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SHORT COMMUNICATION

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Cold priming on pathogen susceptibility in the Arabidopsis *eds1* mutant background requires a functional *stromal Ascorbate Peroxidase*

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ABSTRACT

24 h cold exposure (4°C) is sufficient to reduce pathogen susceptibility in *Arabidopsis thaliana* against the virulent *Pseudomonas syringae* pv. *tomato* (*Pst*) strain even when the infection occurs five days later. This priming effect is independent of the immune regulator Enhanced Disease Susceptibility 1 (EDS1) and can be observed in the immune-compromised *eds1–2* null mutant. In contrast, cold priming-reduced *Pst* susceptibility is strongly impaired in knock-out lines of the stromal and thylakoid ascorbate peroxidases (sAPX/tAPX) highlighting their relevance for abiotic stress-related increased immune resilience. Here, we extended our analysis by generating an *eds1 sapx* double mutant. *eds1 sapx* showed *eds1*-like resistance and susceptibility phenotypes against *Pst* strains containing the effectors avrRPM1 and avrRPS4. In comparison to *eds1–2*, susceptibility against the wildtype *Pst* strain was constitutively enhanced in *eds1 sapx*. Although a prior cold priming exposure resulted in reduced *Pst* titers in *eds1–2*, it did not alter *Pst* resistance in *eds1 sapx*. This demonstrates that the genetic *sAPX* requirement for cold priming of basal plant immunity applies also to an *eds1* null mutant background.

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Short communication

Plants have evolved strategies for improved stress responses based on prior stress experiences. One such strategy that differs from acclimation and adaptation but requires a molecular stress imprint or memory is defined as priming.[1,2] A diverse set of stimuli has been shown for being effective in priming the plant immune system against pathogens.[3,4] This includes abiotic changes and pretreatments with altered environmental conditions as a consequence of activated cross-tolerance. Several short (1.5 h) and repetitive cold (4°C) or heat (38°C) treatments increase the resistance of *Arabidopsis thaliana* (Arabidopsis) against the hemi-biotrophic, virulent pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*).[5] Improved plant resistance was also observed when the light period the day prior to the *Pst* infection is extended from 8 h to 16–32 h as a consequence of photoperiod stress.[6] A 24 h pre-exposure of Arabidopsis to an extended or continuous light phase increases the ability for a strong apoplastic production of reactive oxygen species, boosts pathogen-driven salicylic acid accumulation and signaling, and reduces the capability of *Pst* for inducing so-called water-soaking leasions.[7,8]

Recently, we showed that a 24 h cold exposure (4°C) is sufficient to prime plant immunity for an infection with *Pst* occurring 5 days later and resulting in reduced bacterial titers in cold pre-treated Arabidopsis plants (accession: Col-0) compared to naïve control plants.[9] This effect is independent of the plant immune regulator Enhanced Disease Susceptibility 1 (EDS1) and can be observed in the highly susceptible null mutant *eds1–2*. [9] In contrast, cold priming did not lead to reduced bacterial titers when *Pst* strains delivering the pathogen effector proteins avrRPM1 or avrRPS4 were used.[9] When detected by the host, these strains initiate strong and robust plant immune responses in the context of effector-triggered immunity (ETI).[10–13] While avrRPS4-triggered ETI is strongly EDS1-dependent, defense activation triggered by the recognition of avrRPM1 is mainly EDS1-independent.[14]

EDS1 is part of a small family of nucleocytoplasmic lipase-like proteins.[15–18] Together with its other family members Phytoalexin-Deficient 4 (PAD4) and Senescence-Associated Gene 101 (SAG101), EDS1 forms exclusive heterodimers and functions as a central regulator of ETI, basal immunity, and systemic acquired resistance.[17,19–21] Intracellular immune receptors containing Toll-Interleukin 1 receptor (TIR) domains catalyze ribosylated nucleotide second messengers that specifically bind either to EDS1-PAD4 or to EDS1-SAG101 heterodimers and initiate complex activation.[22–25] Mobilized EDS1 complexes contribute to the activation of pathogen-triggered transcriptional defense reprogramming and cell death, and boost accumulation of immune enhancing metabolites, such as salicylic acid and pipecolic acid derivatives.[20,21,26]

As mentioned above, EDS1-dependent signaling is dispensable for cold priming-enhanced *Pst* resistance. However, functional plastid ascorbate peroxidases (APX) are indispensable.[9] Two APX isoforms reside in the chloroplasts of Arabidopsis and most tracheophytes: a soluble stromal APX (sAPX) and thylakoid-bound APX (tAPX).[27–29] While tAPX specifically resides in the plastids, sAPX is dual targeted to the chloroplast stroma and

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the mitochondrial matrix.[30,31] Based on homologies, a further plastid APX-like protein, named TL29, was identified with location to the thylakoid lumen, but does not possess peroxidase activities.[32,33] The interplay of tAPX and sAPX provides two spatial layers for scavenging photosynthesis-related H_2O_2 in the plastid.[27,28] In this context, sAPX and tAPX have mainly redundant functions for photooxidative protection under abiotic stress situations in mature plants.[34–36] However, also distinct roles are reported. Photoprotection in seedlings rather requires sAPX, while tAPX functions in leaves as central regulator of cold priming mediated-repression of core stress-responsive genes during a second cold phase.[35–38] In the priming control, tAPX-mediated suppression of chloroplast NADPH dehydrogenase subunits resulting in less cyclic electron transport provides a source for altered chloroplast-to-nucleus stress signaling.[39] While cold priming-reduced *Pst* susceptibility is significantly weakened in *tapx-knockout* (KO) lines compared to Col-0, *Pst* titers are similar in cold-pretreated and control *sapx*-KO (hereafter: *sapx*) plants indicating a stronger contribution of sAPX.[9]

To test, whether cold priming-reduced *Pst* susceptibility requires plastid ascorbate peroxidases also in the background of the null mutant *eds1–2*, we generated an *eds1 sapx* line (Figure 1a) using the *eds1–2* null mutant and the *sapx* line (SALK_083737).[9,35,40] We tested EDS1 and sAPX protein abundance in the *eds1 sapx* line using a plastid APX serum[37] and a commercial EDS1 antibody (AS13 2751, Agrisera, Sweden) confirming (in addition to prior genotyping) lack of EDS1 and sAPX in the *eds1 sapx* line (Figure 1b). Growth and developmental phenotype of 5-week-old plants did not differ between *eds1 sapx* and parental lines (Figure 1c).

Next, we analyzed the impact of sAPX for EDS1-dependent and -independent immunity. For this purpose, we infiltrated the *eds1 sapx* double line either with *Pst avrRPM1* or *Pst avrRPS4*. We could neither detect differences in bacterial titers between the wildtype Col-0 and the *sapx* nor between *eds1–2* and *eds1 sapx* (Figure 1d, e). The bacterial titers of *Pst avrRPM1* determined three days after inoculation were equally ~ 0.5 $\log_{10}$ higher in *eds1–2* and *eds1 sapx* compared to Col-0 (Figure 1d). In contrast, bacterial numbers of *Pst avrRPS4* were ~ 3 $\log_{10}$ higher in *eds1–2* and *eds1 sapx* than in Col-0 and *sapx* (Figure 1e). This demonstrates that under stable conditions sAPX does not affect plant immunity and that *eds1 sapx* largely resembles the immune phenotype of *eds1–2*.

Our main aim with this study was to investigate whether sAPX is not only required for cold priming-reduced *Pst* susceptibility in Col-0 but also in *eds1–2*. We repeated the cold priming experiments from our recent study[9] in the exact same way, but compared this time *eds1–2* and *eds1 sapx* (Figure 2a). As shown before,[9] a 24 h lasting cold exposure reduced the enhanced susceptibility of *eds1–2* when the *Pst* inoculation was performed 5 days later (Figure 2b). *Pst* titers were lower in cold-primed *eds1–2* than in the non-primed control group (Figure 2b). *Pst* numbers in *eds1 sapx* were already significantly lower without a pre-cold exposure (Figure 2b), which was similar but not significant between Col-0 and *sapx* in our earlier study.[9] The cold priming exposure did not further

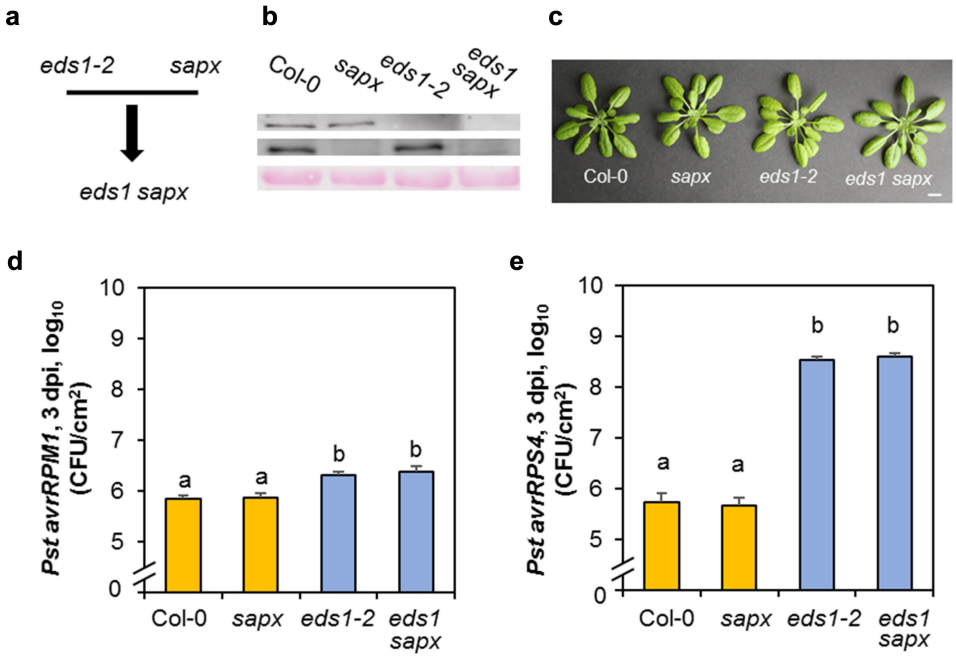

Figure 1. Generation and first analyses of an *eds1 sapx* double mutant line. (a) The *eds1–2* null mutant was crossed with the T-DNA-inserted *sapx*-knockout line (*sapx*) to receive an *eds1 sapx* line. (b) Protein detection of stromal Ascorbate Peroxidase (sAPX) and Enhanced disease Susceptibility1 (EDS1) in leaf extracts of *eds1 sapx* and corresponding single lines. Ponceau S staining of the rubisco large subunit (rbcL) is shown as loading control. (c) Representative picture of rosettes of 5-week-old plants. Scale bar = 1 cm. (d,e) Pathogen-related immune phenotyping of *eds1 sapx* line and parental single lines (5-week-old) was verified by leaf syringe infiltration using *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) strains (OD_{600} = 0.001 in 10 mM $MgCl_2$) delivering either the EDS1-independent effector avrRPM1 (d) or the EDS1-dependent effector avrRPS4 (e). Bacteria were re-isolated 3 days post infection (dpi) and colony-forming units per leaf disk area (CFU/cm^2) were determined. Bars show mean of $\log_{10}$-transformed CFU/cm^2 and standard error (n = 18 from 3 independent experiments). Different letters above the bars denote statistically significant differences (Tukey HSD, $P < .05$).

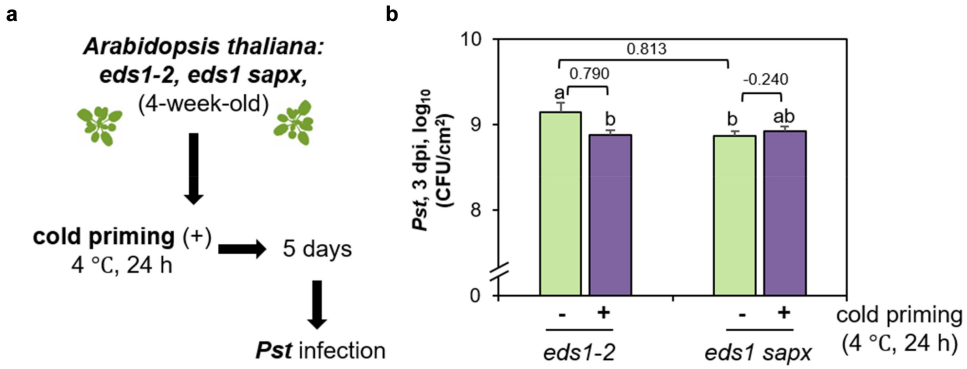

Figure 2. *Pst* titers in *eds1 sapx* after prior cold exposure. (a) Experimental design: 4-week-old *eds1–2* and *eds1 sapx* Arabidopsis plants were cold-treated (4°C) for a full light/dark phase (10 h/14 h) of 24 h starting 2.5 h after the onset of light. After 5 d back at normal temperature conditions (day/night: 22°C/20°C), plants were infiltrated with *Pst* (OD_{600} = 0.001). (b) Bacterial titers of *Pst* (log_{10}-transformed) in (+) cold-primed and (-) control *eds1–2* and *eds1 sapx* plants were determined 3 days post infection (dpi). Bars represent means of log_{10}-transformed colony-forming units (CFU/cm^2) and standard errors calculated from 3 independent experiments (n = 15–18). Different letters above the bars denote statistically significant differences (Tukey HSD, $P < .05$). Numbers between two bars show the effect size between two means according to Cohen's d.

alter *Pst* titers in *eds1 sapx*. This confirms our recent finding, (i) that a prior cold exposure does not alter *Pst* susceptibility when sAPX is lacking. (ii) It additionally shows, that the requirement of sAPX for cold priming-reduced susceptibility also exists in *eds1–2*. (iii) It further highlights, that in the absence of sAPX, *Pst* susceptibility in *eds1 sapx* is constitutively reduced to the level of cold-primed *eds1–2*. As outlined above, EDS1 is required for many different pathogen responses, but not the main player in the cold priming signaling cascade. The generated *eds1 sapx* line provides the opportunity to further analyze the cold priming signaling response on plant immunity in the absence of well-known and strong EDS1-dependent defense responses.

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