Tsuda, K. and Parker, J.E. (2015) Effector-triggered immunity: from pathogen perception to robust defense. *Annu. Rev. Plant Biol.* 66: 487–511.

Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R. and Abrams, S.R. (2010) Abscisic acid: emergence of a core signaling network. *Annu. Rev. Plant Biol.* 61: 651–679.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K. and Scheible, W.-R. (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiol.* 139: 5–17.

De Rybel, B., Audenaert, D., Vert, G., Rozhon, W., Mayerhofer, J., Peelman, F., et al. (2009) Chemical inhibition of a subset of *Arabidopsis thaliana* GSK3-like kinases activates brassinosteroid signaling. *Chem. Biol.* 16: 594–604.

Desprez, T., Vernhettes, S., Fagard, M., Refrégier, G., Desnos, T., Aletti, E., et al. (2002) Resistance against herbicide isoxaben and cellulose deficiency caused by distinct mutations in same cellulose synthase isoform CESA6. *Plant Physiol.* 128: 482–490.

Duval, I. and Beaudoin, N. (2009) Transcriptional profiling in response to inhibition of cellulose synthesis by thaxtomin A and isoxaben in *Arabidopsis thaliana* suspension cells. *Plant Cell Rep.* 28: 811–830.

Duval, I., Brochu, V., Simard, M., Beaulieu, C. and Beaudoin, N. (2005) Thaxtomin A induces programmed cell death in *Arabidopsis thaliana* suspension-cultured cells. *Planta* 222: 820–831.

Ellis, C. and Turner, J.G. (2001) The Arabidopsis mutant *cev1* has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. *Plant Cell* 13: 1025–1033.

Errakhi, R., Dauphin, A., Meimoun, P., Lehner, A., Reboutier, D., Vatsa, P., et al. (2008) An early Ca^{2+} influx is a prerequisite to thaxtomin A-induced cell death in *Arabidopsis thaliana* cells. *J. Exp. Bot.* 59: 4259–4270.

Feys, B.J., Moisan, L.J., Newman, M.-A. and Parker, J.E. (2001) Direct interaction between the *Arabidopsis* disease resistance signaling proteins, EDS1 and PAD4. *EMBO J.* 20: 5400–5411.

Feys, B.J., Wiermer, M., Bhat, R.A., Moisan, L.J., Medina-Escobar, N., Neu, C., et al. (2005) *Arabidopsis* SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. *Plant Cell* 17: 2601–2613.

Fujii, H., Verslues, P.E. and Zhu, J.-K. (2007) Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in *Arabidopsis*. *Plant Cell* 19: 485–494.

Gao, Y., Zeng, Q., Guo, J., Cheng, J., Ellis, B.E. and Chen, J.-G. (2007) Genetic characterization reveals no role for the reported ABA receptor, GCR2, in ABA control of seed germination and early seedling development in Arabidopsis. *Plant J.* 52: 1001–1013.

García, A.V., Blanvillain-Baufumé, S., Huibers, R.P., Wiermer, M., Li, G., Gobbato, E., et al. (2010) Balanced nuclear and cytoplasmic activities of EDS1 are required for a complete plant innate immune response. *PLoS Pathog.* 6: e1000970.

Glazebrook, J. (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43: 205–227.

Goyer, C., Vachon, J. and Beaulieu, C. (1998) Pathogenicity of *Streptomyces scabies* mutants altered in thaxtomin A production. *Phytopathology* 88: 442–445.

Gruner, K., Griebel, T., Návarová, H., Attaran, E. and Zeier, J. (2013) Reprogramming of plants during systemic acquired resistance. *Front. Plant Sci.* 4: 252.

Gupta, R.N. and Spenser, I.D. (1969) Biosynthesis of the piperidine nucleus. The mode of incorporation of lysine into pipecolic acid and into piperidine alkaloids. *J. Biol. Chem.* 244: 88–94.

Halder, V. and Kombrink, E. (2015) Facile high-throughput forward chemical genetic screening by *in situ* monitoring of glucuronidase-based reporter gene expression in *Arabidopsis thaliana*. *Front. Plant Sci.* 6: 13.

Hartmann, M., Kim, D., Bernsdorff, F., Ajami-Rashidi, Z., Scholten, N., Schreiber, S., et al. (2017) Biochemical principles and functional aspects of pipecolic acid biosynthesis in plant immunity. *Plant Physiol.* 174: 124–153.

Hartmann, M., Zeier, T., Bernsdorff, F., Reichel-Deland, V., Kim, D., Hohmann, M., et al. (2018) Flavin monooxygenase-generated N-hydroxypipecolic acid is a critical element of plant systemic immunity. *Cell* 173: 456–469.

Heidrich, K., Wirthmueller, L., Tasset, C., Pouzet, C., Deslandes, L. and Parker, J.E. (2011) *Arabidopsis* EDS1 connects pathogen effector recognition to cell compartment-specific immune responses. *Science* 334: 1401–1404.

Hicks, G.R. and Raikhel, N.V. (2010) Advances in dissecting endomembrane trafficking with small molecules. *Curr. Opin. Plant Biol.* 13: 706–713.

Hicks, G.R. and Raikhel, N.V. (2012) Small molecules present large opportunities in plant biology. *Annu. Rev. Plant Biol.* 63: 261–282.

Jensen, A.B., Raventos, D. and Mundy, J. (2002) Fusion genetic analysis of jasmonate-signalling mutants in *Arabidopsis*. *Plant J.* 29: 595–606.

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., et al. (1999) *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. *Proc. Natl. Acad. Sci. USA* 96: 13583–13588.

Jones, J.D.G. and Dangl, J.L. (2006) The plant immune system. *Nature* 444: 323–329.

Kaschani, F. and van der Hoorn, R. (2007) Small molecule approaches in plants. *Curr. Opin. Chem. Biol.* 11: 88–98.

King, R.R. and Calhoun, L.A. (2009) The thaxtomin phytotoxins: sources, synthesis, biosynthesis, biotransformation and biological activity. *Phytochemistry* 70: 833–841.

King, R.R., Lawrence, C.H. and Clark, M.C. (1991) Correlation of phytotoxin production with pathogenicity of *Streptomyces scabies* isolates from scab infected potato tubers. *Amer. Potato J.* 68: 675–680.

King, R.R., Lawrence, C.H., Clark, M.C. and Calhoun, L.A. (1989) Isolation and characterization of phytotoxins associated with *Streptomyces scabies*. *J. Chem. Soc. Chem. Commun.* 849–850.

Kinkel, L.L., Bowers, J.H., Shimizu, K., Neeno-Eckwall, E.C. and Schottel, J.L. (1998) Quantitative relationships among thaxtomin A production, potato scab severity, and fatty acid composition in *Streptomyces*. *Can. J. Microbiol.* 44: 768–776.

Knight, M.R., Campbell, A.K., Smith, S.M. and Trewavas, A.J. (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* 352: 524–526.

Knoth, C., Salus, M.S., Girke, T. and Eulgem, T. (2009) The synthetic elicitor 3,5-dichloroanthranilic acid induces *NPR1*-dependent and *NPR1*-independent mechanisms of disease resistance in Arabidopsis. *Plant Physiol.* 150: 333–347.

Koch, M., Vorwerk, S., Masur, C., Sharifi-Sirchi, G., Olivieri, N. and Schlaich, N.L. (2006) A role for a flavin-containing mono-oxygenase in resistance against microbial pathogens in Arabidopsis. *Plant J.* 47: 629–639.

Lecourieux, D., Mazars, C., Pauly, N., Ranjeva, R. and Pugin, A. (2002) Analysis and effects of cytosolic free calcium increases in response to elicitors in *Nicotiana plumbaginifolia* cells. *Plant Cell* 14: 2627–2641.

Louis, J., Gobbato, E., Mondal, H.A., Feys, B.J., Parker, J.E. and Shah, J. (2012) Discrimination of Arabidopsis PAD4 activities in defense against green peach aphid and pathogens. *Plant Physiol.* 158: 1860–1872.

Maintz, J., Cavdar, M., Tamborski, J., Kwaaitaal, M., Huisman, R., Meesters, C., et al. (2014) Comparative analysis of MAMP-induced calcium influx

in *Arabidopsis* seedlings and protoplasts. *Plant Cell Physiol*. 55: 1813–1825.
Malo, N., Hanley, J.A., Cerquozzi, S., Pelletier, J. and Nadon, R. (2006) Statistical practice in high-throughput screening data analysis. *Nat. Biotechnol.* 24: 167–175.
Massillon, D., Stalmans, W., van de Werve, G. and Bollen, M. (1994) Identification of the glycogenic compound 5-iodotubercidin as a general protein kinase inhibitor. *Biochem. J.* 299: 123–128.
McCourt, P. and Desveaux, D. (2010) Plant chemical genetics. *New Phytol.* 185: 15–26.
Meesters, C., Mönig, T., Oeljeklaus, J., Krahn, D., Westfall, C.S., Hause, B., et al. (2014) A chemical inhibitor of jasmonate signaling targets JAR1 in *Arabidopsis thaliana*. *Nat. Chem. Biol.* 10: 830–836.
Meggio, F., Deana, A.D., Ruzzene, M., Brunati, A.M., Cesaro, L., Guerra, B., et al. (1995) Different susceptibility of protein kinases to staurosporine inhibition. *Eur. J. Biochem.* 234: 317–322.
Mishina, T.E. and Zeier, J. (2006) The Arabidopsis flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. *Plant Physiol*. 141: 1666–1675.
Morales, J., Kadota, Y., Zipfel, C., Molina, A. and Torres, M.-A. (2016) The Arabidopsis NADPH oxidases *RbohD* and *RbohF* display differential expression patterns and contributions during plant immunity. *J. Exp. Bot.* 67: 1663–1676.
Mosquna, A., Peterson, F.C., Park, S.-Y., Lozano-Juste, J., Volkman, B.F. and Cutler, S.R. (2011) Potent and selective activation of abscisic acid receptors in vivo by mutational stabilization of their agonist-bound conformation. *Proc. Natl. Acad. Sci. USA* 108: 20838–20843.
Mur, L.A.J., Kenton, P., Atzorn, R., Miersch, O. and Wasternack, C. (2006) The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiol*. 140: 249–262.
Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant* 15: 473–497.
Návarová, H., Bernsdorff, F., Döring, A.-C. and Zeier, J. (2012) Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity. *Plant Cell* 24: 5123–5141.
Nawrath, C. and Métraux, J.-P. (1999) Salicylic acid induction-deficient mutants of Arabidopsis express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell* 11: 1393–1404.
Noutoshi, Y., Okazaki, M., Kida, T., Nishina, Y., Morishita, Y., Ogawa, T., et al. (2012) Novel plant immune-priming compounds identified via high-throughput chemical screening target salicylic acid glucosyltransferases in *Arabidopsis*. *Plant Cell* 24: 3795–3804.
Olszak, B., Malinovsky, F.G., Brodersen, P., Grell, M., Giese, H., Petersen, M., et al. (2006) A putative flavin-containing mono-oxygenase as a marker for certain defense and cell death pathways. *Plant Sci.* 170: 614–623.
Park, E., Díaz-Moreno, S.M., Davis, D.J., Wilkop, T.E., Bulone, V. and Drakakaki, G. (2014) Endosidin 7 specifically arrests late cytokinesis and inhibits callose biosynthesis, revealing distinct trafficking events during cell plate maturation. *Plant Physiol*. 165: 1019–1034.
Park, S.-Y., Fung, P., Nishimura, N., Jensen, D.R., Fujii, H., Zhao, Y., et al. (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* 324: 1068–1071.
Pegadaraju, V., Louis, J., Singh, V., Reese, J.C., Bautor, J., Feys, B.J., et al. (2007) Phloem-based resistance to green peach aphid is controlled by Arabidopsis *PHYTOALEXIN DEFICIENT4* without its signaling partner *ENHANCED DISEASE SUSCEPTIBILITY1*. *Plant J.* 52: 332–341.
Ranf, S., Eschen-Lippold, L., Pecher, P., Lee, J. and Scheel, D. (2011) Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns. *Plant J.* 68: 100–113.
Rietz, S., Stamm, A., Malonek, S., Wagner, S., Becker, D., Medina-Escobar, N., et al. (2011) Different roles of Enhanced Disease Susceptibility1 (EDS1) bound to and dissociated from Phytoalexin Deficient4 (PAD4) in Arabidopsis immunity. *New Phytol*. 191: 107–119.
Rigal, A., Ma, Q. and Robert, S. (2014) Unraveling plant hormone signaling through the use of small molecules. *Front. Plant Sci.* 5: 373.
Saijo, Y., Tintor, N., Lu, X., Rauf, P., Pajerowska-Mukhtar, K., Häweker, H., et al. (2009) Receptor quality control in the endoplasmic reticulum for plant innate immunity. *EMBO J.* 28: 3439–3449.
Scheible, W.-R., Fry, B., Kochevenko, A., Schindelasch, D., Zimmerli, L., Somerville, S., et al. (2003) An Arabidopsis mutant resistant to thaxtomin A, a cellulose synthesis inhibitor from *Streptomyces* species. *Plant Cell* 15: 1781–1794.
Schlicht, M. and Kombrink, E. (2013) The role of nitric oxide in the interaction of *Arabidopsis thaliana* with the biotrophic fungi, *Golovinomyces orontii* and *Erysiphe pisi*. *Front Plant Sci.* 4: 351.
Schmittgen, T.D. and Livak, K.J. (2008) Analyzing real-time PCR data by the comparative C_T method. *Nat. Protoc.* 3: 1101–1108.
Schreiber, K., Ckurshumova, W., Peek, J. and Desveaux, D. (2008) A high-throughput chemical screen for resistance to *Pseudomonas syringae* in Arabidopsis. *Plant J.* 54: 522–531.
Serrano, M., Hubert, D.A., Dangl, J.L., Schulze-Lefert, P. and Kombrink, E. (2010) A chemical screen for suppressors of the *avrRpm1-RPM1*-dependent hypersensitive cell death response in *Arabidopsis thaliana*. *Planta* 231: 1013–1023.
Serrano, M., Kombrink, E. and Meesters, C. (2015) Considerations for designing chemical screening strategies in plant biology. *Front. Plant Sci.* 6: 131.
Serrano, M., Robatzek, S., Torres, M., Kombrink, E., Somssich, I.E., Robinson, M., et al. (2007) Chemical interference of pathogen-associated molecular pattern-triggered immune responses in *Arabidopsis* reveals a potential role for fatty-acid synthase type II complex-derived lipid signals. *J. Biol. Chem.* 282: 6803–6811.
Shapiro, A.D. and Zhang, C. (2001) The role of *NDR1* in avirulence gene-directed signaling and control of programmed cell death in Arabidopsis. *Plant Physiol.* 127: 1089–1101.
Song, J.T., Lu, H., McDowell, J.M. and Greenberg, J.T. (2004) A key role for *ALD1* in activation of local and systemic defenses in *Arabidopsis*. *Plant J.* 40: 200–212.
Spoel, S.H. and Dong, X. (2012) How do plants achieve immunity? Defence without specialized immune cells. *Nat. Rev. Immunol.* 12: 89–100.
Straus, M.R., Rietz, S., Ver Loren van Themaat, E., Bartsch, M. and Parker, J.E. (2010) Salicylic acid antagonism of EDS1-driven cell death is important for immune and oxidative stress responses in Arabidopsis. *Plant J.* 62: 628–640.
Stuttmann, J., Hubberten, H.-M., Rietz, S., Kaur, J., Muskett, P., Guerois, R., et al. (2011) Perturbation of *Arabidopsis* amino acid metabolism causes incompatibility with the adapted biotrophic pathogen *Hyaloperonospora arabidopsidis*. *Plant Cell* 23: 2788–2803.
Torres, M.A., Dangl, J.L. and Jones, J.D.G. (2002) *Arabidopsis* gp91^{phox} homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proc. Natl. Acad. Sci. USA* 99: 517–522.
Tóth, R. and van der Hoorn, R.A.L. (2010) Emerging principles in plant chemical genetics. *Trends Plant Sci.* 15: 81–88.
Tsuda, K., Sato, M., Stoddard, T., Glazebrook, J. and Katagiri, F. (2009) Network properties of robust immunity in plants. *PLoS Genet.* 5: e1000772.
Vieten, A., Vanneste, S., Wisniewska, J., Benkova, E., Benjamins, R., Beeckman, T., et al. (2005) Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. *Development* 132: 4521–4531.
Wagner, S., Stuttmann, J., Rietz, S., Guerois, R., Brunstein, E., Bautor, J., et al. (2013) Structural basis for signaling by exclusive EDS1 heteromeric complexes with SAG101 or PAD4 in plant innate immunity. *Cell Host Microbe* 14: 619–630.

Weßling, R. and Panstruga, R. (2012) Rapid quantification of plant–powdery mildew interactions by qPCR and conidiospore counts. *Plant Methods* 8: 35.

Wiermer, M., Feys, B.J. and Parker, J.E. (2005) Plant immunity: the EDS1 regulatory node. *Curr. Opin. Plant Biol.* 8: 383–389.

Wildermuth, M.C., Dewdney, J., Wu, G. and Ausubel, F.M. (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defense. *Nature* 414: 562–565.

Wirthmueller, L., Zhang, Y., Jones, J.D.G. and Parker, J.E. (2007) Nuclear accumulation of the *Arabidopsis* immune receptor RPS4 is necessary for triggering EDS1-dependent defense. *Curr. Biol.* 17: 2023–2029.

Witte, C.-P., Noël, L., Gielbert, J., Parker, J. and Romeis, T. (2004) Rapid one-step protein purification from plant material using the eight-amino acid StrepII epitope. *Plant Mol. Biol.* 55: 135–147.

Xu, F., Zhu, C., Cevik, V., Johnson, K., Liu, Y., Sohn, K., et al. (2015) Autoimmunity conferred by *chs3-2D* relies on *CSA1*, its adjacent TNL-encoding neighbour. *Sci. Rep.* 5: 8792.

Zhang, H., Ning, X., Hang, H., Ru, X., Li, H., Li, Y., et al. (2013) Total synthesis of thaxtomin A and its stereoisomers and findings of their biological activities. *Org. Lett.* 15: 5670–5673.

Zhang, Y., Goritschnig, S., Dong, X. and Li, X. (2003) A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in *suppressor of npr1-1, constitutive 1*. *Plant Cell* 15: 2636–2646.

Zheng, W., Zhai, Q., Sun, J., Li, C.-B., Zhang, L., Li, H., et al. (2006) Bestatin, an inhibitor of aminopeptidases, provides a chemical genetics approach to dissect jasmonate signaling in *Arabidopsis*. *Plant Physiol.* 141: 1400–1413.

Zhou, N., Tootle, T.L., Tsui, F., Klessig, D.F. and Glazebrook, J. (1998) PAD4 functions upstream from salicylic acid to control defense responses in Arabidopsis. *Plant Cell* 10: 1021–1030.

Zhu, S., Jeong, R.-D., Venugopal, S.C., Lapchyk, L., Navarre, D., Kachroo, A., et al. (2011) SAG101 forms a ternary complex with EDS1 and PAD4 and is required for resistance signaling against turnip crinkle virus. *PLoS Pathog.* 7: e1002318.