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Report

Chemical Genetics Reveals Negative Regulation of Abscisic Acid Signaling by a Plant Immune Response Pathway

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Summary

Coordinated regulation of protection mechanisms against environmental abiotic stress and pathogen attack is essential for plant adaptation and survival. Initial abiotic stress can interfere with disease-resistance signaling [1–6]. Conversely, initial plant immune signaling may interrupt subsequent abscisic acid (ABA) signal transduction [7, 8]. However, the processes involved in this crosstalk between these signaling networks have not been determined. By screening a 9600-compound chemical library, we identified a small molecule [5-(3,4-dichlorophenyl)furan-2-yl]-piperidine-1-ylmethanethione (DFPM) that rapidly downregulates ABA-dependent gene expression and also inhibits ABA-induced stomatal closure. Transcriptome analyses show that DFPM also stimulates expression of plant defense-related genes. Major early regulators of pathogen-resistance responses, including *EDS1*, *PAD4*, *RAR1*, and *SGT1b*, are required for DFPM—and notably also for *Pseudomonas*—interference with ABA signal transduction, whereas salicylic acid, *EDS16*, and *NPR1* are not necessary. Although DFPM does not interfere with early ABA perception by PYR/RCAR receptors or ABA activation of SnRK2 kinases, it disrupts cytosolic Ca^{2+} signaling and downstream anion channel activation in a *PAD4*-dependent manner. Our findings provide evidence that activation of *EDS1/PAD4*-dependent plant immune responses rapidly disrupts ABA signal transduction and that this occurs at the level of Ca^{2+} signaling, illuminating how the initial biotic stress pathway interferes with ABA signaling.

Results

Novel Compound DFPM Isolated from a Randomly Synthesized Chemical Library Inhibits Abscisic Acid Signaling

A chemical library of 9600 randomly synthesized compounds was screened using a WT-*RAB18* reporter line grown in 96-well tissue culture plates. Candidate chemicals that antagonized abscisic acid (ABA)-induced gene expression were selected (Figure 1A; see also Figure S1 available online; ID5535396, ID5935873, ID5958440, and ID6015316). Here we report a detailed characterization of the small molecule [5-(3,4-dichlorophenyl)furan-2-yl]-piperidine-1-ylmethanethione (DFPM, ID6015316), which effectively inhibits ABA-induced *RAB18* expression (Figure 1A). In contrast to frequently isolated auxin-related structures in this DIVERSET library, DFPM treatment did not produce auxin-related growth defects or alter auxin induction of the *DR5* promoter expression [9, 10] (Figure S1C). The inhibitory effect of DFPM on ABA-induced gene expression was confirmed using an alternative GUS reporter line under the control of the *RD29B* promoter [11] (Figure 1A). DFPM inhibits ABA induction of gene expression in a dose-dependent manner (IC_{50} = 3 μM and 1.5 μM for inhibition of ABA induction of the endogenous *RD29B* and *RAB18* promoters, respectively) (Figure 1B; Figure S2A). To determine functional relevant residues of the DFPM structure, we analyzed derivatives of DFPM (Figure 1C). Modification of any ring structure and deleting or changing positions of the chloride groups reduced DFPM activity (Figure 1D). Thus DFPM was the most effective among the derivatives analyzed. ATH1 GeneChip microarray analyses showed that DFPM downregulates ABA induction of more than 40% of ABA-responsive genes, showing that DFPM affects a subset of the ABA signaling network (Figure 1E; Figure S3; Table S1).

DFPM also inhibited ABA-mediated physiological responses, including ABA-induced stomatal closure (Figure 1F) and ABA inhibition of stomatal opening (Figure S4C). In contrast, DFPM hardly affected ABA-induced delay in seed germination (Figure S2C), indicating that DFPM does not control the entire ABA signaling network but rather acts preferably on a subset of ABA responses. In addition, ABA content measurements under nonstress conditions or in response to osmotic stress showed that DFPM does not affect endogenous ABA concentrations (Figure S2D), suggesting that DFPM disrupts ABA signaling steps rather than ABA metabolism.

DFPM Inhibition of ABA Responses Requires Plant Immune Signaling

To validate microarray analysis results, expression of several ABA-induced genes was tested by quantitative PCR (qPCR), including *RAB18*, *RD29B*, *Cor15a*, and *ABI1* (Figure 2D; Figure S4B). ABA induction of *RAB18*, *RD29B*, and *Cor15a* was reduced by pretreatment (30 min) with DFPM (Figure 2D). However, DFPM did not affect the ABA induction of *ABI1* in both microarray and q-PCR experiments (Figure S4B).

In addition to the inhibitory effect of DFPM on ABA-responsive gene induction, transcriptome analyses also revealed that DFPM alone regulates the transcript levels of 386 genes (Figure 2A). Signaling pathway impact analysis revealed that DFPM induces components in the plant pathogen signaling network (KEGG: ath04626) (Figure 2B; Table S1). Strong DFPM-induction of typical pathogen-responsive genes *PR5* and *EDS1* [12, 13] were confirmed using q-PCR (Figure 2C).

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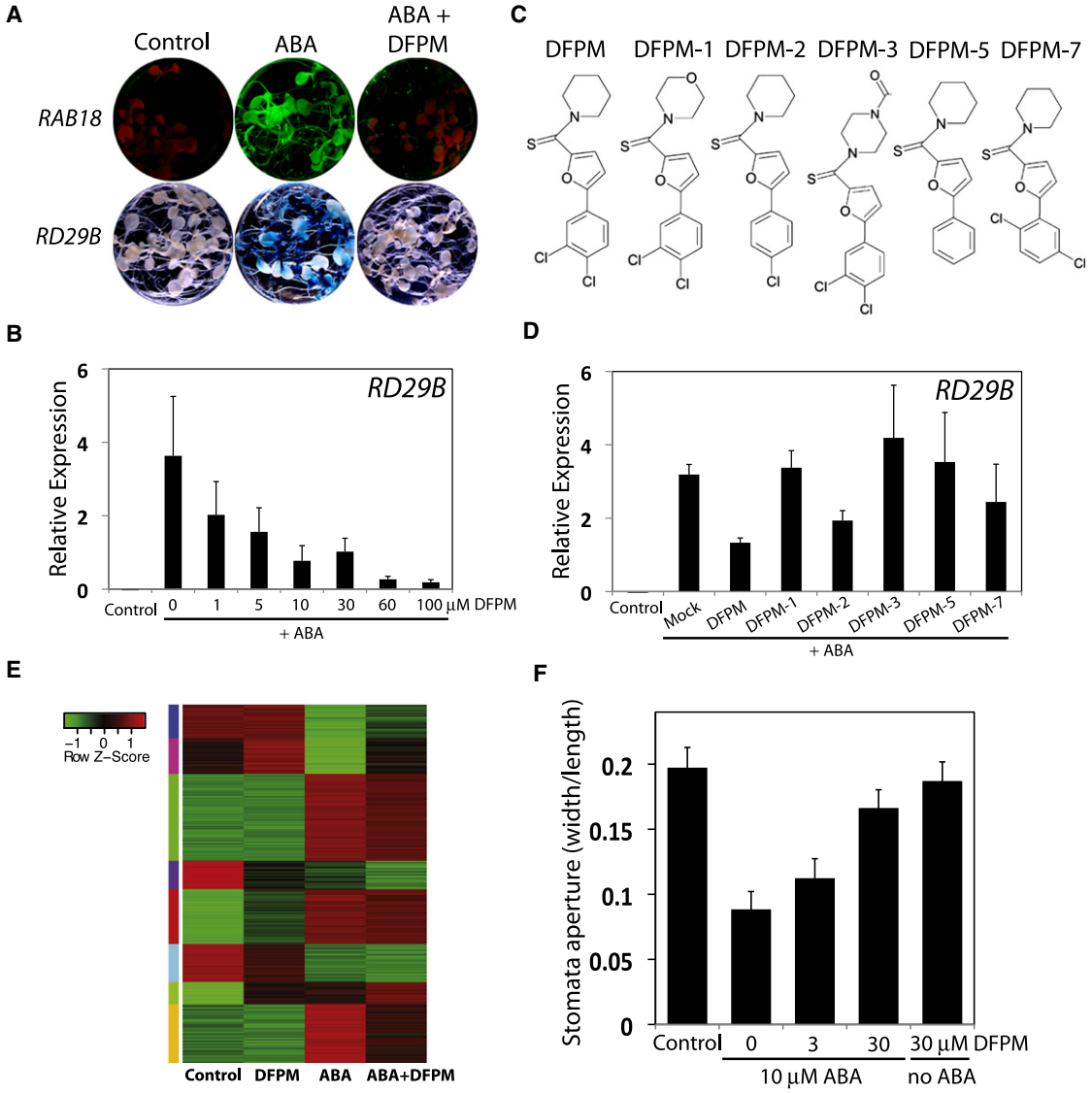

Figure 1. Small Molecule DFPM Inhibits Abscisic Acid-Induced Gene Expression and Stomatal Closing

(A) [5-(3,4-dichlorophenyl)furan-2-yl]-piperidin-1-ylmethanethione (DFPM) treatment reduces ABA-induction of green fluorescent protein (GFP) and β-glucuronidase (GUS) reporter gene expression in *RAB18-GFP* and *RD29B-GUS* promoter reporter lines.

(B) Concentration-dependent effects of DFPM in inhibition of abscisic acid (ABA)-induced *RD29B* gene expression measured by quantitative PCR (qPCR).

(C and D) Structures and test of DFPM derivatives for inhibition of ABA-induced *RD29B* gene expression as quantified by q-PCR.

(E) Transcriptomic analysis shows that groups of ABA-induced genes are downregulated by DFPM (30 µM) (n = 3 microarrays per condition). The heat map contains 470 probe sets regulated by ABA (292 upregulated and 178 downregulated; 45 probe sets are also affected by DFPM, shown in Figure 2A).

(F) DFPM exposure 30 min prior to ABA exposure inhibits ABA-induced stomatal closing. Error bars represent mean ± standard error of the mean (SEM) (n = 3 experiments, 30 stomata per experiment and condition).

ABA was applied at 10 µM in (A)–(F).

To address whether the transcriptional activation of plant defense genes by DFPM is linked to inhibition of ABA signaling, we analyzed genetic mutations in components of plant disease-resistance pathways. Notably, DFPM's inhibitory activity on ABA induction of *RAB18* and *RD29B* expression was compromised in the *eds1-22* [14], *pad4-1* [15], *sgt1b(eta3)* [16, 17], and *rar1-21* [18] mutants (Figure 2D; Figure S4A), indicating that *EDS1*, *PAD4*, *SGT1b*, and *RAR1* are required for the inhibitory activity of DFPM on ABA signal transduction. Because *EDS1*, *PAD4*, *SGT1b*, and *RAR1* are important early components of plant nucleotide-binding leucine-rich repeat (NB-LRR)-triggered immunity [16, 18–20], these data suggest that activation of NB-LRR proteins or early steps of resistance-signaling pathways antagonize ABA signal transduction. *EDS1* and *PAD4* control both salicylic acid (SA)-dependent and SA-independent pathways [21, 22]. A critical SA response regulator, *NPR1* [23], was not required for DFPM disruption of ABA signaling (Figure 2D), suggesting that SA signaling is not involved in the DFPM inhibition.

Preincubation with DFPM for 30 min inhibited the rapid response of ABA-induced stomatal closure (Figure 1F). To test whether DFPM inhibition of this rapid ABA response also requires early pathogen signaling components, we examined ABA-induced stomatal responses of disease-resistance

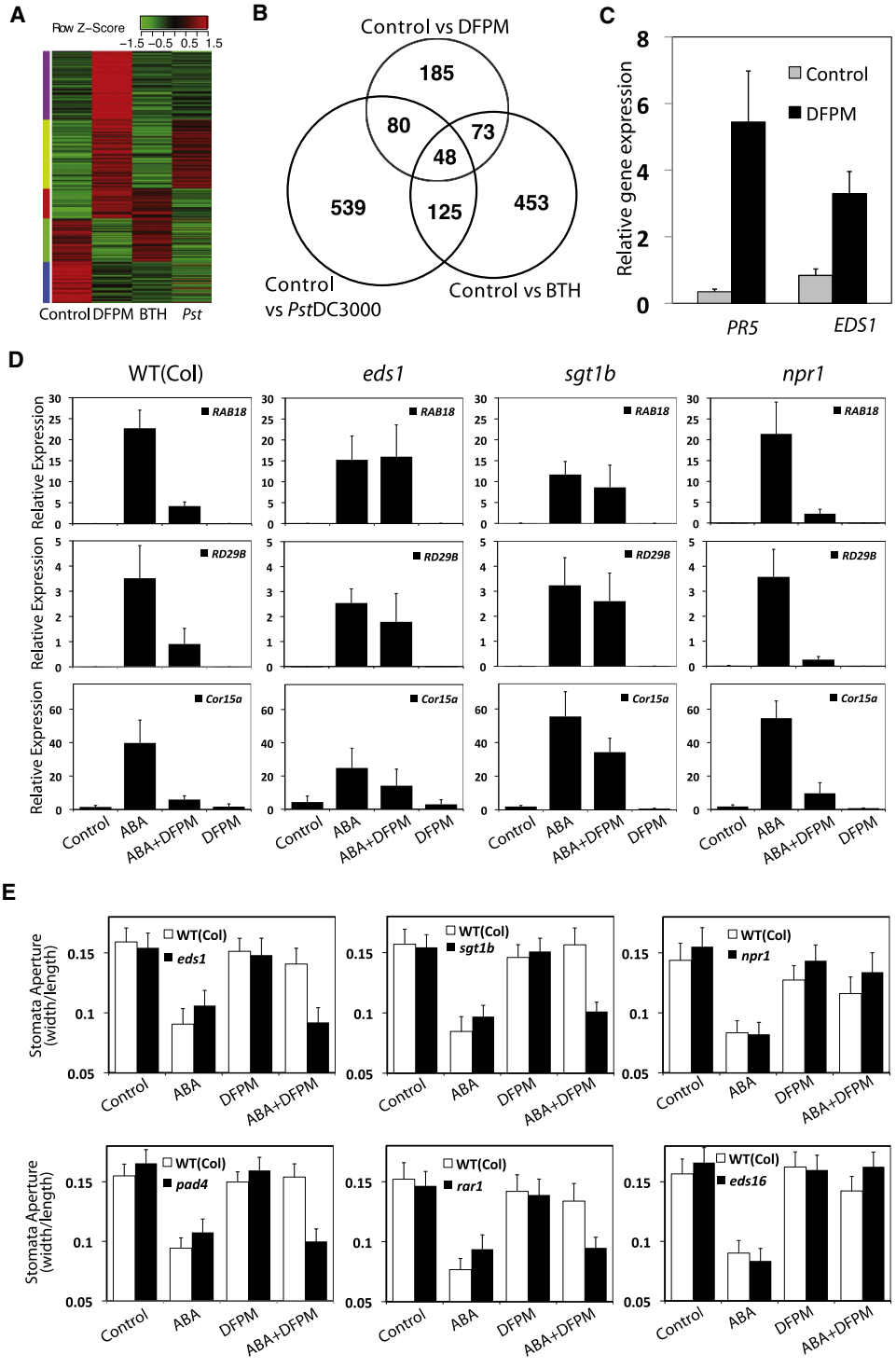

Figure 2. DFPM Inhibition of ABA Signaling Requires Early Signaling Components of Effector-Triggered Immune Signal Transduction

(A) Heat map of 386 probe sets regulated by DFPM.

(B) DFPM-regulated genes overlap with benzothiadiazole (BTH)-regulated and *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000-regulated gene expression.

(C) DFPM induction of *PR5* and *EDS1* gene expression was quantified by q-PCR.

(D) DFPM inhibition of ABA-inducible *RAB18*, *RD29B*, and *COR15a* expression requires functional *EDS1* and *SGT1b* but not *NPR1*. Error bars show ± SEM (n = 3).

(E) DFPM inhibition of ABA-induced stomatal closing requires *EDS1*, *PAD4*, *SGT1b*, and *RAR1* but not *NPR1* or *EDS16*. Error bars represent mean ± SEM (n = 3 blind experiments, 30 stomata per experiment and condition).

DFPM was applied at 30 μM and ABA was applied at 10 μM in (A)–(E).

mutants (Figure 2E). DFPM inhibition of ABA-induced stomatal closure required functional *EDS1*, *PAD4*, *SGT1b*, and *RAR1* but not *NPR1* or the SA biosynthetic gene *EDS16/SID2* [24] (Figure 2E). DFPM also disrupted ABA inhibition of stomatal opening, and the inhibition was impaired in *eds1*, *pad4*, *rar1*, and *sgt1b* mutants but not in *npr1* (Figure S4C). These data suggest that the rapid action of DFPM in disrupting stomatal responses to ABA requires *EDS1*/*PAD4*-dependent signaling but is independent of salicylic acid.

Constitutively Activated NB-LRR Receptor SNC1-1 Inhibits ABA Signaling

The requirement for *EDS1*, *PAD4*, *SGT1b*, and *RAR1* during DFPM inhibition of ABA signaling (Figures 2D and 2E; Figure S4) and the transcriptional activation of defense-related gene expression by DFPM (Figures 2A and 2B) led us to hypothesize that DFPM stimulates immune pathways activated by NB-LRR receptors. We therefore tested whether activation of an NB-LRR protein can also inhibit ABA responses. ABA induction of gene expression and ABA-induced stomatal closure were examined in the *snc1-1* (*suppressor of npr1-1, constitutive1*) mutant [25]. In *snc1-1*, a point mutation in a Toll/interleukin-1 receptor domain (TIR)-NB-LRR protein creates an autoactivated receptor, which triggers constitutive pathogen resistance through EDS1 and PAD4 [25]. ABA induction of *RAB18*, *RD29B*, and *Cor15a* was reduced in *snc1-1* (Figure S5A). *SNC1* is expressed in guard cells [26, 27], and stomata of *snc1-1* were less responsive to ABA during ABA-induced stomatal closing (Figure S5B; two-tailed t test, $p = 0.0059$ for wild-type [WT]+ABA versus *snc1-1*+ABA). These data demonstrate that constitutive activation of an NB-LRR protein antagonizes ABA induction of gene expression and stomatal closure.

Pseudomonas syringae Infection Mimics DFPM Inhibitory Effects on ABA Responses

DFPM-induced EDS1/PAD4-dependent signaling has a negative impact on ABA-induced gene expression and physiological ABA responses. We therefore tested whether EDS1/PAD4 signaling in response to authentic pathogen infection can inhibit ABA signal transduction. ABA induction of *RD29B* gene expression was examined after exposure of *Arabidopsis* seedlings to the virulent *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000, which induces EDS1/PAD4-dependent basal (low-level) immunity, or the avirulent *Pst*DC3000/*avrRps4* strain, which induces EDS1/PAD4-dependent effector-triggered immunity after TIR-NB-LRR receptor activation [19, 21]. Infection by either strain led to a strong reduction of ABA-induced *RD29B* gene expression (Figure 3A).

As reported previously, *P. syringae* infection causes a transient stomatal closing and reopening [28, 29]. ABA-induced stomatal closing was slightly reduced by infection with *Pst*DC3000 or *Pst*DC3000/avrRps4 (Figure 3B), indicating that immune signaling triggered by these pathogens may also downregulate ABA signaling in guard cells. As with the DFPM treatment, *Pst* infection inhibited guard cell ABA responses in *npr1* and *eds16* mutants but failed to do so in *eds1*, *pad4*, *and sgt1b* (Figure 3B). ABA induction of *RD29B* gene expression (Figure 3A) and ABA activation of stomatal closing responses (Figure 3B) were partially inhibited by infection with a *Pst*DC3000(COR-) strain lacking the virulence factor coronatine [30], which mediates stomatal reopening after pathogen-mediated stomatal closing [28, 31]. This result suggests that the inhibition of ABA signaling by *P. syringae* infection observed here occurs in part independently of coronatine production.

Analyses of DFPM Inhibition of Early ABA Signaling Mechanisms

We examined which step in the ABA signal transduction pathway is targeted by DFPM. ABA signal transduction begins with ABA binding to PYR/RCAR receptors and interactions with PP2C protein phosphatases [32, 33]. Coimmunoprecipitation analyses showed that DFPM did not affect ABA-dependent PYR1 interaction with the PP2C ABI1 (Figure 4A), indicating that ABA perception by PYR/RCAR receptors and PYR1-PP2C complex formation are not directly interrupted by DFPM. ABA perception causes activation of three SnRK2 protein kinases [34–36] by deactivation of the negatively regulating PP2Cs [32, 33, 37–40]. DFPM did not interfere with ABA activation of these SnRK2 protein kinases (Figure 4B; Figure S6), indicating that DFPM interferes with downstream processes of SnRK2 kinase activation.

Guard cells enable dissection of further steps in early ABA signal transduction [41]. To further investigate which step of ABA signaling can be impaired by DFPM, we exposed guard cells to four repetitive 5 min Ca^{2+} pulses known to cause Ca^{2+}-induced stomatal closing [42–44]. DFPM partially inhibited imposed repetitive Ca^{2+} pulse-mediated stomatal closing (Figure 4C), indicating that DFPM-triggered signaling disrupts stomatal closing at the level of or downstream of Ca^{2+} signaling.

Elevated ABA enhances the cytosolic [Ca^{2+}] sensitivity of S-type anion-channel activation in *Arabidopsis* guard cells [45]. To test whether DFPM impairs ABA regulation of S-type anion-channel activities, we analyzed ABA activation of S-type anion channels at 2 μM free cytosolic [Ca^{2+}] [43, 45]. DFPM pretreatment significantly reduced ABA-induced Ca^{2+}-activated S-type anion-channel currents (Figure 4D). DFPM inhibition of ABA-induced Ca^{2+}-activated S-type anion-channel activity was significantly impaired in *pad4-1* mutant guard cells (Figure 4E).

Discussion

With the aim of dissecting new mechanisms in the ABA signaling network, a small-molecule antagonist of ABA signaling, DFPM, was identified by screening a 9600-compound-containing chemical library (Figure 1; Figure S1). DFPM effectively inhibits ABA-induced gene expression without producing any noticeable growth and developmental defects (Figure 1; Figure 2). In addition to the long-term inhibitory effect of DFPM on ABA-dependent gene expression, 30 min pretreatment with DFPM interferes with rapid guard cell ABA responses such as ABA-induced and repetitive Ca^{2+} pulse-induced stomatal closing (Figure 1; Figure 4; Figure S4C).

Identification of DFPM as an activator of plant immunity-related gene expression (Figures 2A and 2B) provided evidence that DFPM negatively affects ABA signal transduction through activation of plant immune signaling. Many studies have shown that the converse crosstalk occurs from initial ABA/abiotic stimulation, which subsequently antagonizes plant pathogen/biotic stress signaling [1–6]. Here we show that initial plant disease-resistance signaling by application of the small molecule DFPM or *P. syringae* infection interferes with subsequent ABA signal transduction, indicating that biotic stress responses restrict plant abiotic stress signal transduction.

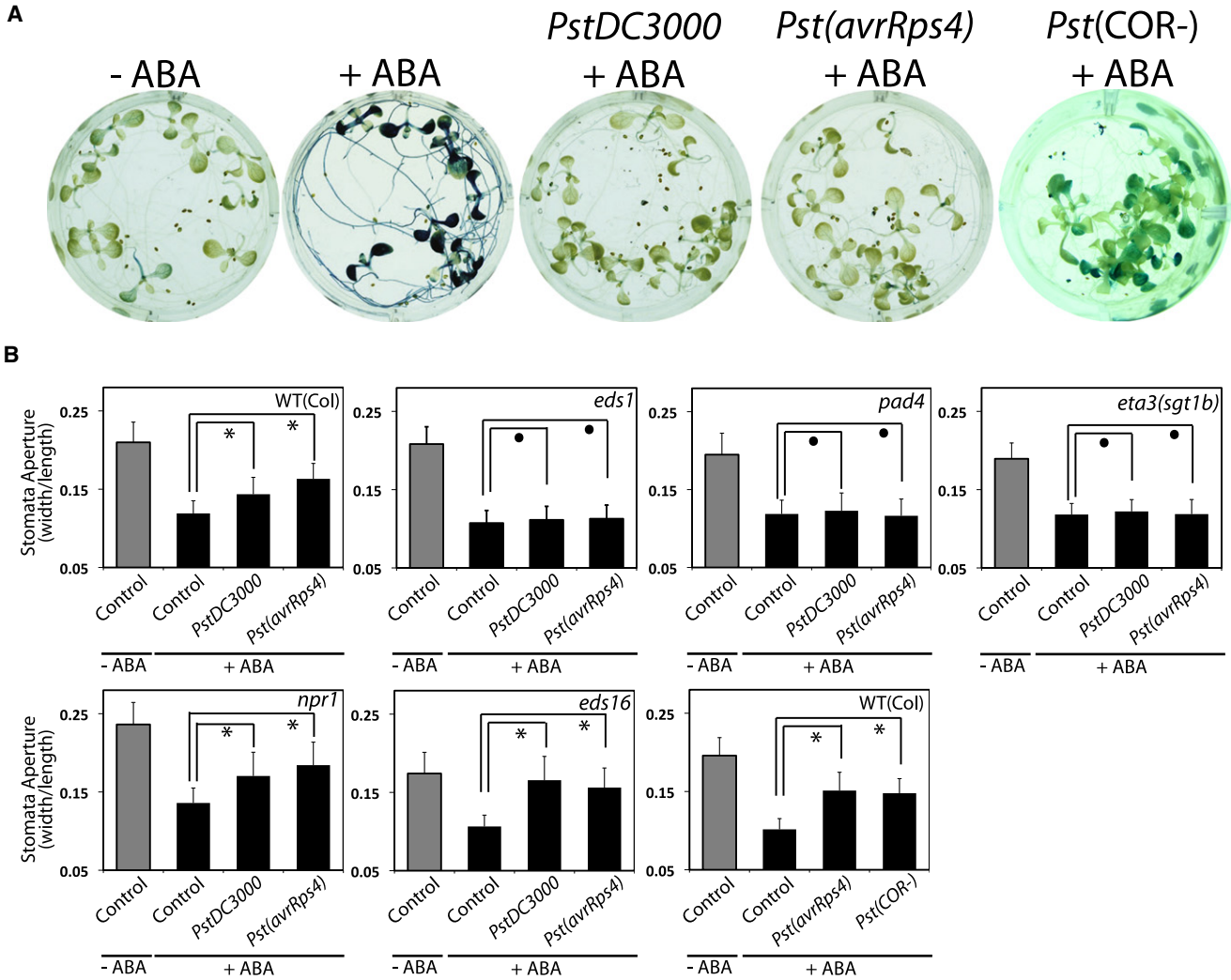

Figure 3. *P. syringae* Infection Inhibits ABA Signaling through the *EDS1/PAD4* Pathway

(A) Infections by *Pseudomonas syringae* pv. *tomato (Pst)* DC3000, *Pst(avrRps4)*, and *Pst*(COR-) inhibit ABA-induced *RD29B* reporter gene expression.
(B) ABA-induced stomatal closing is inhibited by *Pst*DC3000 and *Pst(avrRps4)* infection in an *EDS1/PAD4/SGT1b*-dependent manner but independently of *NPR1* and *EDS16*. Infections by *Pst(*COR-*)* also inhibit ABA-induced stomatal closing. *p < 0.025; •p > 0.2, respectively (n = 3 experiments, 30 stomata per experiment and condition, two-tailed t test). Error bars represent mean ± SEM (n = 3).
ABA was applied at 10 μM in (A) and (B).

Our analyses of defense-signaling mutants reveal that impairment of ABA signal transduction by DFPM pretreatment requires *EDS1* and *PAD4*, major regulators of effector-triggered and basal immunity in plants (Figures 2C and 2D) [19, 21]. Overlap between genes induced by DFPM and the SA analog benzothiadiazole (BTH) (Figures 2A and 2B) suggests that DFPM activates both SA-dependent and SA-independent defenses. However, the dispensability of SA biosynthesis (*eds16*/*sid2*) and downstream signaling (*npr1*) components for DFPM interference with ABA responses (Figures 2D and 2E; Figure S4C) delineates the DFPM effect to an SA-independent branch of the EDS1/PAD4 pathway that is important for both basal and TIR-NB-LRR receptor-triggered resistance responses [21, 22].

Notably, SA is necessary for the "reverse crosstalk," in which initial ABA signal transduction interferes with biotic stress signaling [5, 46], suggesting differences in the underlying mechanisms mediating abiotic-to-biotic signaling interference [1–6, 46]. The EDS1/PAD4-dependent and SA-independent disruption of ABA responses identified here interferes with early ABA signaling mechanisms because DFPM inhibition of both ABA-triggered stomatal closing and ABA inhibition of stomatal opening are strongly reduced in the *eds1* or *pad4* mutants (Figure 2E) and DFPM inhibition of ABA activation of the anion channel is compromised in *pad4* mutant guard cells (Figure 4E).

A requirement for *RAR1* and *SGT1b* in DFPM-mediated negative regulation of ABA-induced responses (Figures 2D and 2E; Figures S4A and S4C) suggests that the antagonism occurs via NB-LRR immune receptors because a major function of RAR1 and SGT1b is to assist the accumulation of plant NB-LRR complexes [47]. This would not, however, explain the effectiveness of virulent *Pst*DC3000 in inhibiting ABA-induced *RD29B* gene expression (Figure 3A), which induces "basal" resistance in the absence of obvious NB-LRR recognition. One possibility is that the EDS1/PAD4 basal immunity barrier

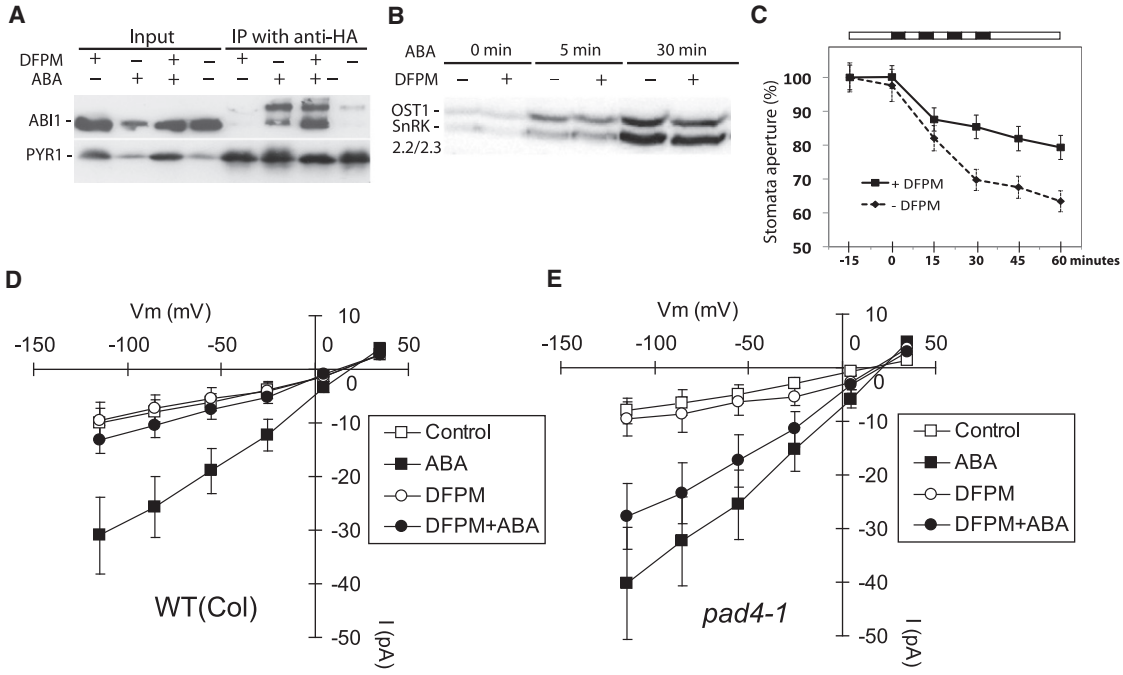

Figure 4. DFPM Inhibits Guard Cell ABA Signal Transduction at the Level of Ca^{2+} Signaling

(A) ABA-dependent protein-protein interaction between the PYR1 ABA receptor and the ABI1 PP2C-type phosphatase is not disrupted by DFPM pretreatment. HA-PYR1 and YFP-ABI1 were coimmunoprecipitated in the presence of ABA (100 μM) and DFPM (50 μM).

(B) ABA activation (10 μM) of SnRK2 kinases OST1, SnRK2.2, and SnRK2.3 [32] was not disrupted by DFPM treatment (50 μM).

(C) DFPM (30 μM) inhibits stomatal closing mediated by repetitive imposed Ca^{2+} transients. Black bars represent periods in which stomata were exposed to buffer containing 1 mM $CaCl_2$+1 mM KCl, and white bars indicate periods with application of 0 mM $CaCl_2$+50 mM KCl [43]. Each black bar corresponds to 5 min timescale. Stomatal apertures at time = 0 (100%) correspond to average stomatal apertures of 4.02 ± 0.25 μm in control treatments and 3.53 ± 0.26 μm in DFPM pretreatments (30 min prior to first Ca^{2+} pulse). Error bars show ± SEM (n = 4 experiments).

(D) ABA activation of S-type anion-channel currents is significantly inhibited by DFPM in Columbia wild-type guard cells (Control: n = 6; 10 μM ABA: n = 10; 30 μM DFPM: n = 4; 30 μM DFPM+10 μM ABA: n = 10; p = 0.032; two-tailed t test).

(E) DFPM inhibition of ABA activation of S-type anion channels is not visible in *pad4-1* guard cells (control: n = 6; 10 μM ABA: n = 10; 30 μM DFPM: n = 6; 30 μM DFPM+10 μM ABA: n = 10; p = 0.314; two-tailed t test). Guard cell protoplasts were pretreated with 0.06% dimethyl sulfoxide (DMSO) (control) or DFPM for 30 min before ABA+DMSO or ABA+DFPM treatment. Error bars show ± SEM.

is triggered by low-activity NB-LRR receptors. Alternatively, SGT1 and RAR1 function at an early intersection between NB-LRR activation and EDS1/PAD4 basal resistance signaling. Either scenario is supported by *sgt1b* and *rar1* defects reported for basal resistance to virulent pathogen infection [48–50]. Together, the data favor inhibition of a sector of ABA signaling proceeding through the plant EDS1/PAD4 basal resistance pathway that can be effectively activated by NB-LRR receptors such as RPS4 and SNC1 (Figure 3; Figure S5).

Investigation of the mechanism mediating DFPM disruption of ABA signal transduction showed that DFPM interferes with events at the level of or downstream of intracellular Ca^{2+} signaling, whereas upstream ABA perception by PYR/RCAR receptors [32, 33] and subsequent activation of the major ABA signaling kinases, OST1, SnRK2.2, and SnRK2.3, were not affected by DFPM treatment (Figure 4; Figure S6). It is notable that intracellular Ca^{2+} has been characterized as an important transducer of plant immunity [51–55]. One hypothesis is that distinct Ca^{2+} signals generated during biotic stress signaling interfere with those produced during ABA signal transduction. Alternatively, depletion of Ca^{2+} binding proteins that are shared by pathogen-induced and ABA responses may limit ABA signal transduction. For example, the Ca^{2+}-dependent protein kinases CPK6, -4, and -11 have been shown to be required for ABA signal transduction [43, 56], and recent research shows that CPK4, -5, -6, and -11 function in flg22-induced resistance to the bacterial pathogen *Pst*DC3000 [54]. However, other associated proteins or mechanisms may also trigger the identified biotic-to-ABA signaling interference identified here (see also Supplemental Discussion).

In summary, our findings define negative regulation of ABA signal transduction by rapid activation of plant innate immune responses by the small molecule DFPM and by *P. syringae* infection in part independently of SA signaling. Combined genetic and guard cell signaling analyses show that activation of resistance signaling antagonistically regulates ABA responses downstream of ABA-activated SnRK2 kinase activation, at the level of or downstream of Ca^{2+} signaling. Further investigation of how the small molecule DFPM modulates Ca^{2+} signaling during ABA signaling will shed light on regulatory mechanisms that adjust plant adaptive responses against combined biotic and abiotic stress exposures.

Accession Numbers

The NCBI-GEO accession number for the microarray data is GSE28800.

Supplemental Information

Supplemental Information includes six figures, one table, Supplemental Discussion, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.04.045.

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