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# 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 ABA signal transduction [7, 8]. However, the processes involved in cross talk between these signaling networks have not been determined. By screening a 9,600 compound chemical library, we identified a small molecule DFPM that rapidly down-regulates 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. While 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 this occurs at the level of $Ca^{2+}$ signaling, illuminating how the initial biotic stress pathway interferes with ABA signaling.



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**Supplementary Information**

Six figures, 1 table, Supplementary Discussion, and Experimental Procedures are available on line (http://www.current-biology.com).

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## Results

### Novel compound DFPM isolated from a randomly synthesized chemical library inhibits ABA signaling

A chemical library of 9,600 randomly synthesized compounds was screened using the WT-*RAB18* reporter line grown in 96 well tissue culture plates. Candidate chemicals that antagonized ABA-induced gene expression were selected (Fig. 1A and S1; ID5535396, ID5935873, ID5958440, and ID6015316). Here we report a detailed characterization of the small molecule [5-(3,4-Dichlorophenyl)Furan-2-yl]-Piperidin-1-ylMethanethione (DFPM, ID6015316), which effectively inhibits ABA-induced *RAB18* expression (Fig. 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] (Fig. 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] (Fig. 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) (Fig. 1B and S2A). To determine functional relevant residues of the DFPM structure, derivatives of DFPM were analyzed (Fig. 1C). Modification of any ring structure and deleting or changing positions of the chloride groups reduced DFPM activity (Fig. 1D). Thus DFPM was the most effective among the derivatives analyzed. ATH1 GeneChip microarray analyses showed that DFPM down-regulates ABA-induction of more than 40% of ABA-responsive genes, showing that that DFPM affects a subset of the ABA signaling network (Fig. 1E, S3 Table S1).

DFPM also inhibited ABA-mediated physiological responses, including ABA-induced stomatal closure (Fig. 1F) and ABA-inhibition of stomatal opening (Fig. S4C). In contrast, DFPM hardly affected ABA-induced delay in seed germination (Fig. 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 non-stress conditions or in response to osmotic stress showed that DFPM does not affect endogenous ABA concentrations (Fig. 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, including *RAB18*, *RD29B*, *Cor15a*, and *ABI1* (Fig. 2D and Fig. S4B). ABA induction of *RAB18*, *RD29B* and *Cor15a* was reduced by pre-treatment (30 min) with DFPM (Fig. 2D). However DFPM did not affect the ABA-induction of *ABI1* in both microarray and q-PCR experiments (Fig. 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 (Fig. 2A). Signaling Pathway Impact Analysis revealed that DFPM induces components in the plant pathogen signaling network (KEGG: ath04626) (Fig. 2B and Table S1). Strong DFPM-induction of typical pathogen responsive genes *PR5* and *EDS1* [12, 13] were confirmed using q-PCR (Fig. 2C).

To address whether the transcriptional activation of plant defense genes by DFPM is linked to inhibition of ABA signaling, genetic mutations in components of plant disease resistance pathways were analyzed. 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 (Fig. 2D and 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 NB-LRR (nucleotide binding-leucine rich repeat)-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 (Fig. 2D), suggesting that SA signaling is not involved in the DFPM inhibition.

Pre-incubation with DFPM for 30 minutes inhibited the rapid response of ABA-induced stomatal closure (Fig. 1F). To test whether DFPM inhibition of this rapid ABA response also requires early pathogen signaling components, ABA-induced stomatal responses of disease resistance mutants were examined (Fig. 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] (Fig. 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* (Fig. 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 (Fig. 2D, 2E, S4), and the transcriptional activation of defense-related gene expression by DFPM (Fig. 2A, 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 TIR (Toll/Interleukin-1 Receptor domain)-NB-LRR protein creates an auto-activated receptor, which triggers constitutive pathogen resistance through EDS1 and PAD4 [25]. ABA-induction of *RAB18*, *RD29B*, and *Cor15a* was reduced in *snc1-1* (Fig. S5A)). *SNC1* is expressed in guard cells [26, 27] and stomata of *snc1-1* were less responsive to ABA during ABA-induced stomatal closing (Fig. S5B; 2-tail T-test p=0.0059 for WT+ABA vs. *snc1-1*+ABA). These data demonstrate that constitutive activation of a NB-LRR protein antagonizes ABA-induction of gene expression and stomatal closure.

## *Pseudomonas syringae* infection mimics DFPM inhibitory effects on ABA responses

We have shown that chemical activation of 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 strong reduction of ABA-induced *RD29B* gene expression (Fig. 3A).

As reported previously, *P. syringae* infection causes a transient stomatal closing and reopening [28, 29]. ABA-induced stomatal closing was significantly reduced by infection with *Pst*DC3000 or *Pst*DC3000/avrRps4 (Fig. 3B), indicating that immune signaling triggered by these pathogens can also down-regulate 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* (Fig. 3B). ABA induction of *RD29B*

gene expression (Fig. 3A) and ABA activation of stomatal closing responses (Fig. 3B) were also inhibited by infection with a *Pst*DC3000(COR-) strain lacking the virulence factor coronatine [30] which mediates stomatal re-opening after pathogen-mediated stomatal closing [28, 31]. This result suggests that the inhibition of ABA signaling by *P. syringae* infection observed here occurs largely 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]. Co-immunoprecipitation analyses showed that DFPM did not affect ABA-dependent PYR1 interaction with the PP2C ABI1 (Fig. 4A), indicating that ABA perception by PYR/RCAR receptors and PYR1-PP2C complex formation is not directly interrupted by DFPM. ABA perception causes activation of three SnRK2 protein kinases [34–36] by de-activation of the negatively regulating PP2Cs [32, 33, 37–40]. DFPM did not interfere with ABA-activation of these SnRK2 protein kinases (Fig. 4B, 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, guard cells were exposed 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 (Fig. 4C), indicating that DFPM-triggered signaling disrupts stomatal closing at the level 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, ABA-activation of S-type anion channels was analyzed at 2 μM free cytosolic [$Ca^{2+}$] [43, 45]. DFPM pre-treatment significantly reduced ABA-induced $Ca^{2+}$-activated S-type anion channel currents (Fig. 4D). DFPM inhibition of ABA-induced $Ca^{2+}$-activated S-type anion channel activity was significantly impaired in *pad4-1* mutant guard cells (Fig. 4E).

## Discussion

With the aim to dissect new mechanisms in the ABA signaling network, a small molecule antagonist of ABA signaling, DFPM, was identified by screening a 9,600 compound-containing chemical library (Fig.1, S1). DFPM effectively inhibits ABA-induced gene expression without producing any noticeable growth and developmental defects (Fig. 1, 2). In addition to the long-term inhibitory effect of DFPM on ABA-dependent gene expression, 30 minute pretreatment with DFPM interferes with rapid guard cell ABA responses such as ABA-induced and repetitive $Ca^{2+}$ pulse-induced stomatal closing (Fig. 1, 4, S4C).

Identification of DFPM as an activator of plant immunity-related gene expression (Fig. 2A, B) provided evidence that DFPM negatively affects ABA signal transduction through activation of plant immune signaling. Many studies have shown that the converse cross-talk 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.

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 (Fig. 2C, D) [19, 21]. Overlap between genes induced by DFPM and the SA analogue BTH (Fig. 2A, 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 (Fig. 2D, E, S4C) delineates the DFPM effect to an SA-independent branch of the EDS1/PAD4 pathway which is important for both basal and TIR-NB-LRR receptor-triggered resistance responses [21, 22].

Notably, salicylic acid is necessary for the “reverse cross-talk”, 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 since DFPM inhibition of both ABA-triggered stomatal closing and ABA-inhibition of stomatal opening are strongly reduced in the *eds1* or *pad4* mutants (Fig. 2E) and DFPM inhibition of ABA-activation of anion channel is compromised in *pad4* mutant guard cells (Fig. 4E).

A requirement for *RAR1* and *SGT1b* in DFPM-mediated negative regulation of ABA-induced responses (Fig. 2D, E, S4A, S4C) suggests that the antagonism occurs via NB-LRR immune receptors since a major function of RAR1 and SGT1b is to assist the accumulation and activation of plant NB-LRR complexes [47]. This would not, however, explain the effectiveness of virulent *Pst*DC3000 in inhibiting ABA-induced *RD29B* gene expression (Fig. 3A), which induces ‘basal’ resistance in the absence of obvious NB-LRR recognition. One possibility is that the EDS1/PAD4 basal immunity barrier 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 (Fig. 3, 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 (Fig. 4, S6). It is notable that intracellular $Ca^{2+}$ has been proposed 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.

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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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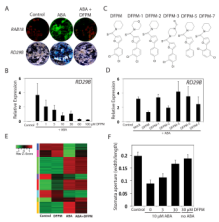

**Figure 1. Small molecule DFPM inhibits ABA-induced gene expression and stomatal closing**

**(A)** DFPM treatment reduces ABA-induction of GFP and GUS reporter gene expression in *RAB18-GFP (green fluorescence protein)* and *RD29B-GUS (β-glucuronidase*) promoter reporter lines. **(B)** Concentration-dependent effects of DFPM in inhibition of ABA-induced *RD29B* gene expression measured by quantitative-PCR (q-PCR). **(C–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 down-regulated by DFPM (30 μM) (n=3 microarrays per condition). The heat map contains 470 probesets regulated by ABA (292 up-regulated / 178 down-regulated; 45 probesets are also affected by DFPM shown in Fig. 2A) **(F)** DFPM exposure 30 min prior to ABA exposure inhibits ABA-induced stomatal closing. Error bars mean ± s.e.m. (n=3 experiments, 30 stomata per each experiment and condition). ABA was applied at 10 μM (A–F).

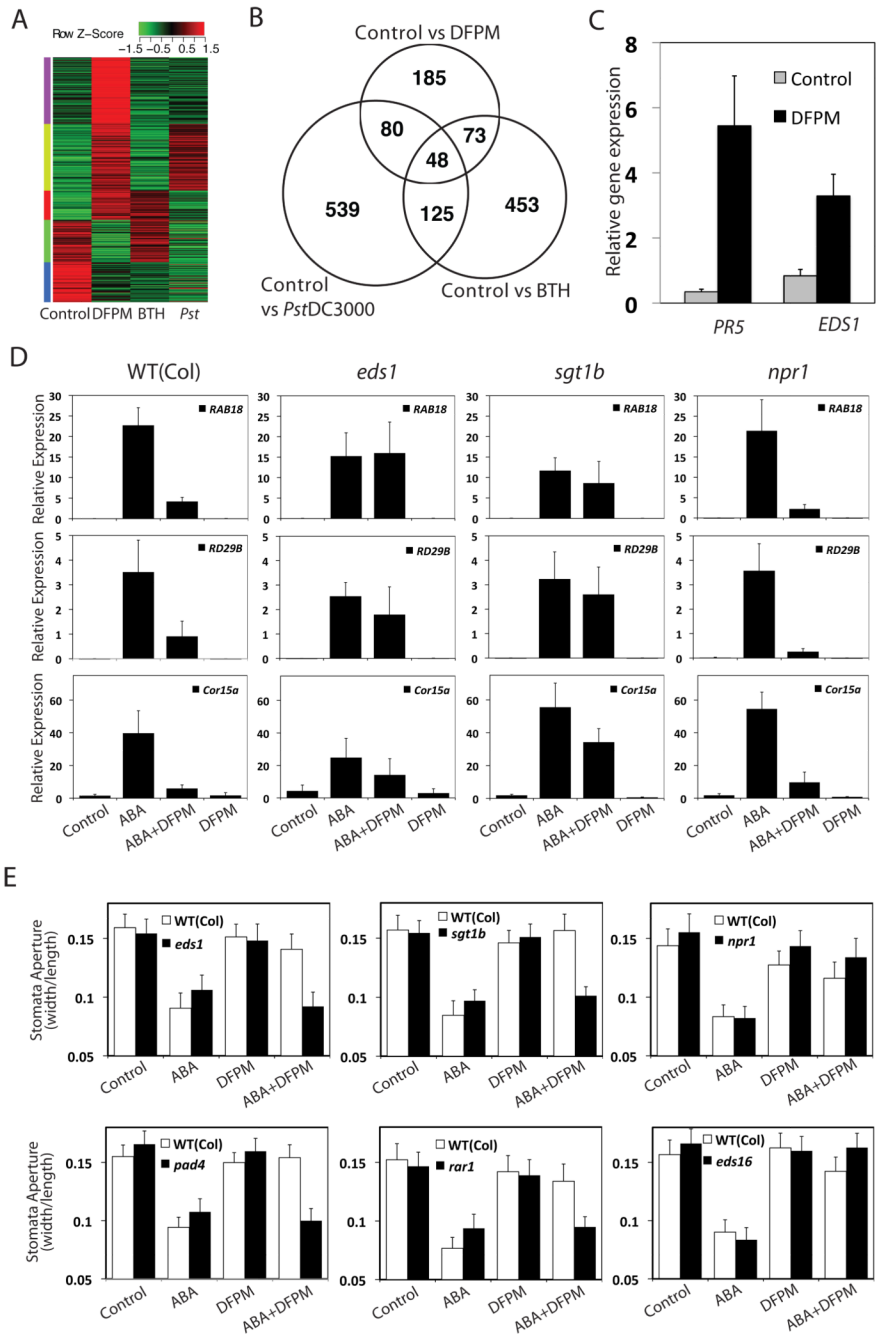


**Figure 2. DFPM-inhibition of ABA signaling requires early signaling components of effector-triggered immune signal transduction**

**(A)** Heat map of 386 probesets regulated by DFPM. **(B)** DFPM-regulated genes overlap with BTH (benzothiadiazole)-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 ± s.e.m (n=3). **(E)** DFPM-inhibition of ABA-induced stomatal closing requires *EDS1*, *PAD4*, *SGT1b*, and *RAR1* but not *NPR1* or *EDS16*. Error bars mean ± s.e.m. (n=3 experiments, 30

stomata per experiment and condition). DFPM was applied at 30 μM and ABA was applied at 10 μM (A–E).

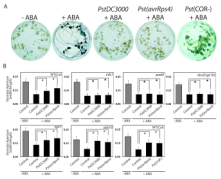

**Figure 3. *P. syringae* infection inhibits ABA signaling through the *EDS1/PAD4* pathway**

**(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. Symbols * and • represent $p < 0.025$ and $p > 0.2$, respectively (n=3 experiments, 30 stomata per experiment and condition, 2-tail T-test). Error bars mean ± s.e.m. (n=3). ABA was applied at 10 μM (A–B).

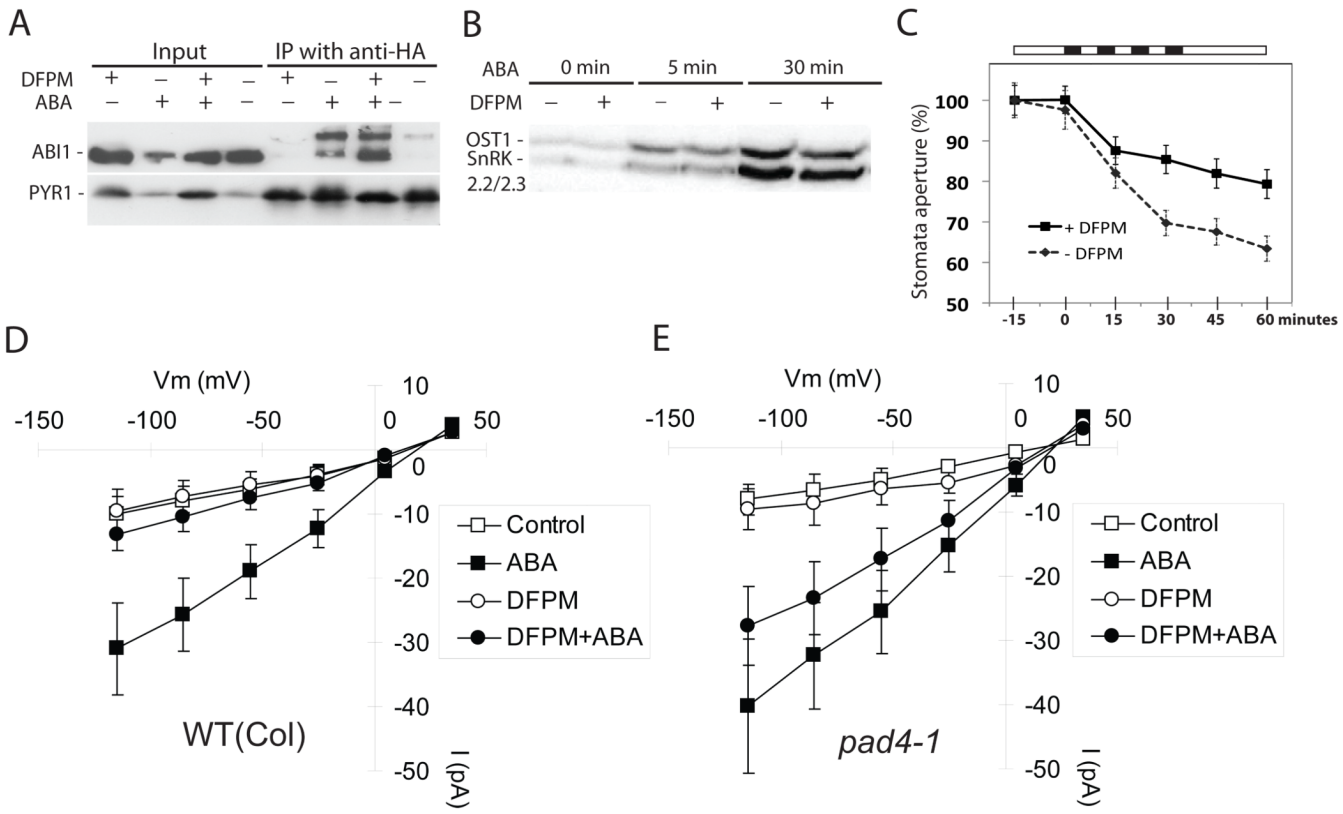


**Figure 4. DFPM inhibits guard cell ABA signal transduction at the level of $Ca^{2+}$ signaling**

**(A)** ABA-dependent protein-protein interaction between the PYR1 ABA receptor and the ABI1 PP2C-type phosphatase is not disrupted by DFPM pre-treatment. HA-PYR1 and YFP-ABI1 were co-immunoprecipitated 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 minute time scale. Stomatal apertures at time = 0 (100%) correspond to average stomatal apertures of 4.02±0.25μm in control treatments and 3.53±0.26μm in DFPM pre-treatments (30 min prior to first $Ca^{2+}$ pulse). Error bars show ± s.e.m (n=4 experiments). **(D)** ABA activation of S-type anion channel currents is significantly inhibited by DFPM in Columbia wildtype 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, 2-tail 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; 2-tail T-test). Guard cell protoplasts were pretreated with 0.06% DMSO (Control) or DFPM for 30 min before ABA+DMSO or ABA+DFPM treatment. Error bars show ± s.e.m.