Letter

Mutant Muddle: Some Arabidopsis *eds5* Mutant Lines Have a Previously Unnoticed Second-Site Mutation in *FAH1*[OPEN]

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Salicylic acid (SA) is produced by the enzyme isochorismate synthase (ICS) within the chloroplast and is subsequently exported to the cytosol by the multidrug and toxic compound extrusion transporter ENHANCED DISEASE SUSCEPTIBILITY5 (EDS5; Nawrath et al., 2002; Serrano et al., 2013). The ICS pathway is the major source of SA during plant responses to various fungal and bacterial pathogens (Nawrath and Métraux, 1999; Wildermuth et al., 2001). Accordingly, Arabidopsis (*Arabidopsis thaliana*) mutants defective in *EDS5* are impaired in the SA-dependent establishment of resistance against *Pseudomonas syringae* pv. *tomato* (Nawrath and Métraux, 1999). Several SA-deficient *eds5* loss-of-function alleles have been established. The mutant alleles *eds5-1* and *eds5-3* (Glazebrook et al., 1996; Nawrath and Métraux, 1999) are widely used in plant pathology research, whereas *eds5-2* (Volko et al., 1998) is less frequently used. We discovered that the *eds5-3*, *syp121-1 syp122-1 eds5-3*, and *syp121-1 syp122-1 eds5-3 sid2-1* mutants all carried an unnoticed second-site mutation in *FERULIC ACID 5-HYDROXYLASE1* (*FAH1*). The *eds5-1* mutant lines might harbor a similar mutation in *FAH1*. Therefore, it is strongly recommended to test all stocks of *eds5-3* and *eds5-1* for the *FAH1* background mutation before use.

eds5 mutants do not have an obvious growth phenotype. However, under UV-A illumination, leaves of *eds5-3* exhibit red chlorophyll fluorescence rather than the blue fluorescence observed in wild-type (Col-0) plants (Fig. 1A). UV-excited blue fluorescence is mainly emitted by sinapoylmalate, which is a major phenylpropanoid in vacuoles of the leaf upper epidermis, where it serves as a protective barrier against harmful UV irradiation (Fraser and Chapple, 2011). *eds5-1* mutant seedlings grown from two different seed stocks (the Mario Serrano laboratory stock and the Arabidopsis Biological Resource Center stock CS3735, donated by the Frederick Ausubel laboratory) had similar levels of blue fluorescence as the wild type (Fig. 1A), suggesting that the defect in *EDS5* expression did not cause the lack of sinapoylmalate in *eds5-3*. NahG *sid2-1* plants cannot accumulate SA due to a mutation in the ICS coding gene *ICS1/SID2* and transgenic expression of the SA hydroxylase gene NahG from *Pseudomonas putida* (Delaney et al., 1994; Supplemental Materials and Methods S1). Under UV, NahG *sid2-1* was indistinguishable from wild-type plants, underpinning again that SA deficiency does not affect sinapoylmalate biosynthesis (Fig. 1A).

The *eds5-3* mutant had a similar UV phenotype as reported in the previously identified *reduced epidermal fluorescence* mutants, including the *fah1-2* line (Fraser and Chapple, 2011; Fig. 1A). *fah1-2* has a defect in *FAH1*, which encodes ferulate-5-hydroxylase, an essential enzyme for the conversion of ferulate into sinapates such as sinapoylmalate (Chapple et al., 1992; Fraser and Chapple, 2011). Ultra-performance liquid chromatography coupled to mass spectrometry (Tzin et al., 2012) was employed for molecular phenotyping of the mutants (Supplemental Materials

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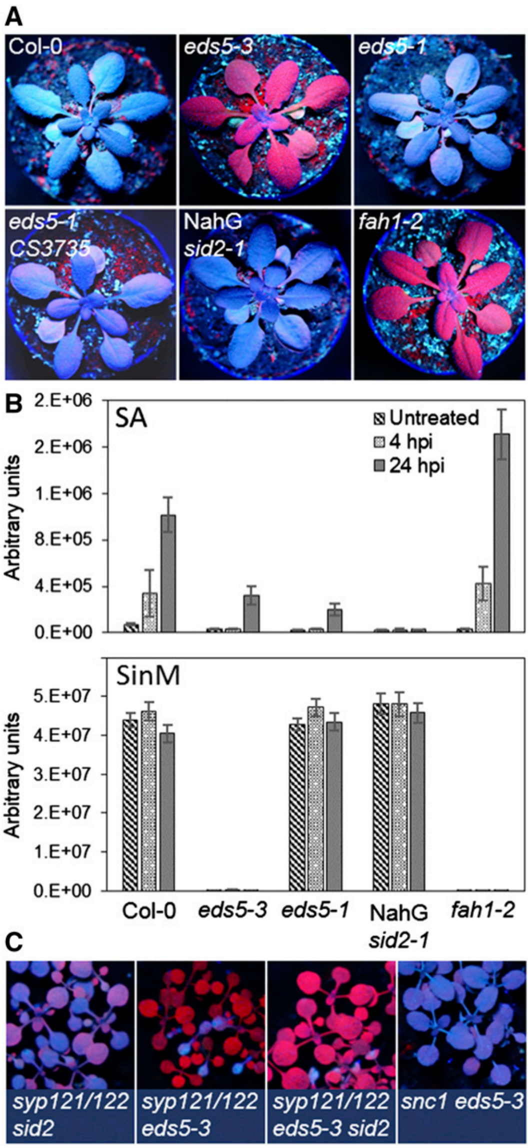

Figure 1. *eds5-3* carries a *FAH1* mutation. A, Detection of blue fluorescent sinapoylmalate under UV-A illumination (365-nm wavelength). Mutant lines lacking sinapoylmalate emit red fluorescence under UV. B, Semiquantification of SA and sinapoylmalate (SinM) by ultra-performance liquid chromatography coupled to mass spectrometry. Plants were infected with *P. syringae* pv. *tomato AvrRpm1*, and leaf extracts were sampled at 4- and 24-h post infection (hpi). Error bars indicate the SD ($n = 4$–5). C, The *syp121-1 syp122-1 eds5-3* and *syp121-1 syp122-1 eds5-3 sid2-1* mutants carry the *fah1-2* background mutation as evidenced by their red fluorescence phenotype. *snc1-1 eds5-3* showed blue fluorescence under UV illumination, suggesting that the *fah1-2* mutation had been crossed out. Refer to Supplemental Materials and Methods S1 for a detailed description of the mutant lines and methods.

and Methods S1). Pathogen infection with avirulent *P. syringae* pv *tomato AvrRpm1* triggered the accumulation of SA at 4 h and more pronouncedly at 24 h after infection in wild-type and *fah1-2* plants (Fig. 1B). By contrast, the SA peak was reduced in *eds5-3* and *eds5-1*, and absent in NahG *sid2-1*. Sinapoylmalate levels were high and not strongly regulated upon pathogen infection in wild type, *eds5-1*, and NahG *sid2-1*. However, sinapoylmalate was not detectable in *eds5-3* and *fah1-2* (Fig. 1B). Collectively, these results point to sinapoylmalate biosynthesis being disrupted in *eds5-3* independently of the *EDS5* mutation.

This prompted us to consider whether an unknown second-site mutation caused the unexpected *eds5-3* phenotype. In more recent reports, Col-0 was given as the background of *eds5-3* (Nawrath et al., 2002; Serrano et al., 2013). Only the initial publications, describing the generation, identification, and characterization of *eds5* mutants, provided an explanation for the sinapoylmalate deficiency of *eds5-3*. In these publications, it was mentioned that *eds* mutants were generated by EMS mutagenesis of *fah1-2* rather than Col-0 seeds (Glazebrook et al., 1996; Rogers and Ausubel, 1997). The *fah1-2* mutant (in the Col-0 background) was chosen because the red fluorescence phenotype is a useful marker in genetic crosses (Glazebrook et al., 1996). *eds5-1* and *eds5-3* both originated from the same mutant population. The *fah1-2* and *eds5* mutations are only 10 centimorgans apart (Reuber et al., 1998), which rendered removal of the *fah1-2* mutation by backcrossing difficult due to genetic linkage. *eds5-1* was successfully backcrossed with Col-0 before submission to the Arabidopsis Biological Resource Center (Fig. 1; J. Glazebrook, personal communication), whereas *eds5-3* still carries the *fah1-2* background mutation (Fig. 1; Nawrath and Métraux, 1999). *eds5-2* originated from an independent screen, not involving *fah1-2* (Volko et al., 1998).

eds5 lines were crossed with various mutants to study the interaction of SA signaling with other defense mechanisms (Zhang et al., 2008; Venugopal et al., 2009; Dong et al., 2016). Inspecting multiple mutants under UV revealed that *syp121-1 syp122-1 eds5-3* and *syp121-1 syp122-1 eds5-3 sid2-1* (mutants from H.T.-C.'s laboratory; Zhang et al., 2008) displayed the red fluorescence phenotype, whereas in *snc1-1 eds5-3* (mutant from Xin Li's laboratory; Dong et al., 2016) the *fah1-2* mutation was seemingly outcrossed (Fig. 1C). Thus, due to genetic linkage, *fah1-2* can be an unnoticed background mutation in multiple mutants containing *eds5-3*. Some laboratories could have stocks of the original *eds5-1* line with the *fah1-2* background. However, we have yet to test multiple mutants containing *eds5-1*, such as *acd11 eds5-1* and *ssi2 eds1-2 eds5-1* (Brodersen et al., 2005; Venugopal et al., 2009).

Previous studies provided evidence that the lack of sinapates and syringyl lignin in *fah1-2* increased susceptibility to the fungal pathogens *Botrytis cinerea* and *Verticillium longisporum* (Lloyd et al., 2011; Demkura and Ballaré, 2012; König et al., 2014). Hence, the unnoticed *fah1-2* background mutation could influence pathogen resistance of *eds5-3* and possibly *eds5-1* plants in a SA-independent manner, thereby leading to false conclusions on the role of EDS5 and SA in plant–pathogen interactions. For this reason, it is strongly recommended to check all lab stocks of *eds5-3*, *eds5-1*, and multiple mutants containing one of these mutations under UV-A illumination for the characteristic red fluorescence phenotype of *fah1-2*, followed by molecular confirmation of the genotype by PCR with *FAH1*-specific primers as described in Weng et al. (2010).

Supplemental Data

The following supplemental materials are available.

Supplemental Materials and Methods S1. Description of the mutant lines, materials, and methods used.

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