Enhanced Disease Susceptibility 5 (EDS5) is required for *N*-hydroxy pipecolic acid formation

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Abstract

Pipecolic acid is essential for the establishment of systemic acquired resistance in plants. It is synthesized in the plastid and further processed in the cytosol to its active form *N*-hydroxy pipecolic acid. Here we provide strong evidence that the exporter Enhanced Disease Susceptibility 5 is not only required for the biosynthesis of salicylic acid, but also of *N*-hydroxy pipecolic acid, suggesting that it represents a convergent point of plant immunity.

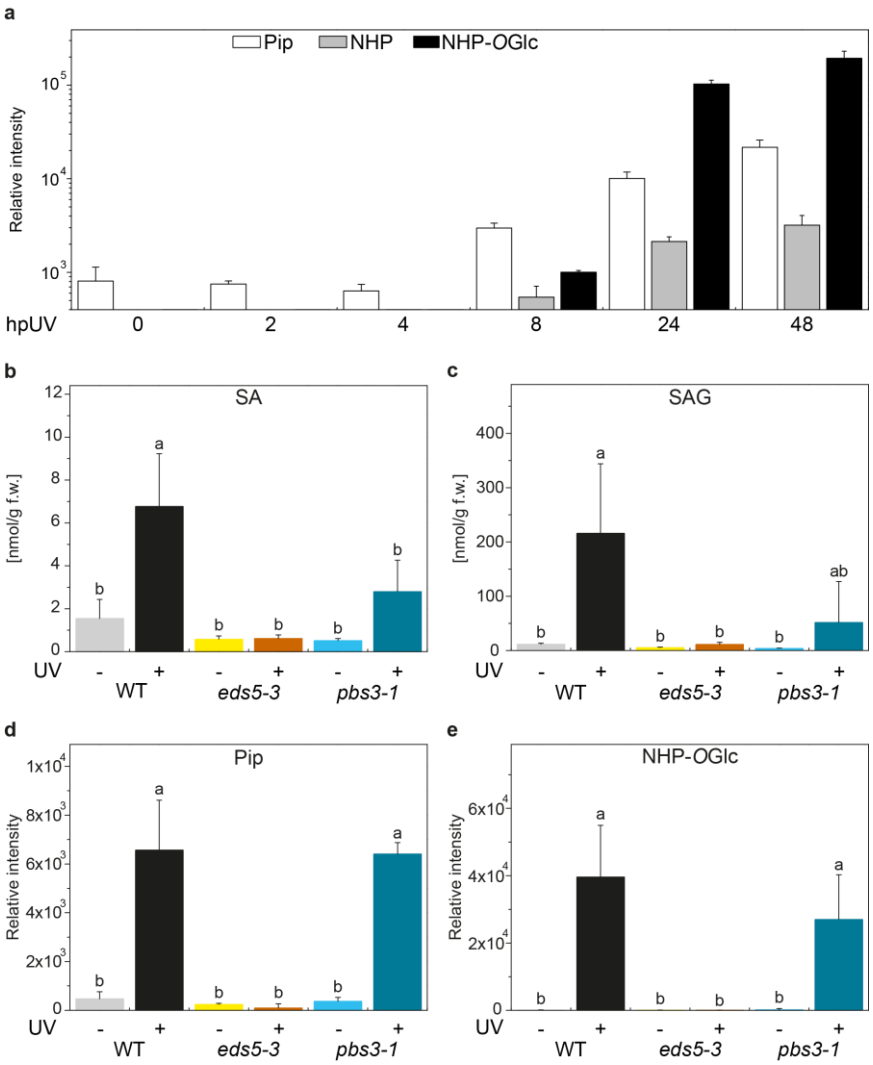
Plants have to adapt to biotic and abiotic challenges in nature. In order to cope with these risks, they produce signaling molecules which are critical for the activation of their defense system[1]. The accumulation of salicylic acid (SA) and pipecolic acid (Pip) at the site of infection as well as in systemic

31 tissues is a key event for establishing the immune response against biotrophic pathogens[2]. The first
32 step in SA biosynthesis is the conversion of chorismic acid (CA) to isochorismic acid (IC) by
33 Isochorismate Synthase 1 (ICS1) in plastids[3]. We recently showed that Enhanced Disease Susceptibility
34 5 (EDS5) is essential for the export of IC from plastids into the cytosol[4]. IC is further processed to
35 isochorismate-9-glutamate by avrPphB Susceptible 3 (PBS3) that subsequently decomposes to SA in a
36 non-enzymatic process. Loss of any of these three genes leads to a drastic reduction of pathogen-
37 induced SA production and to enhanced disease susceptibility[5,6].
38 Pip has been shown to be as crucial as SA for plant immunity[7]. Its biosynthesis occurs in plastids starting
39 from lysine[8,9]. First, the α-aminotransferase AGD2-like Defense Response Protein 1 (ALD1) catalyzes
40 the formation of ε-amino α-keto caproic acid, which spontaneously cyclizes to Δ^1-piperideine-2-
41 carboxylic acid (P2C) in solution. The ketimine reductase SAR-Deficient 4 (SARD4) catalyzes
42 subsequently the formation of Pip from P2C[10,11]. Pip-based signaling relies on Flavin-dependent
43 Monooxygenase 1 (FMO1), which is responsible for the *N*-hydroxylation of Pip to yield *N*-hydroxy
44 pipecolic acid (NHP)[12,13]. This newly discovered compound was proposed to be a crucial regulator of
45 systemic acquired resistance (SAR)[12]. Although the exact subcellular localization of FMO1 has not been
46 determined yet, studies of other FMOs strongly suggest a localization on the cytoplasmic face of the
47 endoplasmic reticulum[14], implying the need for a transporter from the site of Pip biosynthesis to the
48 location where it is further processed. Both SA and NHP can be further glycosylated to form SA-β-
49 glucoside (SAG), SA-glucose ester (SGE)[15] and NHP-glycoside (NHP-*O*Glc)[13], respectively. Glycosylation
50 has been proposed to inactivate plant hormone signaling and is typically facilitated by cytosolic UDP-
51 dependent glycosyltransferases (UGTs)[16].
52 Not only pathogen infection, but also abiotic stresses like ozone or UV-C treatment stimulate the
53 biosynthesis of SA and SAG[17], leading to comparable changes in gene expression[18]. In order to have a
54 fast and reproducible experimental setup for Pip synthesis, we examined the possibility to induce Pip
55 production by UV-C stress. Indeed, Pip accumulates over time in *Arabidopsis thaliana* leaves in a similar
56 course of time (Fig. 1a), as it was described for *Pseudomonas syringae* infection before[12]. Beside Pip,

57 we also observed increased amounts of NHP and NHP-*O*Glc (of which the MS/MS fragmentation
58 patterns are depicted in Supplementary Fig. 1)[13]. This indicates that Pip oxidation and glycosylation
59 also occur after UV-C treatment. To exclude that the observed synthesis of Pip and NHP-*O*Glc is
60 activated by UV-C-triggered SA accumulation, we examined Pip and NHP-*O*Glc contents in the SA-
61 deficient mutants, *eds5, pbs3* (Fig. 1b-e) and Salicylic acid Induction Deficient 2 (*sid2),* harboring a
62 mutation in the *ICS1* gene (Supplementary Figure 2a and b). As expected, the mutant lines, but not
63 wild type plants, lack both SA and SAG 24 hours after being exposed to UV-C for 20 min (Fig. 1b and c).
64 On the other hand Pip and NHP-*O*Glc accumulation was observed only in wild type, *pbs3* and *sid2*, but
65 surprisingly not in *eds5* plants (Fig. 1d and e). Similar to pathogen-treated plants[12], UV-C stress-induced
66 Pip-biosynthesis and -catabolism do not depend on the presence of SA in UV-C treated plants. The
67 absence of Pip and NHP-*O*Glc in *eds5* raised the question whether EDS5 is responsible for the export
68 of not only the SA precursor IC, but also of Pip. Recently, it was shown that *fmo1* mutants are impaired
69 in the hydroxylation of Pip to NHP. Instead, *fmo1* accumulates large amounts of Pip upon UV-C
70 irradiation (Supplementary Fig. 3), again in a similar extent as described for pathogen assays[12]. In line
71 with FMO1 functioning downstream of Pip synthesis, these results corroborate that EDS5 acts
72 upstream of FMO1 by exporting Pip to the cytosol.
73 The assumed block in Pip export did not result in an enrichment of Pip in *eds5* plants, despite the
74 induction of Pip biosynthesis on the transcriptional level: Both *SARD4* and *EDS5* were strongly
75 upregulated upon UV-C treatment in the SA deficient *sid2* mutant and wild type plants (Supplementary
76 Figure 2c and d), supporting our metabolite profiling results. In analogy, we observed increased levels
77 of IC in *pbs3*, but not in *eds5* mutant plants upon UV-C treatment (Supplementary Fig. 4). It is likely
78 that a diversion of the metabolic flux prevents a harmful accumulation of both Pip and IC in plastids[19].
79 Pip may either inhibit its own biosynthesis via a feedback loop or feed into the lysine degradation
80 pathway towards the Krebs cycle[20]. IC on the other hand might be channeled towards the synthesis of
81 aromatic amino acids.

82 In order to exclude that the observed absence of NHP and NHP-*O*Glc in *eds5* mutants is due to their
83 inability to accumulate SA, we tested whether external SA supply could induce Pip and NHP-*O*Glc
84 production in *eds5* and *pbs3* plants. As reported, SA treatment triggers the biosynthesis of Pip and
85 NHP-*O*Glc in wild type plants (Fig. 2a)[7]. SA biosynthesis is impaired in the *pbs3* mutant, but drenching
86 the soil with SA still initiated Pip biosynthesis in these plants (Fig. 2a). However, as with UV-C
87 treatment, external SA also did not lead to Pip accumulation in *eds5* mutants. This is consistent with
88 EDS5 representing a plastidial exporter of Pip independent of SA. When Pip irrigation was used to
89 trigger *in planta* Pip biosynthesis, we detected increased levels of the downstream product NHP-*O*Glc
90 in wild type plants (Fig. 2b). In *eds5* and the Pip biosynthesis mutant *sard4*, the levels of NHP-*O*Glc are
91 reduced by more than 60%. This suggests that in the mutants only the external Pip was metabolized
92 further, whereas *de novo* Pip biosynthesis did not occur. The ability of *eds5* plants to convert
93 exogenously applied Pip into NHP-*O*Glc supports FMO1 being active outside of plastids and not
94 affected by the loss of function of *EDS5*.
95 Taken together, we show that UV-C treatment is sufficient to induce the production of Pip and its
96 metabolites. This process does not require the presence of SA and can thus be used to study the SA-
97 independent branch of plant defense signaling. Moreover, we identified here a previously unknown
98 connection between Pip and EDS5, a protein that, so far, was only recognized for its involvement in SA
99 biosynthesis[17]. Most likely EDS5 is also responsible for the export of Pip from plastids into the cytosol,
100 where it is further processed by FMO1. This spatial separation of Pip biosynthesis and the site of
101 activation to NHP adds an additional layer for regulation. Our study implies that EDS5 serves as the
102 central hub in the biosynthesis of two major defense signaling molecules, SA and NHP (Fig. 2c). It is
103 surprising that no pathogen effector has been found to target EDS5 and thereby exploit this key point
104 in plant immunity.
105

106 **Figures**



107

108 **Figure 1 UV-C treatment of *Arabidopsis thaliana* leads to an accumulation of the signaling**
109 **compounds SA and Pip and their corresponding glycosides. a,** Relative amounts (counts per second)
110 of pipecolic acid (Pip, white bars) and its downstream products *N*-hydroxy pipecolic acid (NHP, grey
111 bars) and *N*-hydroxy pipecolic acid glycoside (NHP-*O*Glc, black bars) in *Arabidopsis* wild type leaves
112 (Col-0) at different time points after UV-C light treatment. **b-e,** Relative amounts of salicylic acid (SA)
113 and its glycoside SAG (nmol g^{-1} leaf fresh weight [f.w.]) and of Pip and its glycoside NHP-*O*Glc (counts
114 per second) in leaves of wild type (Col-0), respectively *eds5*-3 and *pbs3*-1 mutant plants 24 hours after
115 UV-C treatment in comparison to untreated plants. Bars represent the mean ± STD of three biological
116 replicates. Statistical differences among replicates are labeled with different letters ($P < 0.05$, one-way
117 ANOVA and post hoc Tukey's Test; n = 3). The experiments were repeated twice with similar results.

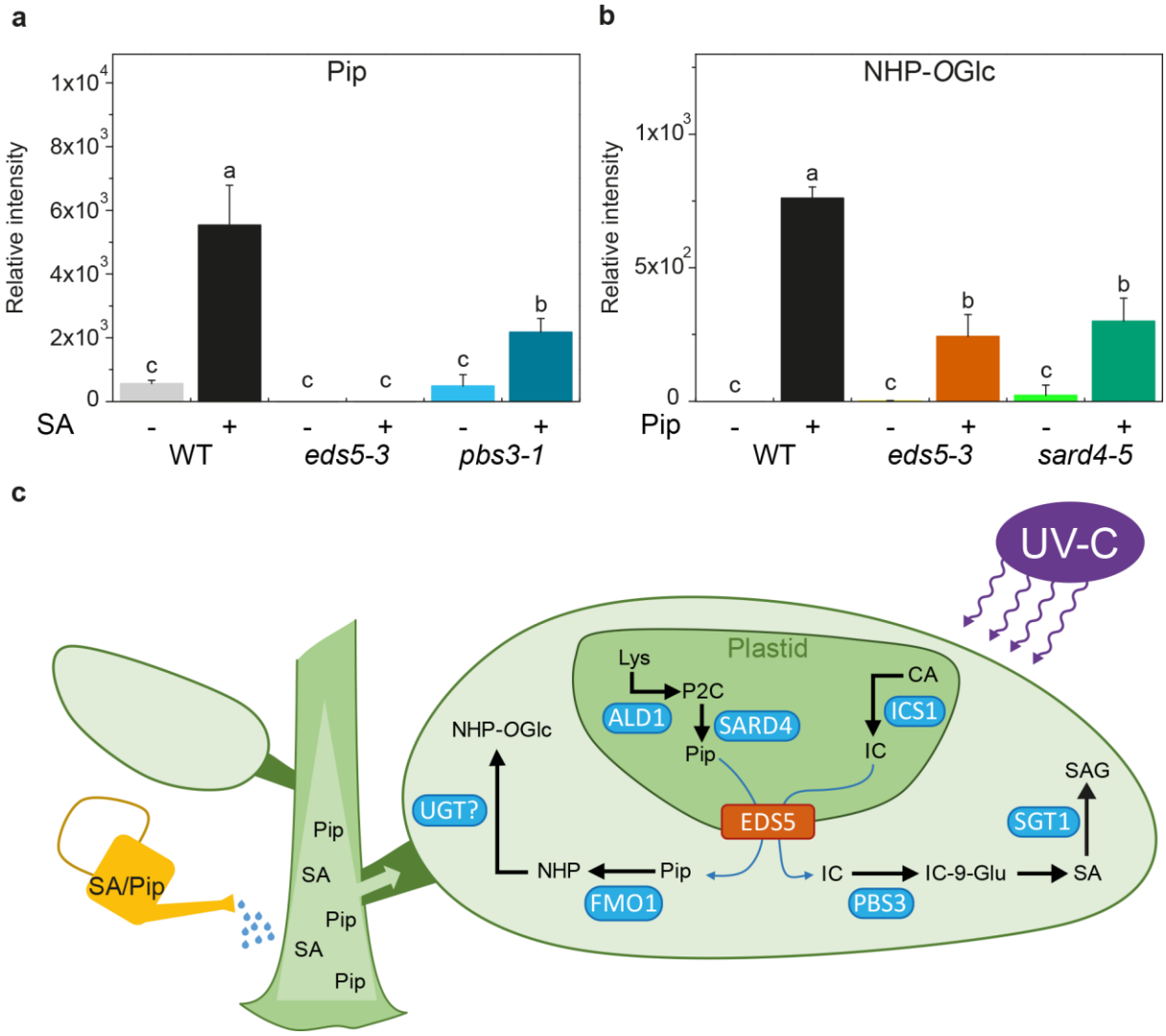


Figure 2 Root application of either SA or Pip induces Pip biosynthesis and processing in leaves. a, Relative amounts (counts per second) of pipecolic acid (Pip) in leaves of wild type (Col-0) *eds5*-3 and *pbs3*-1 plants 24 hours after soil drenching with water (-) or SA (+). **b,** Relative amounts (counts per second) of *N*-hydroxy pipecolic acid glycoside (NHP-*O*Glc) in leaves of wild type (Col-0), *eds5*-3 and *sard4-5* plants 24 hours after soil drenching with water (-) or Pip (+). Bars represent the mean ± STD of three biological replicates. Statistical differences among replicates are labeled with different letters ($P < 0.05$, one-way ANOVA and post hoc Tukey's Test; n = 3). The experiment was repeated twice with similar results. **c,** A working model depicting EDS5 as the central hub in the biosynthesis of the signaling compounds SA and NHP. The induction of these pathways was facilitated here by UV-C light treatment.

129 ## Methods

130 **Plant material.** *Arabidopsis* plants were grown in a chamber at 22 °C with a 16 h light period and 60%
131 relative humidity for 4-5 weeks. For our experiments, we use *Arabidopsis* ecotype Col-0 and the
132 following mutants in this background: *eds5-3*[21], *pbs3-1*[6], *sard4-5*[10], and fmo1-1[12], which were described
133 previously.

134

135 **UV-C and soil drench treatment.** For the UV-C treatment, we followed previous protocol[17]. In short, 4-
136 5 week old *Arabidopsis* plants were exposed to UV-C light (254 nm) for 20 min at 50 cm distance to the
137 lamp (TUV T8 30W, Philips) for the induction of SA and Pip biosynthesis. For the treatment with SA and
138 Pip, we followed previously described protocols[7,21]. 4-5 week old plants, were soil drenched with either
139 10 mL water, 10 mL of a 5 mM Pip solution (P45850, Sigma) or 10 mL of a 5 mM SA solution (S5922,
140 Sigma), equals to 50 µM final concentration. Samples were collected 24 hours after treatment and
141 metabolites were extracted and analyzed as described earlier[10,22]. The deviation of exact mass to
142 accurate mass for Pip, NHP and NHP-*O*Glc was less than ±2 mDa in the untargeted metabolite analysis.
143 SA and SAG were quantified based on internal D4-SA standard (C/D/N Isotopes Inc., Pointe-Claire,
144 Canada). The NHP standard was chemical synthesized as described in Hartmann et al., 2018[12]. The
145 MS/MS spectra of NHP and NHP-*O*Glc corresponds to the results from Chen et al., 2018[13].

146

147 **Quantitative real-time PCR analysis.** To analyze the expression of *SARD4* (At5g52810) and *EDS5*
148 (AT4G39030) after UV-C treatment, total RNA was isolated from frozen leaves with the Spectrum™
149 Plant Total RNA Kit (Sigma) following the manufacturer's instruction. One microgram of RNA was
150 treated with DNase I (Thermo Fisher Scientific) and cDNA was synthesized using Revert Aid H Minus
151 Reverse Transcriptase (Thermo Fisher Scientific). Quantitative RT-PCR was performed using Takyon No
152 ROX SYBR Mastermix blue dTTP (Kaneka Eurogentec) in reaction volume 20 µl. The gene ACTIN8
153 (At1g49240) was used as a control. Each reaction was performed with material from plants harvested

154 from three independent samples in iQ5 real time detection system (Bio-Rad). Primers are depicted in
155 Supplementary Table 1.
156

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165 Author contributions

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