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Running Title: PAD4 activities in plant defense

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Discrimination of Arabidopsis PAD4 activities in defense against green peach aphid and pathogens[1]

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FOOTNOTES

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ABSTRACT

The Arabidopsis lipase-like protein PHYTOALEXIN DEFICIENT4 (PAD4) is essential for defense against green peach aphid (GPA; *Myzus persicae* Sülzer) and the pathogens *Pseudomonas syringae* (*Pst*) and *Hyaloperonospora arabidopsidis* (*Hpa*). In basal resistance to virulent strains of *Pst* and *Hpa*, PAD4 functions together with its interacting partner ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) to promote salicylic acid (SA)-dependent and SA-independent defenses. By contrast, dissociated forms of PAD4 and EDS1 signal effector-triggered immunity (ETI) to avirulent strains of these pathogens. PAD4-controlled defense against GPA requires neither EDS1 nor SA. Here we show that resistance to GPA is unaltered in an *eds1 sid2* double mutant, indicating that redundancy between *EDS1* and *SID2*-dependent SA previously reported for ETI conditioned by certain NB-LRR receptors, does not explain dispensability of *EDS1* and *SID2* in defense against GPA. Mutation of a conserved serine (S118) in the predicted lipase catalytic triad of PAD4 abolished PAD4-conditioned antibiosis and deterrence against GPA feeding but S118 was dispensible for deterring GPA settling and promoting senescence in GPA-infested plants, and for pathogen resistance. These results highlight distinct molecular activities of PAD4 determining particular aspects of defense against aphids and pathogens.

INTRODUCTION

Plants have evolved complex defense mechanisms to counter infection by pathogens and insects. In *Arabidopsis thaliana*, *PHYTOALEXIN DEFICIENT4* (*PAD4*) is an important modulator of resistance to pathogens and the green peach aphid (GPA; *Myzus persicae* Sülzer) (Glazebrook, 2005; Wiermer et al., 2005; Goggin, 2007; Walling, 2008). Genetic studies identified *PAD4* as an essential component of Arabidopsis basal immunity against virulent pathogens that have a biotrophic phase in their infection cycle and for promoting accumulation of the defense signaling hormone salicylic acid (SA) and phytoalexin, camalexin (Zhou et al., 1998; Jirage et al., 1999; Feys et al., 2001). *PAD4* also contributes to effector-triggered immunity (ETI) involving local pathogen containment and host cell death conditioned by intracellular TIR-NB-LRR (Toll-interleukin receptor- nucleotide-binding-leucine-rich-repeat) receptors (Zhou et al., 1998; Jirage et al., 1999; Feys et al., 2001; Rusterucci et al., 2001; Rietz et al., 2011). In addition to local defenses, *PAD4* drives activation of systemic acquired resistance (SAR) which protects leaves against subsequent infection (Rusterucci et al., 2001; Rietz et al., 2011).

PAD4 physically interacts with the sequence-related EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1) protein (Feys et al., 2001, 2005; Reitz et al., 2011). This stabilizes PAD4 and resulting EDS1-PAD4 complexes accumulate in the nucleus and cytoplasm of leaf cells (Feys et al., 2005; Rietz et al., 2011). Interaction with EDS1 was found to be necessary for PAD4 function in basal immunity since pathogen growth and severity of disease caused by virulent isolates of the oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) and the bacterial pathogen *Pseudomonas syringae* pv *tomato* (*Pst*) strain DC3000 were enhanced in stable transgenic plants expressing an eds1^{L262P} amino acid exchange mutant that fails to bind PAD4 (Rietz et al., 2011). Pathogen infection-induced up-regulation of *PAD4* expression as well as SA accumulation and SAR were disrupted in *eds1*^{L262P} plants (Rietz et al., 2011). By contrast, TIR-NB-LRR-conditioned ETI against avirulent strains of *Hpa* and local programmed cell death were not compromised in *eds1*^{L262P} plants (Rietz et al., 2011), implying that physical interaction between EDS1 and PAD4 is not critical for ETI (Rietz et al., 2011). Since dissociated EDS1 and PAD4 proteins are required for ETI (Rusterucci et al., 2001; Rietz et al., 2011), it was suggested that two different molecular states of PAD4 condition basal immunity and ETI against pathogens

(Rietz et al., 2011). Furthermore, because EDS1-PAD4 association is required for SA accumulation and SA drives up-regulation of *PAD4* and *EDS1* expression in pathogen-infected leaves, a feed-forward loop involving EDS1-PAD4 complexes has been proposed to lead to defense amplification (Jirage et al., 1999; Feys et al., 2001; Rietz et al., 2011).

Arabidopsis *PAD4* is also required for controlling GPA infestation (Pegadaraju et al., 2005, 2007). GPA is a phloem sap-sucking insect pest of plants and vector for several viral diseases (Kennedy et al., 1962; Matthews, 1991; Blackman and Eastop, 2000). Aphids use their slender stylets to penetrate largely intercellularly to reach the sieve elements. Unlike chewing insects, this feeding strategy does not cause extensive wounding of plant tissue (Walling, 2000; Howe and Jander, 2008). In resistance to GPA, PAD4 controls antibiosis, which curtails insect growth, development and reproduction. A PAD4 dependent antibiotic activity is present in Arabidopsis petiole exudate (enriched in vascular sap) that adversely impacts insect fecundity (Louis et al., 2010b). Also, PAD4 conditions antixenotic defenses that deter GPA settling on plants and feeding from the sieve elements. Moreover, PAD4 promotes premature leaf senescence in GPA-infested plants, characterized by chlorophyll loss, cell death and the elevated expression of a subset of *SENESENCE ASSOCIATED GENES* (*SAG*) genes (Pegadaraju et al., 2005, 2007). In support of a role for a senescence-like mechanism in controlling GPA infestation, *PAD4*-dependent constitutive *SAG13* expression in the Arabidopsis *ssi2* (*suppressor of SA-insensitivity2*) mutant correlated with enhanced resistance to GPA (Louis et al., 2010b).

Studies with Arabidopsis *sid2* (*salicylic acid induction deficient2*) and *pad3* mutant plants which are defective in SA and camalexin synthesis, respectively, and the *npr1* (*nonexpresser of PR genes1*) mutant defective in SA signaling, indicated that SA and camalexin are not required for defense against GPA, suggesting that the role of PAD4 in defense against GPA is different from its activities in pathogen resistance (Pegadaraju et al., 2005). Also, *PAD4* involvement in limiting GPA infestation is independent of *EDS1* since GPA populations were restricted to a similar extent in *eds1* mutant and wild-type (WT) plants (Pegadaraju et al., 2007). Moreover, constitutive over-expression (OE) of *PAD4* from the *Cauliflower mosaic virus* (*CaMV*) *35S* promoter in an *eds1* mutant background was sufficient to confer enhanced GPA resistance to a level comparable to that observed in *35S:PAD4* transgenic plants containing a WT

EDS1 allele (Pegadaraju et al., 2007). Thus, several distinct PAD4-conditioned mechanisms seem to contribute to Arabidopsis defenses against pathogens and GPA. Further studies reinforced the notion of discrete PAD4-controlled outputs contributing to GPA resistance. For example, constitutive OE of *PAD4* from the *35S* promoter enhanced antixenosis (Pegadaraju et al., 2007) but did not cause an increase in antibiotic activity contained in petiole exudates (Louis et al., 2010b). Similarly, elevated *SAG13* expression and antibiosis activity against GPA in *ssi2* mutant was not accompanied by increased antixenosis activity against GPA. Also, *PAD4* expression which is up-regulated in GPA-infested WT Arabidopsis (Pegadaraju et al., 2005), was not constitutively elevated in the *ssi2* mutant (Louis et al., 2010b), suggesting that basal *PAD4* transcript levels are sufficient for antibiosis against GPA but induced *PAD4* expression in GPA-infested plants contributes to antixenosis.

PAD4 and EDS1 share homology in their N-terminal halves to α/β-fold acyl hydrolase enzymes that include lipases and esterases (Zhou et al., 1998; Jirage et al., 1999) although hydrolase activity has not been demonstrated for either protein (Wiermer et al., 2005). A predicted catalytic triad consisting of the amino acids serine (S), aspartic acid (D) and histidine (H) is present in PAD4 at positions 118, 178 and 229, respectively. X-ray crystallography studies indicated that a comparable triad of S, D and H form part of the active site in eukaryotic lipases (Brady et al., 1990; Winkler et al., 1990). Mutational analysis of human pancreatic lipase indicates that these amino acids are required for lipase activity (Winkler et al. 1990; Lowe, 1992). More direct evidence of the S in catalysis came from crystal structure of a fungal lipase complexed with the substrate analog *n*-hexylphosphate ethyl ester, which formed a covalent bond with the S residue (Brzozowski et al., 1991). In this study, we used Arabidopsis transgenic plants expressing mutant versions of PAD4 in which these residues were individually replaced by alanine (A) to test whether the conserved amino acids are important for PAD4 involvement in defense against pathogens or GPA. We find that each of the predicted catalytic residues are dispensible for resistance to *Pst* and *Hpa*. Alanine substitution at S118 severely compromises antibiosis and feeding deterrence against GPA but does not interfere with PAD4-determined deterrence of insect settling or promotion of *SAG13* expression and chlorophyll loss in response to GPA infestation. Thus, S118 is necessary for a subset of PAD4-modulated defenses against GPA. We further show that unlike as shown by Venugopal et al. (2009) for ETI, genetic

redundancy between *EDS1* and *SID2* does not explain dispensibility of these signaling components in *PAD4*-conditioned resistance to GPA. Our results suggest that PAD4 is able to adopt a number of molecularly and mechanistically different forms determining particular PAD4 sub-functions in plant defense against pathogens and GPA.

RESULTS

EDS1 and *SID2* do not act redundantly in limiting GPA infestation

Previously, we concluded that the involvement of PAD4 in curtailing GPA infestation was independent of EDS1 since resistance against GPA was not compromised in *eds1* null mutants of accessions Columbia (Col) or Wassilewskija (Ws), and constitutive OE of *PAD4* enhanced resistance against GPA in an *eds1* mutant background (Pegadaraju et al., 2007). When *EDS1* was constitutively over-expressed from the *35S* promoter this slightly enhanced resistance against GPA compared to WT Ws and *eds1-1* plants in a no-choice assay (Fig. 1A). In the no-choice assay, twenty apterous (wingless) GPA were released on each plant and the GPA population size (adults + nymphs) was determined 2 days post infestation (dpi). PAD4 transcript accumulation was comparable between the *EDS1* OE and WT plants (Fig. 1B). The slight resistance enhancing effect of constitutive *EDS1* OE may be due to the ability of the constitutively over-expressed EDS1 to stabilize PAD4 protein (Feys et al., 2001, 2005; Rietz et al., 2011). Since the impact of EDS1 OE on enhancing resistance against GPA was not as strong as that observed in plants constitutively over-expressing PAD4 alone, and basal resistance against GPA was not compromised in the *eds1-1* mutant, we conclude that EDS1 is not rate-limiting in PAD4-mediated defense against GPA.

Redundant functions of *EDS1* and *SID2*, which is involved in SA biosynthesis, in ETI triggered by the *Pst* effectors *AvrRpt2* and *AvrRps4* and the *Hpa* effector *Atr8*, were uncovered when both pathways were disabled in an *eds1 sid2* double mutant (Venugopal et al., 2009). Therefore, we thought it possible that the lack of effect of *eds1* alleles or SA biosynthesis and signaling mutants on Arabidopsis defense against GPA (Pegadaraju et al., 2005, 2007) may be

due to redundancy between *EDS1*- and *SID2*-regulated pathways. This might also explain why resistance to GPA was marginally enhanced in the *EDS1* OE plants (Fig. 1A). To test this hypothesis, a no-choice assay was conducted with the *eds1-1 sid2-1* and *eds1-22 sid2-1* double mutants, and corresponding single mutant and WT plants. The *eds1-1* allele is in Arabidopsis accession Ws and the *eds1-22* and *sid2-1* alleles are in accession Col. We found that GPA numbers on the *eds1-1 sid2-1* and the *eds1-22 sid2-1* double mutants were similar to those on WT and single mutant plants, whereas GPA numbers were higher on Col *pad4-1* and Ws *pad4-5* mutants that were used as controls (Fig. 1C). These results indicate that any redundancy that may exist between *EDS1* and the *SID2*-dependent pathways is not important for controlling GPA infestation.

S118 is required for PAD4-controlled restriction of aphid infestation

S118 in PAD4 is embedded within the GHSTG sequence that resembles the GXSXG motif of several eukaryotic lipases (Fig. 2A and 2B). Several amino acids flanking S118 are also conserved between PAD4 and these eukaryotic lipases (Fig. 2B). Serine is the key catalytic residue and an aspartic acid (D178 in PAD4) and histidine (H229 in PAD4) complete the catalytic triad of many lipases (Fig. 2B) (Blow, 1990; Brady et al., 1990; Winkler et al., 1990; Brzozowski et al., 1991; Lowe, 1992). Replacement of S153 in human pancreatic lipase by alanine (A) resulted in loss of lipase activity, but did not impact its ability to bind the lipid substrate, thus confirming that S153 in pancreatic lipase is essential for catalysis (Lowe, 1992). Similarly, replacement of S at amino acid 423 by A in the rat hormone-sensitive lipase resulted in loss of lipase activity (Holm et al., 1994). Mutations at D703 and H733, the other two active site residues, also resulted in loss of enzymatic activity (Østerlund et al., 1997). To determine whether S118, D178 or H229 of PAD4 have a role in defense against GPA, *PAD4* constructs were made in which the S, D and H at these positions were individually substituted with A to produce corresponding pad4^{S118A}, pad4^{D178A} and pad4^{H229A} proteins driven by the native *PAD4* promoter and fused N-terminally to a cMyc epitope tag. The three mutant constructs and non-mutated *cMyc-PAD4* (*PAD4*^{WT}) expressed from the *PAD4* native promoter were transformed into the *pad4-5* null mutant in Arabidopsis accession Ws and independent homozygous

transgenic lines selected for each construct. Whereas *PAD4* expression was undetectable in GPA-infested *pad4-5* mutant plants, *PAD4* transcript level increased over time in GPA-infested leaves of transgenic plants expressing *PAD4*^{WT} (Fig. 3A), indicating that *PAD4*^{WT} complements the *pad4-5* mutant defect in promoting *PAD4* expression in response to GPA infestation (Fig. 3A) (Louis et al., 2010a). We determined whether the pad4^{S118A}, pad4^{D178A} and pad4^{H229A} proteins could also restore *PAD4* up-regulation in GPA-infested plants. As shown in Figure 3A, *PAD4* expression was induced in GPA-infested leaves of the *pad4*^{S118A}, *pad4*^{D178A} and *pad4*^{H229A} transgenic plants, indicating that S118, D178 and H229 of PAD4 are not required for increased *PAD4* expression. Western blot probed with α-cMyc antibodies indicated that leaves of the transgenic *PAD4*^{WT}, *pad4*^{S118A}, *pad4*^{D178A} and *pad4*^{H229A} plants accumulated PAD4 protein (Fig. 3B). However, compared to un-infested leaves, no increases in the PAD4^{WT} or pad4 mutant proteins were detected in GPA-infested leaves of the transgenic plants (Fig. 3B). These results suggest that the Arabidopsis response to GPA involves translational control of PAD4 protein synthesis and/or PAD4 turnover.

A no-choice assay was conducted with two independent transgenic lines for each of the control *PAD4*^{WT} or *pad4*^{S118A}, *pad4*^{D178A} and *pad4*^{H229A} mutant constructs to determine if expression of the pad4 mutant forms complements the *pad4-5* mutant defect in controlling GPA infestation. As found previously (Pegadaraju et al., 2007), GPA population size was higher on the *pad4-5* mutant compared to WT (Fig. 4A). Whereas expression of the *PAD4*^{WT} construct complemented the *pad4-5* defect (Fig. 4A), insect numbers on the *pad4*^{S118A} transgenic lines were similar to those on *pad4-5* (Fig. 4A), suggesting that S118 is a key residue for PAD4 function in Arabidopsis defense against GPA. Insect numbers were also significantly higher on the *pad4*^{D178A} lines compared to *PAD4*^{WT} and non-transgenic WT Ws plants (Fig. 4A). However, loss of resistance to GPA in *pad4*^{D178A} was not as extreme as in *pad4*^{S118A} plants (Fig. 4A). By contrast, GPA infestation was effectively controlled on *pad4*^{H229A} plants, suggesting that H229 is not critical for PAD4-mediated resistance to GPA.

PAD4-dependent accumulation of antibiosis activity in vascular sap requires S118

Compared to vascular sap-enriched petiole exudates collected from Col WT plants that reduce insect fecundity when added to a synthetic diet, petiole exudates from the *pad4-1* mutant in accession Col lack an antibiosis activity (Louis et al., 2010b). Petiole exudates collected from the *pad4-5* mutant in accession Ws also lack antibiosis activity (Fig. 4B). To determine whether S118 is required for PAD4 controlled antibiosis activity, insects were reared on synthetic diet supplemented with petiole exudates collected from leaves of the *pad4*^{S118A} transgenic lines. Compared to insect numbers on Ws WT or *PAD4*^{WT} petiole exudate-supplemented diets, insect numbers on the *pad4*^{S118A} diets were significantly higher and similar to those feeding on *pad4-5*-derived petiole exudate-supplemented or control diets (Fig. 4B). Therefore, S118 is required for PAD4 promotion of antibiosis activity.

PAD4^{S118} is essential for limiting GPA feeding from sieve elements

The Electrical Penetration Graph (EPG) technique provides a sensitive tool to monitor insect feeding behavior on plants (van Helden and Tjallingii, 2000). In EPG, a wired insect which forms part of a low voltage circuit is allowed to feed on the plant. Different waveforms generated by the insect provide a signature for the amount of time it spends in different activities, including the time required to reach first sieve element phase (f-SEP), the time spent feeding from sieve elements (sieve element phase; SEP), the time spent drinking from the xylem (xylem phase; XP), the non-probing phase (NP) when the insect stylet is not inserted into the plant tissue, and the pathway phase (PP) when the stylet is inserted in the plant tissue, but outside the vasculature. EPG comparison of GPA feeding behavior between WT and *pad4* plants had demonstrated previously that GPA spends more time in the SEP on *pad4* than on WT, thus suggesting that PAD4 deters insect feeding from sieve elements (Pegadaraju et al., 2007). To determine if S118 was required for PAD4 involvement in deterring GPA feeding from the sieve elements, GPA behavior was compared between *PAD4*^{WT} and *pad4*^{S118A} plants. Representative EPG waveforms resulting from GPA activity on these genotypes are shown in Figure S1. No differences were observed between *PAD4*^{WT} and *pad4*^{S118A} in the time taken for GPA to first probe the plants (FP), to reach the first SEP (f-SEP), or time spent in the xylem phase (XP) and the non-probing phase (NP) (Table 1). However, the sum of time spent in SEP (s-SEP) by GPA was significantly

longer on the *pad4*^{S118A} plants compared to *PAD4*^{WT} (Table 1), indicating that PAD4^{S118} is necessary for controlling insect feeding from the sieve element. The %SEP [the percentage of available SEP (a-SEP) actually spent in SEP] was also greater on *pad4*^{S118A} plants compared to *PAD4*^{WT} (Table 1), further indicating that S118 is required for PAD4-conditioned limitation of time spent by the insect feeding from the sieve elements. A corresponding reduction in the total duration of the pathway phase (PP), during which the insect attempts to locate subsequent sieve elements, was also observed on *pad4*^{S118A} plants compared to *PAD4*^{WT}. Thus, S118 in PAD4 is essential for Arabidopsis to limit GPA feeding from the sieve elements.

PAD4 function in limiting insect settling on Arabidopsis does not require S118

When given a choice between the WT and the *pad4* mutant, more numbers of the released insects tend to stay on the *pad4* mutant, suggesting that PAD4 deters insect settling on WT Arabidopsis (Pegadaraju et al., 2007). Since this deterrence effect of PAD4 was most prominent at 48 hpi, and was not observed prior to 12 hpi, it is unlikely that the insects are more attracted to the *pad4* mutant than the WT plants. Instead, this difference is likely due to the ability of the insects to stay longer on the *pad4* mutant compared to the WT plant. Considering that the insects have begun feeding from sieve-elements within this period, we had previously suggested that this deterrence effect of PAD4 on insect settling is exerted after the insect has started to feed (Pegadaraju et al., 2007). This extended time period may be required for the accumulation of a PAD4-dependent factor that deters continued stay by insects on the WT as compared to the *pad4* mutant. Alternatively, compared to the WT plant, on the *pad4* mutant GPA may be more competent in altering host physiology to make the mutant plant more suitable for continued infestation. To determine if the alanine substitution at S118 impacts this behavior of the insect on Arabidopsis, the insects were given the choice of *PAD4*^{WT} or *pad4*^{S118A} plants. Twenty adult aphids were released at the center of each pot containing one *PAD4*^{WT} and one *pad4*^{S118A} plant and the number of adult aphids that were present on each plant was counted 2 days later to determine if the insects preferred one genotype over the other. This experiment was conducted simultaneously with the following pairs of genotypes: WT Ws and *pad4-5* mutant, WT Ws and *PAD4*^{WT}, WT Ws and *pad4*^{S118A}, *PAD4*^{WT} and *pad4-5* mutant, and *pad4*^{S118A} and *pad4-5* mutant.

As expected, the average number of GPA settling on *pad4-5* was higher than on WT Ws or *PAD4*^{WT} plants and insect numbers were similar between WT Ws and *PAD4*^{WT} (Fig. 5A and S2A), confirming that transgenic *PAD4*^{WT} complements the *pad4-5* defect. Aphids did not discriminate between the *PAD4*^{WT} and *pad4*^{S118A} plants (Fig. 5A and S2A). Even at earlier time points (12 and 24 hpi), no differences in insect settling on *PAD4*^{WT} versus *pad4*^{S118A} were noticeable (Fig. S3A and S3B). The similar numbers of insects found on both genotypes suggests that S118 is not required for PAD4 involvement in deterring insect settling on Arabidopsis, distinguishing this function from activities requiring PAD4^{S118} for effective antibiosis and controlling the time spent by insect feeding from sieve elements (Fig. 4B and Table 1). This conclusion was further supported in choice assays between the *pad4-5* and *pad4*^{S118A} plants in which aphids preferred settling on *pad4-5* over *pad4*^{S118A} plants (Fig. 5A and Fig. S2A). Similarly D178 and H229 are not required for PAD4 involvement in limiting the number of insects that had settled on Arabidopsis (Fig. 5B and Fig. S2B).

S118 is dispensible for PAD4-controlled premature leaf senescence in response to aphid attack

PAD4-conditioned restriction of GPA infestation in WT Arabidopsis is accompanied by premature leaf senescence characterized by chlorophyll loss and elevated expression of the *SAG13* gene (Fig. 3A, 6A and 6B) (Pegadaraju et al., 2005; Louis et al., 2010b). The loss in chlorophyll content was reduced and up-regulation of *SAG13* expression was delayed in GPA-infested *pad4-5* leaves compared to leaves of GPA-infested WT plants (Fig. 3A and 6B) (Louis et al., 2010b). We determined whether the alanine substitution at S118 in *pad4*^{S118A} plants attenuated chlorophyll loss by measuring chlorophyll contents in GPA-infested leaves of *pad4*^{S118A} and *PAD4*^{WT}, as well as WT Ws and *pad4-5* plants. As shown in Figure 6B, GPA infestation caused a similar reduction in chlorophyll content in *pad4*^{S118A} plants as in *PAD4*^{WT} or WT Ws plants. Also, the temporal pattern of *SAG13* expression was comparable between GPA-infested WT Ws, *PAD4*^{WT} and *pad4*^{S118A} plants compared to the *pad4-5* mutant (Fig. 3A). These results indicate that PAD4^{S118} is not critical for GPA-induced up-regulation of *SAG13* or

premature leaf senescence. Similarly, PAD4^{D178} and PAD4^{H229} are also not critical for the up-regulation of *SAG13* expression and chlorophyll loss in GPA-infested plants (Fig. 3A and 6B).

PAD4^{S118A} exchange does not compromise resistance to leaf-infecting pathogens

We have shown above that PAD4^{S118} is required for antibiosis against GPA and for deterring GPA feeding from sieve elements. PAD4 is also important for resistance to pathogens that have a biotrophic phase in their life cycle (Glazebrook, 2005; Wiermer et al., 2005). Previous studies (Jirage et al., 1999; Feys et al., 2001; Pegadaraju et al., 2005, 2007; Rietz et al., 2011) and experiments with the *eds1 sid2* double mutant presented above (Fig. 1C) suggest that different mechanisms and/or molecular configurations contribute to PAD4 functions in defense against GPA and pathogens. We tested whether the *pad4*^{S118A}, *pad4*^{D178A} and *pad4*^{H229A} transgenic lines complemented the loss of basal immunity in *pad4-5* to virulent *Pst* strain DC3000. *PAD4*^{WT} expressing transgenic lines and WT Ws plants served as positive (complementing) controls and *pad4-5* and *eds1-1* mutants as negative controls in the bacterial infection assay. As anticipated, expression of *PAD4*^{WT} restored basal resistance to *Pst* DC3000 since bacterial titers in the *PAD4*^{WT} transgenic lines were similar to those in WT Ws at 3 dpi and significantly lower than those in the *pad4-5* and *eds1-1* single mutants or a *eds1-1 pad4-5* double mutant (Fig. 7A). Basal resistance to *Pst* DC3000 was also restored in plants expressing the *pad4*^{S118A}, *pad4*^{D178A} or *pad4*^{H229} variants (Fig. 7A). The extent of leaf chlorosis associated with *Pst* DC3000 infection was similarly reduced in *PAD4*^{WT} and the *pad4*^{S118A}, *pad4*^{D178A} and *pad4*^{H229A} plants compared to the *pad4-5* or *eds1-1* mutants (Fig. 7B). These results indicate that the predicted lipase site catalytic residues are not critical for PAD4-conditioned basal immunity to *Pst* DC3000. We found that PAD4^{S118} is also dispensible in Arabidopsis ETI mediated by *TIR-NB-LRR* genes at the *RPP1* locus (Aarts et al., 1998; Botella et al., 1998) to the avirulent Noco2 biotype of *Hpa*. In *Hpa* infection assays, even minor changes in the plant local resistance response can be detected by trypan blue staining inoculated leaves to reveal host cell death and pathogen infection structures (Rusterucci et al., 2001; Rietz et al., 2011). Leaves of WT Ws, *PAD4*^{WT} and *pad4*^{S118A} produced discrete necrotic lesions which prevented Noco2 hyphal extension from infection foci whereas the *pad4-5* mutant displayed a typical trailing necrotic phenotype due to a partial loss of

ETI (Fig. 7C and Fig. S4, and Table 2) (Rietz et al., 2011). The *pad4*^{D178A} and *pad4*^{H229A} transgenic lines were also fully resistant to *Hpa* Noco2 (Fig. 7C and Fig. S4, and Table 2).

DISCUSSION

Arabidopsis *PAD4* is required for defense against GPA and the biotrophic pathogens *Pst* and *Hpa* (Jirage et al., 1999; Feys et al., 2001; Pegadaraju et al., 2005, 2007). The feeding strategy of these parasites minimizes mechanical wounding of the host compared to chewing insect or necrotrophic pathogen attack (Walling, 2000; Glazebrook, 2005; Howe and Jander, 2008). Results presented here and our previous studies (Feys et al., 2005; Pegadaraju et al., 2005, 2007; Wiermer et al., 2005; Rietz et al., 2011) allow us to discriminate a number of molecularly and mechanistically separable PAD4 activities that contribute to defense against GPA and pathogens. As depicted in Figure 8, ETI in response to avirulent pathogen attack involves an acute local reaction in which low levels of dissociated PAD4 and EDS1 promote a hypersensitive response (HR) characterized by cell death at infection sites (Reitz et al., 2011). The surrounding cells undergo a 'reinforcement' phase, requiring an EDS1-PAD4 complex to promote accumulation of SA and transcriptional amplification of defenses involving SA (Reitz et al., 2011). By contrast, PAD4 activities in defense against GPA do not involve EDS1 (Fig. 1A; Pegadaraju et al., 2007) or SA (Fig. 1c; Pegadaraju et al., 2005). Furthermore, our analysis of *eds1 sid2* double mutants demonstrates that redundancy between *EDS1* and *SID2*, which was reported to underlie ETI conditioned by certain NB-LRR receptors (Venugopal et al., 2009), does not contribute to PAD4 limitation of GPA infestation (Fig. 1C).

Our analysis of transgenic plants expressing the pad4^{S118A} variant in which serine at position 118 in the PAD4 putative lipase catalytic triad (Fig. 2A and 2B) was exchanged with alanine suggests further bifurcation of PAD4 activities between defenses against pathogens and GPA. S118 is necessary for defense against GPA (Fig. 4A and 4B and Table 1) but not for PAD4-mediated pathogen resistance (Fig. 7A, 7B, 7C and Fig. S4, and Table 2). The inability of *pad* ^{S118A} transgenic plants to control GPA infestation correlated with an absence of antibiotic activity in petiole exudates of the *pad4*^{S118A} compared to *PAD4*^{WT} and WT Ws plants (Fig. 4B)

and a failure to control insect feeding from sieve elements (Table 1). However, PAD4-dependent deterrence of insect settling, promotion of chlorophyll loss and induced expression of *SAG13* in response to GPA infestation were unaffected in the *pad4*^{S118A} plant. It is plausible that different thresholds of PAD4 activity are required for the different functions of PAD4 in Arabidopsis defense against GPA. Replacement of S118 by A could have a quantitative effect on PAD4 activity and thus show defects in some PAD4 functions in defense against GPA, but not other outputs. However, considering that replacement of the equivalent S in other eukaryotic lipases by A resulted in loss of lipase activity (Lowe, 1992; Holm et al., 1994), and the fact that PAD4 exists in different molecular pools (Feys et al., 2005; Rietz et al., 2011), we propose that two distinct PAD4 activities operating independently of EDS1 determine different defenses against GPA, as depicted in Figure 8. The first activity, which requires S118, limits insect feeding from the sieve elements (Table 1) and promotes accumulation of an antibiotic activity in vascular sap (Fig. 4B). The second PAD4 activity, which does not require S118, enables deterrence of insect settling on the plant (Fig. 5), and drives premature leaf senescence which is associated with chlorophyll loss and induction of *SAG13* expression (Fig. 3A, 6A and 6B). Notably, the second PAD4 activity is also required for the feed-forward auto-regulation of *PAD4* expression in GPA-infested plants (Fig. 3A). However, it is unlikely to be molecularly equivalent to PAD4-mediated transcriptional amplification of defenses in basal resistance or ETI to pathogens because the latter employs a PAD4-EDS1 complex and SA (Rietz et al., 2011).

The PAD4-dependent antibiosis factor is present in petiole exudates of uninfested plants (Fig. 3B) (Louis et al. 2010b). Hence, the accumulation of this antibiosis activity does not require the activation of premature leaf senescence. This is further supported by our studies of the *pad4*^{S118A} plant, which although not affected in its ability to activate leaf senescence in response to GPA infestation, has lower levels of the antibiosis activity in petiole exudates. However, previous studies indicated that hyper-senescence in the Arabidopsis *ssi2* mutant was accompanied by enhanced antibiosis against GPA (Louis et al. 2010b). Therefore, although not essential for accumulation of the *PAD4*-dependent antibiosis activity, senescence activated in response to GPA infestation could potentially further enhance antibiosis activity against GPA. However, since GPA can successfully colonize Arabidopsis and manipulate host physiology, as

the infestation progresses it is possible that GPA suppresses the accumulation of this antibiosis activity or counters its activity, thus allowing it to successfully colonize Arabidopsis.

Previously we had shown that the time taken by GPA to reach the f-SEP was comparable on the WT and *pad4* mutant (Pegadaraju et al., 2007), suggesting that presence of PAD4 does not hamper the insect's ability to find the sieve elements. Furthermore, when given the choice between the WT and *pad4* mutant plants, GPA did not exhibit any difference in settling on these genotypes during the first 12 hours of infestation (Pegadaraju et al., 2007). The difference in plant choice was only observed later during infestation (Pegadaraju et al., 2007). Hence we suggested that the difference in insect settling behavior on WT versus *pad4* mutant was exerted at a stage after the insect had begun feeding (Pegadaraju et al., 2007). Results presented here indicate that S118 is required for PAD4's contribution in controlling the total time spent by GPA feeding from the sieve elements (Table 1). Insects spent more time in SEP on *pad4*^{S118A} plants than on plants expressing WT *PAD4*. However, GPA did not exhibit preferential settling on transgenic *pad4*^{S118A} plants compared to *PAD4*^{WT} plants expressing the WT *PAD4* transgene, unlike with the *pad4-5* null mutant (Fig. 5A and S2). Taken together, these results suggest that although the effect of *PAD4* on insect settling behavior is likely exerted after it has begun feeding from sieve elements, the length of time the insect spends in the sieve elements is not critical for PAD4 to exert this settling deterrence.

The GHSTG sequence containing S118 in PAD4 resembles the GXSXG motif that is part of an Asp-His-Ser triad in a large family of α/β fold hydrolases that includes lipases and esterases (Brady et al., 1990; Winkler et al., 1990). The serine in this triad is the nucleophilic residue essential for catalysis (Brady et al., 1990; Winkler et al., 1990). The importance of this conserved S118 for a subset of biological functions of PAD4, suggests that PAD4 may possess a hydrolase activity. This is strengthened by the fact that aspartic acid at position 178, another predicted catalytic residue, is also required for controlling aphid infestation (Fig. 4A). It is possible that H229 is not as critical as S118 and D178 for any hydrolase activity that PAD4 may possess. However, no hydrolase activity has been detected for PAD4 (Steffen Rietz and Jane E Parker, unpublished) and the possibility that these residues serve a structural rather than enzymatic function cannot be ruled out. Also, discrimination of other PAD4 biological functions

not requiring S118 implies that PAD4 can have additional biochemical attributes and/or conformational states. Possession of more than one biochemical function, a phenomenon termed 'moonlighting', has been reported for other proteins (Jeffery, 1999; Moore, 2004). Further dissection of the PAD4 protein and its associations should provide important insights to its role in diverse defense outputs.

We noted that although *PAD4* transcript abundance increased during the course of aphid infestation of WT plants (Fig. 3A), there was no corresponding increase in PAD4 protein content (Fig. 3B). A lack of correlation between mRNA and protein accumulation is not uncommon and has been associated with steps impacting synthesis and/or turnover of individual proteins under different environmental or developmental conditions (Greenbaum et al., 2003). It is possible that increased *PAD4* transcription does not translate to a corresponding increase in synthesis of the PAD4 protein due to translational control. Alternatively, newly produced PAD4 protein may be turned over faster in GPA-infested tissues compared to the uninfested plants. Arabidopsis might compensate for increased turnover of PAD4 protein in GPA-infested tissues by increasing *PAD4* transcription and thereby the amount of fresh PAD4 protein synthesized. Destabilization of the PAD4 protein in aphid-infested plants might also explain why constitutive OE of *EDS1* results in a small increase in resistance against GPA (Fig. 1A). EDS1 directly stabilizes PAD4 (Feys et al., 2005; Rietz et al., 2011) and therefore EDS1 OE might increase the amount of PAD4 protein available for defenses against aphid feeding. However, EDS1-mediated stabilization of the PAD4 protein is unlikely to be a limiting factor in PAD4 defense against GPA since GPA numbers were similar in WT and *eds1* mutant plants in no-choice assays (Fig. 1A; Pegadaraju et al., 2007).

CONCLUSION

Replacement of serine with alanine at amino acid position 118 in PAD4 provides molecular evidence for distinct PAD4 activities regulating diverse Arabidopsis defenses against GPA and pathogens. Encompassing several molecular attributes in one protein either singly or in association with other components, as observed for PAD4, might increase the plant's signaling

repertoire and enable it to respond to diverse biotic stresses using an existing regulatory framework.

MATERIALS AND METHODS

Aphid propagation and plant growth conditions

GPA was reared on an equal mix of commercially available mustard (Early scarlet globe) and radish (Florida broadleaf) in a growth chamber set at 22˚C and programmed for a 14 h light (100 µE m^{-2} s^{1}) and 10 h dark cycle. Same conditions were used for cultivating Arabidopsis. All plants were cultivated in autoclaved composed-peat based planting mixture Premier Pro Mix-BX (Premier Tech Horticulture, http://www.pthorticulture.com/).

Arabidopsis mutants and transgenic lines

The *pad4-5, eds1-1* and *pad4-5 eds1-1* mutants are in accession Ws-0 (Ws; Feys et al., 2001, 2005; Glazebrook et al., 1997) and *eds1-22* and *sid2-1* are in accession Col-0 (Col; Nawrath and Métraux, 1994; Yang and Hua, 2004). The *eds1-1 sid2-1* and *eds1-22 sid2-1* double mutants have been described previously (Venugopal et al., 2009). Arabidopsis accession Col contains two tandem repeats (*At3g48090* and *At3g48080*) of *EDS1* (Yang and Hua, 2004). The *eds1-22* mutant contains a T-DNA insertion in *At3g48090* and hence is not a complete loss-of -function mutation (Yang and Hua, 2004). The *PAD4*-OE line (#1) (Pegadaraju et al., 2007) expresses *PAD4* cDNA under control of the *35S* promoter and fused to a C-terminal StrepII (SII) epitope tag on a pXCSG-Strep binary vector (Witte et al., 2004) in the *pad4-5* background. The *EDS1*-OE transgenic line over-expresses *EDS1-SII* under control of the *35S* promoter on the pXCSG-Strep vector (Witte et al., 2004) in the *eds1-1* background. α-EDS1 antibodies (Feys et al., 2001) were used in Western blots for monitoring EDS1 OE. A functional cMyc epitope-tagged *PAD4* cDNA driven by 1kb of 5' *PAD4* promoter sequence (Feys et al., 2001) was used as template for introducing S118A, D178A and H229A mutations using a QuickChange mutagenesis kit (Stratagene). All constructs were verified by DNA sequencing before cloning into a Basta-resistant binary pSLJ5515 vector and transforming *pad4-5* with *Agrobacterium tumefaciens*

strain GV3101. Two independent single locus transgenic lines per construct were made homozygous before further testing.

No-choice and choice tests with GPA

No-choice and choice bioassays were performed as previously described (Pegadaraju et al., 2005; Louis et al., 2010b). In no-choice assays, twenty adult apterous (wingless) GPA were released on each plant and the total numbers of nymphal plus adult GPAs were counted 2 days post-infestation (dpi). In choice tests, twenty adult apterous aphids were released at the center of the pot containing one WT and one mutant/transgenic line and numbers of adult GPA on each plant were counted 2 dpi.

Analysis of GPA feeding behavior

The electro-physiological Electrical Penetration Graph technique (van Helden and Tjallingii, 2000) was used to monitor feeding behavior of GPA on *PAD4*^{WT} and *pad4*^{S118A} plants as previously described (Pegadaraju et al., 2007). Ten replications were performed and the mean time spent by aphids on various activities was analyzed by the non-parametric Kruskal–Wallis test ($P < 0.05$).

Petiole exudate collection and feeding trials

Petiole exudates enriched in vascular sap were collected from 80-120 leaves (~25-30 plants) as previously described (Chaturvedi et al., 2008). Feeding trial bioassays were performed using a synthetic diet (Mittler and Dadd, 1965) as described (Louis et al., 2010b). Three adult apterous aphids were introduced into the feeding chamber and allowed to feed on the diet that was mixed with plant petiole exudates. Total numbers of nymphal plus adult GPAs were determined 4 days later.

Pathogen infection assays

Infections with *Pseudomonas syringae* pv *tomato* strain DC3000 (*Pst*) were conducted by dipping leaves of 4-week-old plants into a freshly prepared bacterial suspension (1×10^7 cfu ml^{-1}) in 10 mM $MgCl_2$. Bacterial numbers inside leaves were counted at 0 (3 hpi) and 3 dpi, as previously described (Birker et al., 2009). Infections with *Hyaloperonospora arabidopsidis*

(*Hpa*) biotype Noco2, which is avirulent on Arabidopsis accession Ws, were done by spraying 16 day-old plants with a conidiospore suspension in distilled water (4×10^4 spores ml^{-1}) (Feys et al., 2005). Six days after inoculation the development of host responses and *Hpa* infection structures was monitored under a light microscope after lactophenol trypan blue staining of infected leaves (Aarts et al., 1998) or observing plants under a binocular microscope with UV illumination.

RNA extraction and RT-PCR analysis

RNA for RT-PCR analysis was extracted from Arabidopsis leaves (Pegadaraju et al., 2005). Gene-specific PCR primers used for *ACT8* (At1g49240), *PAD4* (At3g52430), *EDS1* (At3g48090) and *SAG13* (At2g29350) were as previously described (Pegadaraju et al., 2007, Louis et al., 2010b). PCR conditions used were as follows: 95°C for 5 min, followed by 30 cycles (for Fig. 3A) and 25 cycles (for Fig. 1B) of 95°C for 30 sec, 55°C for 45 sec, and 72°C for 1 min, with a final extension at 72°C for 5 min.

Western blot analysis

Protein extraction from Arabidopsis leaves was performed as previously described (Feys et al., 2001). 50 µg of protein isolated from leaves of WT Ws, *pad4-5*, and *pad4-5* complemented with cMyc-tagged $PAD4^{WT}$ driven from its native promoter or mutated versions of cMyc-tagged $pad4^{S118A}$, $pad4^{D178A}$ and $pad4^{H229A}$ were separated on a 4 to 20% SDS-PAGE gel and then transferred to a nitrocellulose membrane. After protein transfer, the membrane was blocked for 2 h at room temperature with 5% non-fat dry milk in TBST (Tris-buffered saline [10 mM Tris, 150 mM NaCl, pH 7.5]) containing 0.2% Tween-20) and then incubated overnight at 4ºC with α-cMyc antibody (1: 2500; Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was washed 3 times with TBST and then incubated with alkaline phosphatase-linked α-rabbit IgG (1: 3000; Santa Cruz Biotechnology, Santa Cruz, CA) as a secondary antibody for 1 h at room temperature. After three washes with TBST, the reaction was visualized by 5-bromo-4-chloro-3-indol phosphate (BCIP)/*p*-Nitro-Blue tetrazolium chloride (NBT) staining.

Chlorophyll Quantitation

Chlorophyll extraction and quantitation were conducted as previously described (Lichtenthaler, 1987; Pegadaraju et al., 2005).

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SUPPLEMENTAL MATERIAL

Figure S1. Electrical Penetration Graph analysis of GPA feeding behavior on the Arabidopsis *pad4*^{S118A} transgenic plant.

Figure S2. S118, D178 and H229 are not essential for the *PAD4*-determined deterrence of insect settling on Arabidopsis.

Figure S3. S118 is not essential for the *PAD4*-determined deterrence of insect settling on Arabidopsis.

Figure S4. Leaves of *pad4*^{S118A}, *pad4*^{D178A} and *pad4*^{H229A} exchange mutant lines exhibit wild type effector-triggered immunity to *Hyaloperonospora arabidopsidis*.

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FIGURE LEGENDS

Figure 1. Redundancy between *EDS1* and *SID2* is not important for controlling GPA infestation.

A, Constitutive over-expression of *EDS1* curtails GPA population. No-Choice assay: GPA numbers on WT Ws, *eds1-1*, *pad4-5* and *eds1-1 pad4-5* mutants, and plants constitutively over-expressing *EDS1* (*EDS1*-OE) or *PAD4* (*PAD4*-OE) from the *35S* promoter. This experiment was conducted thrice with similar results.

B, RT-PCR analysis of *PAD4* and *EDS1* expression in leaves of GPA-infested (+GPA) plants of the indicated genotypes. Un-infested (-GPA) plants provided negative controls. *ACT8* expression served as a control for RT-PCR. This experiment was conducted twice with similar results. hpi, hours post-infestation.

C, Aphid population size is not impacted by simultaneous deficiency of *EDS1* and *SID2*. No-Choice assays: GPA numbers on WT accessions Ws and Col, *pad4-5*, *eds1-1*, *sid2-1* and *eds1-1 sid2-1* double mutant plants (Top panel), and WT Col, *pad4-1*, *eds1-22*, *sid2-1* and *eds1-22 sid2-1* double mutant plants in the accession Col (Lower panel). The *pad4-5* and *eds1-1* alleles, and *sid2-1* are in the Ws and Col backgrounds, respectively. These experiments were conducted twice with similar results.

In A and C, GPA population size was determined 2 days post infestation (dpi) (n=10). Error bars represent SE. ANOVA of GPA populations on different plant genotypes were conducted using PROC GLM (SAS Institute). Means were separated using least significant difference procedure. Different letters above bars indicate values that are significantly different ($P<0.05$) from each other.

Figure 2. Amino acid sequence of PAD4 and homology to key regions of fungal lipases.

A, Amino acid sequence of PAD4. Residues S118, D178 and H229 are in bold. The underlined sequence corresponds to the GXSXG motif.

B, Conservation of amino acid sequences around the S118, D178 and H229 residues between PAD4 and other putative fungal lipases. S118, D178 and H229 residues in PAD4 are underlined, invariant residues are in bold, and asterisks (*) identify conserved amino acids. *Rh*TGL, triacylglcyerol lipase precursor 1 from *Rhizomucor miehei; Fh*TGL, triacylgylcerol lipase from *Fusarium heterosporum; Tl*LIP: lipase from *Thermomyces lanuginosus.*

Figure 3. *PAD4* and *SAG13* transcript and PAD4 protein accumulation in plants expressing pad4^{S118A}, pad4^{D178A} and pad4^{H229A} variants.

A, Time course of *PAD4* and *SAG13* transcript accumulation in un-infested (-GPA) and GPA-infested (+GPA) leaves of WT Ws, *pad4-5*, and *pad4-5* mutant plants transformed with the *PAD4*^{WT} (*P4*^{WT}) or the *pad4*^{S118A} (*p4*^{S118A}), *pad4*^{D178A} (*p4*^{D178A}) or *pad4*^{H229A} (*p4*^{H229A}) mutant constructs expressed from the *PAD4* promoter. *ACT8* expression served as a control for RT-PCR. Hpi, hours post infestation.

B, Western blot analysis of PAD4 protein. Total protein extracted from leaves of un-infested and GPA-infested (24 hpi) WT Ws, *pad4-5*, *PAD4*^{WT} (*P4*^{WT}), *pad4*^{S118A} (*p4*^{S118A}), *pad4*^{D178A} (*p4*^{D178A}) and *pad4*^{H229A} (*p4*^{H229A}) plants, were used for monitoring accumulation of the transgene-encoded cMyc epitope-tagged PAD4 variants. An anti-cMyc antibody was used as the primary antibody. Coomasie blue stained Rubisco large subunit (RbcL) is shown as a loading control. MW, molecular weight markers in kD. Experiments in A and B were conducted twice with similar results.

Figure 4. S118 in PAD4 is required for controlling GPA infestation.

A, No-Choice assay: GPA numbers on WT Ws, *pad4-5*, and two independently derived transgenic *pad4-5* mutant lines expressing the *PAD4*^{WT} (*P4*^{WT}), *pad4*^{S118A} (*p4*^{S118A}), *pad4*^{D178A} (*p4*^{D178A}) and *pad4*^{H229A} (*p4*^{H229A}) constructs from the *PAD4* promoter. GPA population size was determined 2 dpi (n=12). This experiment was conducted thrice with similar results.

B, GPA numbers on a synthetic diet containing petiole exudate from *PAD4*^{WT} (*P4*^{WT}) and *pad4*^{S118A} (*p4*^{S118A}) plants. Diet containing petiole exudate collected from the WT Ws and the *pad4-5* mutant, and the buffer used to collect petiole exudates, provided controls for this experiment. Three adult aphids were introduced into each feeding chamber and allowed to feed on the diet and the total numbers of aphids (nymphs plus adults) in each chamber determined four days later (n=3). This experiment was conducted thrice with similar results. In A and B, error bars represent SE. See legend to Figure 1 for details on statistical analysis. Different letters above the bars indicate values that are significantly different (P<0.05) from each other.

Figure 5. S118, D178 and H22 are not essential for the *PAD4*-determined deterrence of insect settling on Arabidopsis.

A and B, Choice test: Insects were given the choice of settling between plants of two genotypes by releasing twenty adult apterous GPA at the center of a pot containing one plant of each indicated genotypes. The total number of adult GPA that had settled on eight plants of each genotype was determined 48 h later. Equal preference for each pair of genotypes was tested using the pooled chi-square test. An asterisk (*) indicates values that are significantly different (P<0.05) from the other genotype. This experiment was conducted thrice with similar results. Also refer to Figure S2A and S2B for mean number of insects per plant with error bars for visual reference.

Figure 6. S118, D178 and H229 are not essential for the *PAD4*-determined chlorosis in GPA-infested plants.

A, Picture of leaves of the WT WS, *pad4-5*, and transgenic *pad4-5* plants expressing the *PAD4*^{WT} (*P4*^{WT}) or *pad4*^{S118A} (*p4*^{S118A}), *pad4*^{D178A} (*p4*^{S118A}) and *pad4*^{H229A} (*p4*^{H229A}) transgenes, 5 days after release of 20 GPA on each plant. Un-infested plants provided the negative controls. This experiment was conducted thrice with similar results.

B, Relative chlorophyll content in GPA-infested leaves of plants of the indicated genotypes, 5 days after release of 20 aphids on each plant. Values are relative to the chlorophyll content in un-infested plants of the corresponding genotype (n=5). Error bars represent SE. Different letters above the bars indicate values that are significantly different (P<0.05) from each other. This experiment was conducted twice with similar results.

Figure 7. S118, D178 and H229 are not required for PAD4-mediated resistance to virulent or avirulent pathogens.

A, Growth of virulent *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 on WT Ws, *eds1-1*, *pad4-5*, *eds1-1*, *PAD4*^{WT} (*P4*^{WT}), *pad4*^{S118A} (*p4*^{S118A}), *pad4*^{D178A} (*p4*^{D178A}) or *pad4*^{H229A} (*p4*^{H229A}) plants. Pathogen growth was monitored in two independently-derived transgenic lines of each genotype. Pathogen-inoculated leaves were harvested at 0 and 3 dpi and bacterial numbers determined by plating dilutions of leaf extracts on selective medium. Bacterial numbers are represented as the Log_{10} of colony forming units per unit area (cfu cm^{-2}) of leaf (n=3). Error bars

represent SE. An asterisk (*) above a bar indicates values that are significantly different ($P<0.05$; *t*-test) from WT Ws at the equivalent time point.

B, Representative *Pst* DC3000-inoculated leaves from plants of indicated genotypes harvested 3 dpi. The extent of chlorosis is an indication of disease severity.

C, Resistance to avirulent *Hyaloperonospora arabidopsidis* biotype Noco2 on WT Ws, *pad4-5*, and transgenic *PAD4*^{WT} (*P4*^{WT}), *pad4*^{S118A} (*p4*^{S118A}), *pad4*^{D178A} (*p4*^{D178A}) or *pad4*^{H229A} (*p4*^{H229A}) plants. Sixteen day-old seedlings of the indicated genotypes were inoculated. At 6 dpi, 21trypan blue-stained leaves per genotype were scored under the microscope for the presence of discrete HR lesions (identified by red arrows) at infection sites or trailing necrosis (TN; identified by black arrows). Whereas extensive TN was observed in ~50% of *pad4-5* leaves (see Table 2), only HR was observed in the transgenic lines and Ws-0 (representing >120 infection sites per line). Photographs of representative samples are shown.

All infection assays were repeated at least twice with similar results.

Figure 8. Model for different PAD4 molecular activities in Arabidopsis interaction with pathogen and GPA.

At least two molecular activities of PAD4 are implicated in Arabidopsis interactions with biotrophic pathogens. PAD4, dissociated from EDS1, is required for ETI conditioned by TIR-NB-LRR type receptors. Here, PAD4 and EDS1 activate a HR involving localized host cell death and restriction of pathogen growth. A different activity of PAD4 bound to EDS1 in a complex promotes expression of SA biosynthetic and other genes (including *PAD4* itself) leading to defense amplification (e.g. transcription of the *PATHOGENESIS-RELATED1* [*PR1*] gene) in basal resistance against virulent pathogens. In Arabidopsis interactions with GPA, PAD4 confers defenses without measurable EDS1 involvement. One PAD4 activity that does not require Ser118 deters insect settling and promotes leaf senescence, characterized by chlorophyll loss and increased *SAG13* expression. This activity also promotes *PAD4* expression in GPA-infested tissues. A different PAD4^{S118}-dependent activity deters insect feeding from the sieve elements and promotes the accumulation of an antibiosis factor in petiole exudates.

Table 1. Time spent by GPA on various activities on the Arabidopsis *PAD4*^{WT} and *pad4*^{S118A} plants.

Activity	*PAD4*^{WT}	*pad4*^{S118A}	*P* value
Time to first probe (FP)	0.19 ± 0.02	0.2 ± 0.04	0.8206
Total duration of pathway phase (PP)	3.96 ± 0.12	3.47 ± 0.14	0.0191*
Total duration of non-probing phase (NP)	2.55 ± 0.23	2.34 ± 0.29	0.8798
Time to first Sieve Element Phase (f-SEP)	1.99 ± 0.3	1.71 ± 0.13	0.1306
Sum of SEP duration time in a total of 8 h recording time (s-SEP)	1.07 ± 0.09	1.94 ± 0.22	0.0025*
Total duration of xylem phase (XP)	0.42 ± 0.1	0.25 ± 0.07	0.2532
Available SEP from the beginning of the first SEP until the end of recording time (a-SEP)	5.69 ± 0.19	6.11 ± 0.13	0.1306
Percentage of available SEP actually spent in SEP (%SEP)	20.25 ± 2.5	29.66 ± 3.15	0.0343*

Values represent mean time (h) ± SE spent by GPA on various activities in each 8 h of recording (n=10). An asterisk (*) represents significant difference (P<0.05, Kruskal–Wallis test) in the time spent by GPA for the indicated activity on the *PAD4*^{WT} and *pad4*^{S118A} plants.

Table 2. S118, D178 and H229 are not essential for resistance to *Hyaloperonospora arabidopsidis* biotype Noco2.

Genotype[a]	Expt. 1		Expt. 2	
	HR	TN	HR	TN
Ws-0	207	nd	380	nd
pad4-5	136	75	191	35
$P4^{WT}$	163	nd	236	1
	135	nd	203	1
$p4^{S118A}$	122	nd	176	nd
	129	nd	186[b]	nd
$p4^{D178A}$	122	nd	285	1
	115	nd	303	2
$p4^{H229A}$	143	nd	231	nd
	67	nd	153	nd

[a]Two independently-derived transgenic lines for each of the $P4^{WT}$, $p4^{S118A}$, $p4^{D178A}$ and $p4^{H229A}$ constructs in the *pad4-5* mutant background were evaluated. In each experiment, 21 inoculated leaves for each line were harvested 6 dpi, stained with trypan blue and examined under the microscope for infection sites exhibiting a hypersensitive response (HR) indicating full resistance and trailing necrosis (TN) indicating a partial breakdown of resistance.

[b]20 inoculated leaves were examined for this line in experiment 2.

nd, none detected.

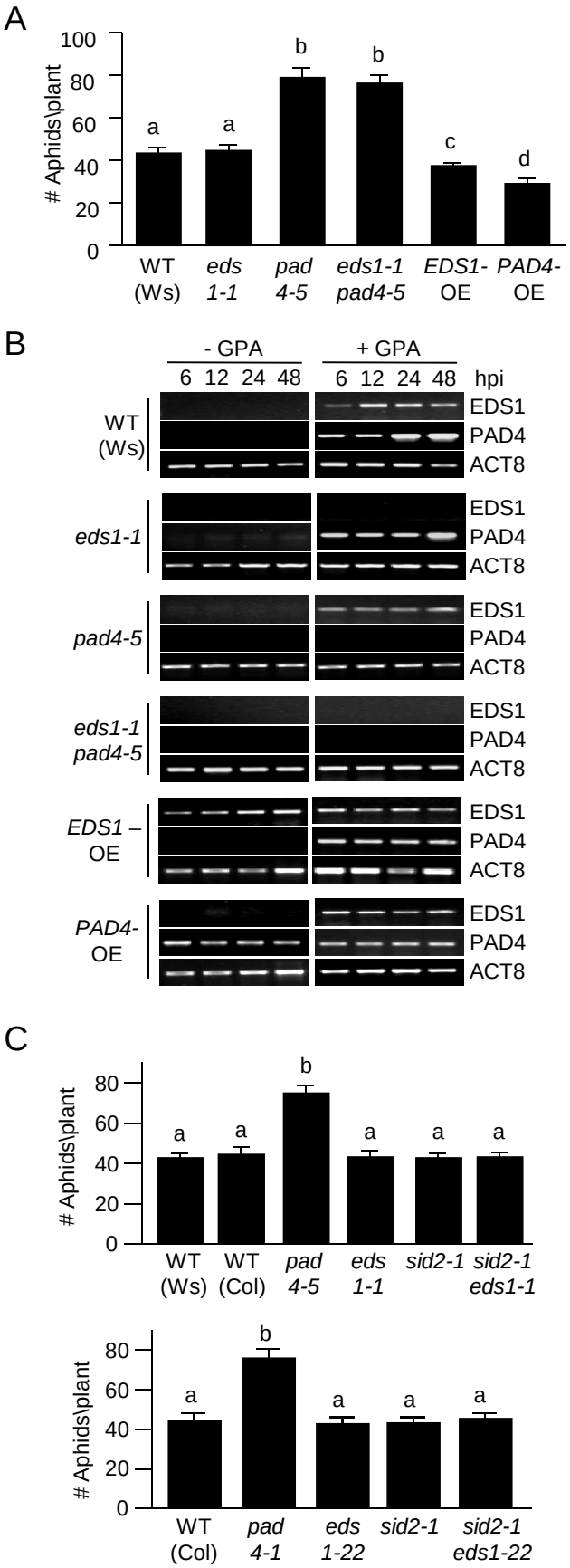
A
Aphids\plant
100
80
60
40
20
0
a
a
b
b
c
d
WT (Ws)
eds 1-1
pad 4-5
eds1-1 pad4-5
EDS1-OE
PAD4-OE
B
- GPA
+ GPA
6 12 24 48
6 12 24 48
hpi
WT (Ws)
eds1-1
pad4-5
eds1-1 pad4-5
EDS1 – OE
PAD4-OE
EDS1
PAD4
ACT8
C
Aphids\plant
80
60
40
20
0
a
a
b
a
a
a
WT (Ws)
WT (Col)
pad 4-5
eds 1-1
sid2-1
sid2-1 eds1-1
Aphids\plant
80
60
40
20
0
a
b
a
a
a
WT (Col)
pad 4-1
eds 1-22
sid2-1
sid2-1 eds1-22

Figure 1. Redundancy between *EDS1* and *SID2* is not important for controlling GPA infestation.

A, Constitutive over-expression of *EDS1* curtails GPA population. No-Choice assay: GPA numbers on WT Ws, *eds1-1*, *pad4-5* and *eds1-1 pad4-5* mutants, and plants constitutively over-expressing *EDS1* (*EDS1*-OE) or *PAD4* (*PAD4*-OE) from the *35S* promoter. This experiment was conducted thrice with similar results.

B, RT-PCR analysis of *PAD4* and *EDS1* expression in leaves of GPA-infested (+GPA) plants of the indicated genotypes. Un-infested (-GPA) plants provided negative controls. *ACT8* expression served as a control for RT-PCR. This experiment was conducted twice with similar results. hpi, hours post-infestation.

C, Aphid population size is not impacted by simultaneous deficiency of *EDS1* and *SID2*. No-Choice assays: GPA numbers on WT accessions Ws and Col, *pad4-5*, *eds1-1*, *sid2-1* and *eds1-1 sid2-1* double mutant plants (Top panel), and WT Col, *pad4-1*, *eds1-22*, *sid2-1* and *eds1-22 sid2-1* double mutant plants in the accession Col (Lower panel). The *pad4-5* and *eds1-1* alleles, and *sid2-1* are in the Ws and Col backgrounds, respectively. These experiments were conducted twice with similar results.

In A and C, GPA population size was determined 2 days post infestation (dpi) (n=10). Error bars represent SE. ANOVA of GPA populations on different plant genotypes were conducted using PROC GLM (SAS Institute). Means were separated using least significant difference procedure. Different letters above bars indicate values that are significantly different ($P<0.05$) from each other.

A

```
  1 MDDCRFETSE LQASVMISTP LFTDSWSSCN TANCNGSIKI HDIAGITYVA
 51 IPAVSMIQLG NLVGLPVTGD VLFPGLSSDE PLPMVDAAIL KLFLQLKIKE
101 GLELELLGKK LVVITGHSTG GALAAFTALW LLSQSSPPSF RVFCITFGSP
151 LLGNQSLSTS ISRSRLAHNF CHVVSIHDLV PRSSNEQFWP FGTYLFCSDK
201 GGVCLDNAGS VRLMFNILNT TATQNTEEHQ RYGHYVFTLS HMFLKSRSFL
251 GGSIPDNSYQ AGVALAVEAL GFSNDDTSGV LVKECIETAT RIVRAPILRS
301 AELANELASV LPARLEIQWY KDRCDASEEQ LGYYDFFKRY SLKRDFKVNM
351 SRIRLAKFWD TVIKMVETNE LPFDFHLGKK WIYASQFYQL LAEPLDIANF
401 YKNRDIKTGG HYLEGNRPKR YEVIDKWQKG VKVPEECVRS RYASTTQDTC
451 FWAKLEQAKE WLDEARKESS DPQRRSLLRE KIVPFESYAN TLVTKKEVSL
501 DVKAKNSSYS VWEANLKEFK CKMGYENEIE MVVDESDAME T
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B

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     PAD4:  VITGHSTGGALAA   VVSIHDLVPRS   NTEEHQRYGH
   RhTGL:  AVTGHSLGGATAL   TNNERDIVPHL   SVLDHLSYFG
   FhTGL:  VVTGHSLGGAVAT   VTHGDDPVPRL   DILAHITYFQ
   TlLIP:  VFTGHSLGGALAT   ITHTNDIVPRL   DIPAHLWYFG
           **        *     *     *  *
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Figure 2. Amino acid sequence of PAD4 and homology to key regions of fungal lipases.

A, Amino acid sequence of PAD4. Residues S118, D178 and H229 are in bold. The underlined sequence corresponds to the GXSXG motif.

B, Conservation of amino acid sequences around the S118, D178 and H229 residues between PAD4 and other putative fungal lipases. S118, D178 and H229 residues in PAD4 are underlined, invariant residues are in bold, and asterisks (*) identify conserved amino acids. *Rh*TGL, triacylglcyerol lipase precursor 1 from *Rhizomucor miehei; Fh*TGL, triacylgylcerol lipase from *Fusarium heterosporum; Tl*LIP: lipase from *Thermomyces lanuginosus.*

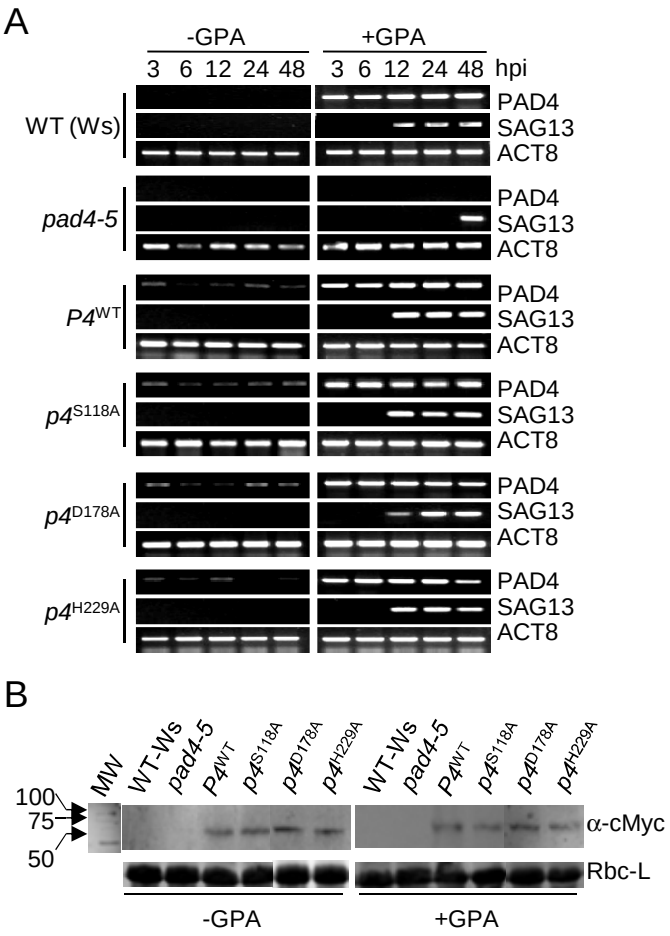


Figure 3. *PAD4* and *SAG13* transcript and PAD4 protein accumulation in plants expressing pad4^{S118A}, pad4^{D178A} and pad4^{H229A} variants.

A, Time course of *PAD4* and *SAG13* transcript accumulation in un-infested (-GPA) and GPA-infested (+GPA) leaves of WT Ws, *pad4-5*, and *pad4-5* mutant plants transformed with the *PAD4*^{WT} (*P4*^{WT}) or the *pad4*^{S118A} (*p4*^{S118A}), *pad4*^{D178A} (*p4*^{D178A}) or *pad4*^{H229A} (*p4*^{H229A}) mutant constructs expressed from the *PAD4* promoter. *ACT8* expression served as a control for RT-PCR. Hpi, hours post infestation.

B, Western blot analysis of PAD4 protein. Total protein extracted from leaves of un-infested and GPA-infested (24 hpi) WT Ws, *pad4-5*, *PAD4*^{WT} (*P4*^{WT}), *pad4*^{S118A} (*p4*^{S118A}), *pad4*^{D178A} (*p4*^{D178A}) and *pad4*^{H229A} (*p4*^{H229A}) plants, were used for monitoring accumulation of the transgene-encoded cMyc epitope-tagged PAD4 variants. An anti-cMyc antibody was used as the primary antibody. Coomasie blue stained Rubisco large subunit (RbcL) is shown as a loading control. MW, molecular weight markers in kD. Experiments in A and B were conducted twice with similar results.

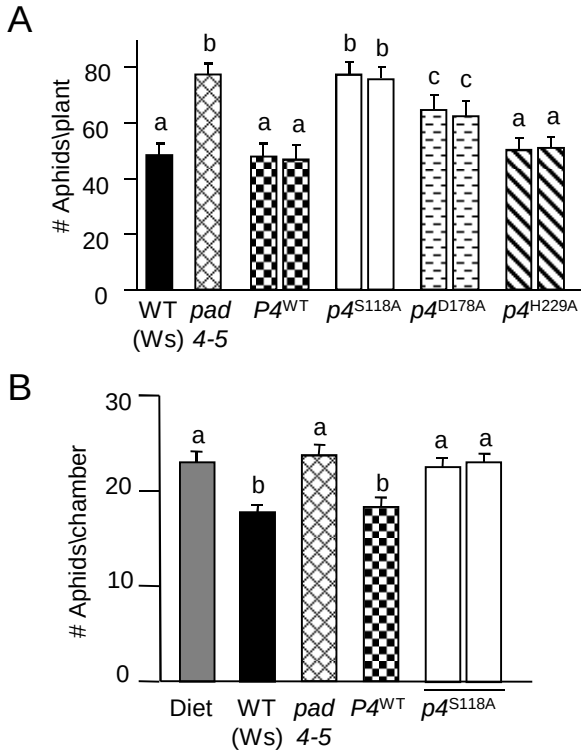
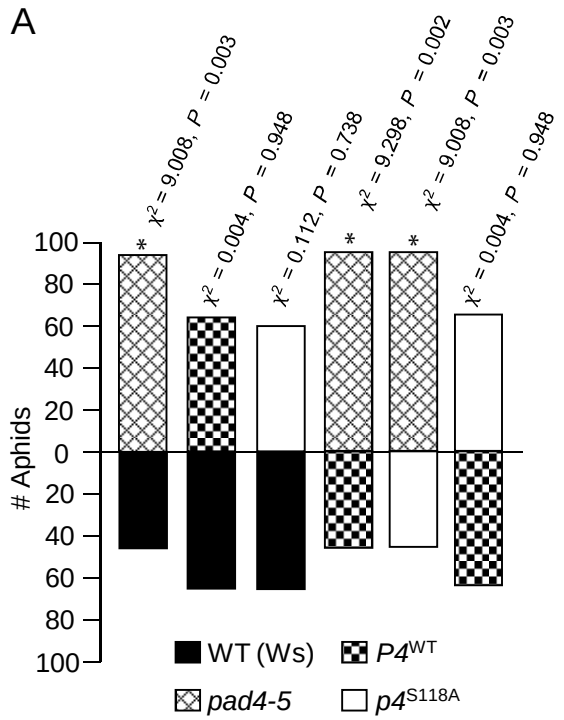


Figure 4. S118 in PAD4 is required for controlling GPA infestation.

A, No-Choice assay: GPA numbers on WT Ws, *pad4-5*, and two independently derived transgenic *pad4-5* mutant lines expressing the *PAD4*^{WT} (*P4*^{WT}), *pad4*^{S118A} (*p4*^{S118A}), *pad4*^{D178A} (*p4*^{D178A}) and *pad4*^{H229A} (*p4*^{H229A}) constructs from the *PAD4* promoter. GPA population size was determined 2 dpi (n=12). This experiment was conducted thrice with similar results.

B, GPA numbers on a synthetic diet containing petiole exudate from *PAD4*^{WT} (*P4*^{WT}) and *pad4*^{S118A} (*p4*^{S118A}) plants. Diet containing petiole exudate collected from the WT Ws and the *pad4-5* mutant, and the buffer used to collect petiole exudates, provided controls for this experiment. Three adult aphids were introduced into each feeding chamber and allowed to feed on the diet and the total numbers of aphids (nymphs plus adults) in each chamber determined four days later (n=3). This experiment was conducted thrice with similar results. In A and B, error bars represent SE. See legend to Figure 1 for details on statistical analysis. Different letters above the bars indicate values that are significantly different (P<0.05) from each other.


A
χ^2 = 9.008, P = 0.003
χ^2 = 0.004, P = 0.948
χ^2 = 0.112, P = 0.738
χ^2 = 9.298, P = 0.002
χ^2 = 9.008, P = 0.003
χ^2 = 0.004, P = 0.948
*
*
*
100
80
60
40
20
0
20
40
60
80
100
Aphids
WT (Ws)
P4WT
pad4-5
p4S118A

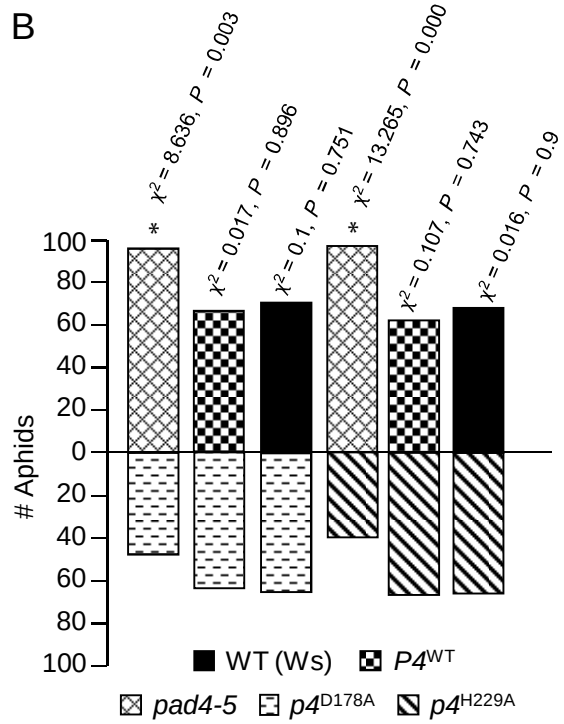

B
χ^2 = 8.636, P = 0.003
χ^2 = 0.017, P = 0.896
χ^2 = 0.1, P = 0.751
χ^2 = 13.265, P = 0.000
χ^2 = 0.107, P = 0.743
χ^2 = 0.016, P = 0.9
*
*
100
80
60
40
20
0
20
40
60
80
100
Aphids
WT (Ws)
P4WT
pad4-5
p4D178A
p4H229A

Figure 5. S118, D178 and H229 are not essential for the *PAD4*-determined deterrence of insect settling on Arabidopsis.

A and B, Choice test: Insects were given the choice of settling between plants of two genotypes by releasing twenty adult apterous GPA at the center of each pot containing one plant of each indicated genotypes. The total number of adult GPA that had settled on eight plants of each genotype was determined 48 h later. Equal preference for each pair of genotypes was tested using the pooled chi-square test. An asterisk (*) indicates values that are significantly different ($P<0.05$) from the other genotype. This experiment was conducted thrice with similar results. Also refer to Figure S2A and S2B for mean number of insects per plant with error bars for visual reference.


A
-GPA
+GPA
WT (Ws)
pad4-5
P4WT
p4S118A
p4D178A
p4H229A
B
Rel. chlorophyll content
100
80
60
40
20
0
a
b
a
a
a
a
WT (Ws)
pad4-5
P4WT
p4S118A
p4D178A
p4H229A

Figure 6. S118, D178 and H229 are not essential for the *PAD4*-determined chlorosis in GPA-infested plants.

A, Picture of leaves of the WT WS, *pad4-5*, and transgenic *pad4-5* plants expressing the *PAD4*^{WT} (*P4*^{WT}) or *pad4*^{S118A} (*p4*^{S118A}), *pad4*^{D178A} (*p4*^{D178A}), *pad4*^{H229A} (*p4*^{H229A}) transgenes, 5 days after release of 20 GPA on each plant. Un-infested plants provided the negative controls. This experiment was conducted thrice with similar results.

B, Relative chlorophyll content in GPA-infested leaves of plants of the indicated genotypes, 5 days after release of 20 aphids on each plant. Values are relative to the chlorophyll content in un-infested plants of the corresponding genotype (n=5). Error bars represent SE. Different letters above the bars indicate values that are significantly different (P<0.05) from each other. This experiment was conducted twice with similar results.

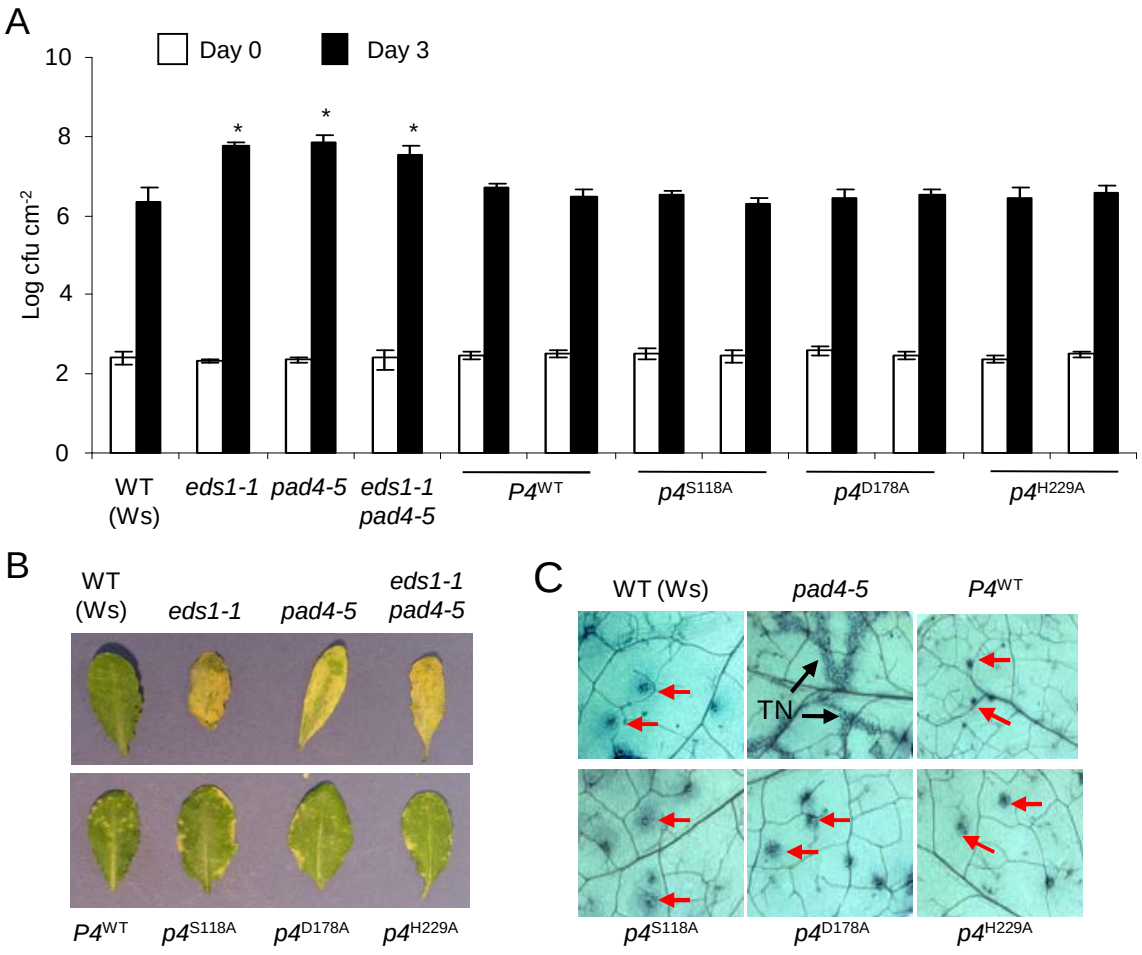
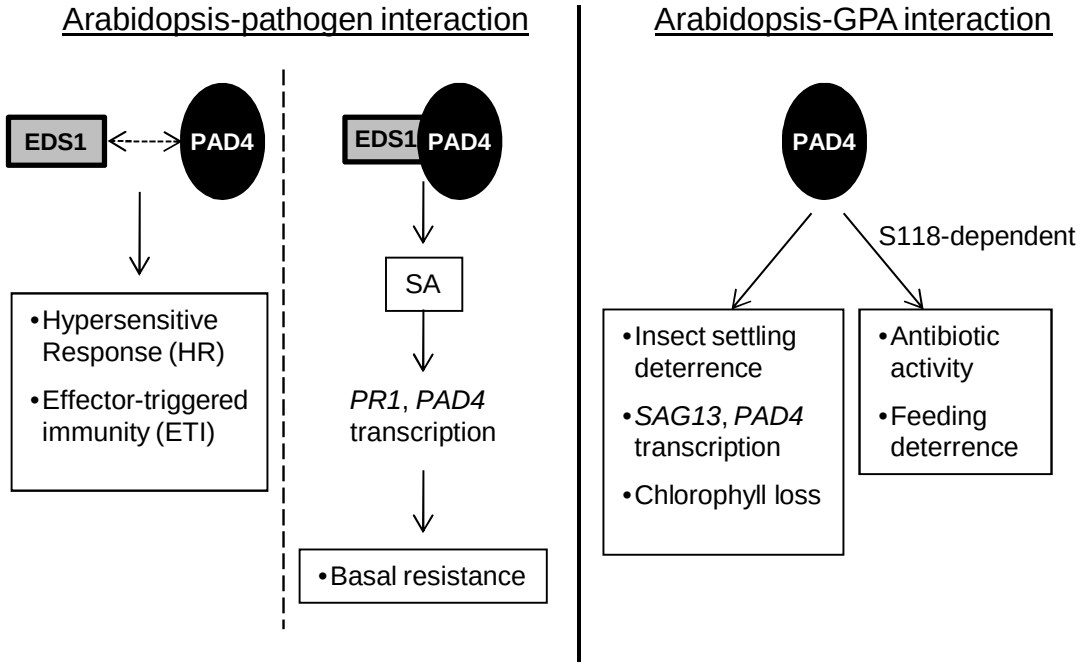

A
Day 0
Day 3
Log cfu cm-2
10
8
6
4
2
0
*
*
*
WT (Ws)
eds1-1
pad4-5
eds1-1 pad4-5
P4WT
p4S118A
p4D178A
p4H229A
B
WT (Ws)
eds1-1
pad4-5
eds1-1 pad4-5
P4WT
p4S118A
p4D178A
p4H229A
C
WT (Ws)
pad4-5
P4WT
TN
p4S118A
p4D178A
p4H229A

Figure 7. S118 is not essential for PAD4 mediated resistance to virulent or avirulent pathogens.
A, Growth of *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 which is virulent on the Ws accession, in WT Ws, *eds1-1*, *pad4-5*, *eds1-1*, *PAD4*^{WT} (*P4*^{WT}), *pad4*^{S118A} (*p4*^{S118A}), *pad4*^{D178A} (*p4*^{D178A}) or *pad4*^{H229A} (*p4*^{H229A}) plants. Pathogen growth was monitored in two independently-derived transgenic lines of each genotype. Pathogen-inoculated leaves were harvested at 0 and 3 dpi, and bacterial numbers determined by plating leaf extracts on selective medium. Bacterial numbers are represented as the Log_{10} of colony forming units per unit area (cfu cm^{-2}) of leaf (n=3). Error bars represent SE. An asterisk above a bar indicates values that are significantly different ($P<0.05$; *t*-test) from the WT Ws at the equivalent time point.
B, Pictures of *Pst* DC3000-inoculated leaves from plants of indicated genotypes harvested 3 dpi. The extent of chlorosis is an indication of disease severity.
C, Resistance to avirulent *Hyaloperonospora arabidopsidis* biotype Noco2 on WT Ws, *pad4-5*, and transgenic *PAD4*^{WT} (*P4*^{WT}), *pad4*^{S118A} (*p4*^{S118A}), *pad4*^{D178A} (*p4*^{D178A}) or *pad4*^{H229A} (*p4*^{H229A}) plants. Sixteen day-old seedlings of the indicated genotypes were inoculated and at 6 dpi 21trypan blue-stained leaves per genotype were scored under the microscope for the presence of discrete HR lesions (identified by red arrows) at infection sites or trailing necrosis (TN; identified by black arrows). Whereas extensive TN was observed in 50% of *pad4-5* leaves (see Table S2), only HR was observed in the transgenic lines and Ws-0 (representing >120 infection sites per line). Photographs of representative samples are shown.
All infection assays were repeated at least twice with similar results.



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At least two molecular activities of PAD4 are implicated in Arabidopsis interactions with biotrophic pathogens. PAD4, dissociated from EDS1, is required for ETI conditioned by TIR-NB-LRR type receptors. Here, PAD4 and EDS1 activate a HR involving localized host cell death and restriction of pathogen growth. A different activity of PAD4 bound to EDS1 in a complex promotes expression of SA biosynthetic and other genes (including *PAD4* itself) leading to defense amplification (e.g. transcription of the *PATHOGENESIS-RELATED1* [*PR1*] gene) in basal resistance against virulent pathogens. In Arabidopsis interactions with GPA, PAD4 confers defenses without measurable EDS1 involvement. One PAD4 activity that does not require Ser118 deters insect settling and promotes leaf senescence, characterized by chlorophyll loss and increased *SAG13* expression. This activity also promotes *PAD4* expression in GPA-infested tissues. A different PAD4^{S118}-dependent activity deters insect feeding from the sieve elements and promotes the accumulation of an antibiosis factor in petiole exudates.

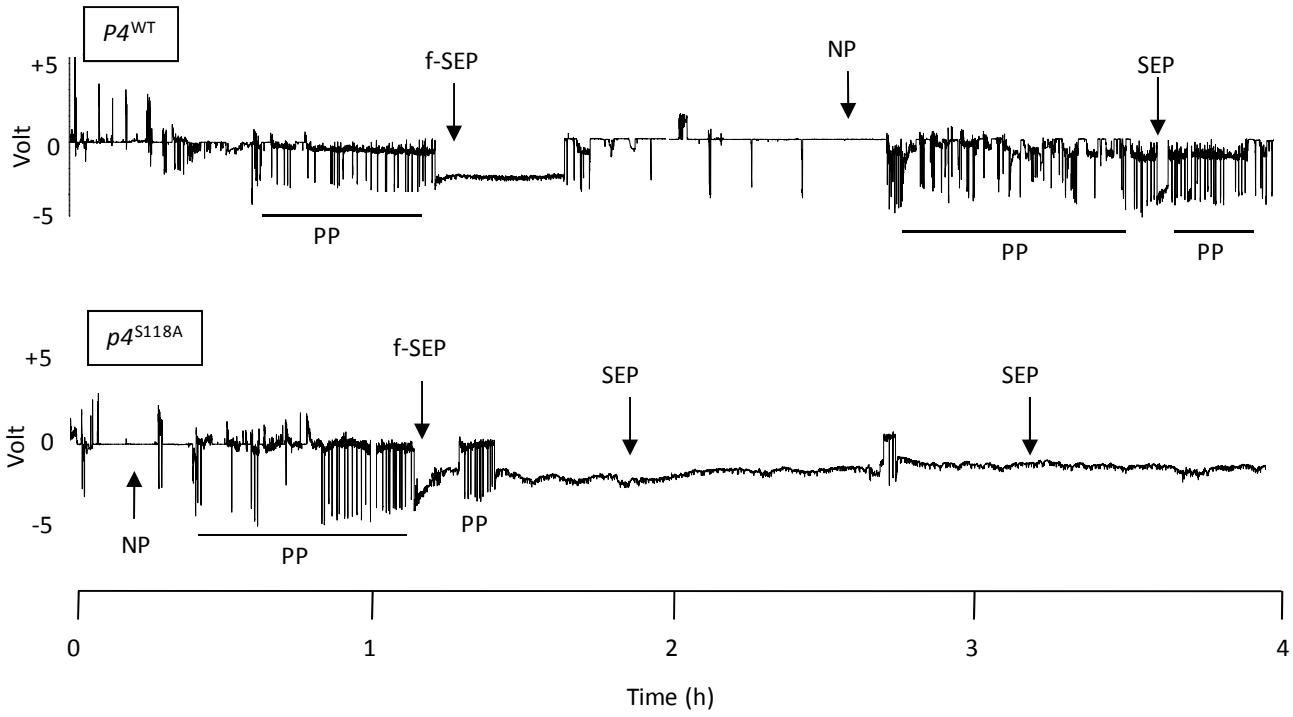

Figure S1. Electrical Penetration Graph analysis of GPA feeding behavior on Arabidopsis *pad4*^{S118A} transgenic plant.

Representative waveform patterns over a 4 h period of GPA feeding on a leaf of a *pad4-5* mutant plant expressing either the *PAD4*^{WT} (*P4*^{WT}) or the *pad4*^{S118A} (*p4*^{S118A}) construct. The plant and insect were held inside a Faraday cage during the recording at an ambient temperature of ~22˚C. PP, pathway phase, f-SEP, first sieve element phase; SEP, sieve element phase; NP, non probing phase.

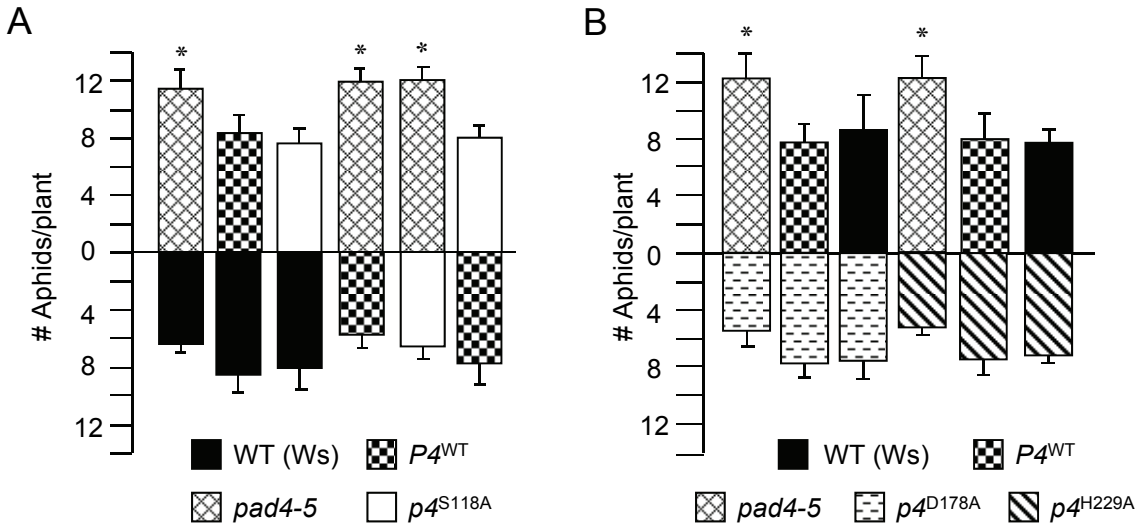

Figure S2. S118, D178 and H229 are not essential for the *PAD4*-determined deterrence of insect settling on Arabidopsis.

A and B, Choice test: Data from Figure 5A and 5B in the main text were plotted as mean # aphids per plant with error bars for visual reference only. Insects were given the choice of settling between plants of two genotypes by releasing twenty adult apterous GPA at the center of each pots containing one plant of each indicated genotypes. The number of adult GPA that had settled on each plant of each genotype was determined 48 h later. Asterisks (*) indicate values that were found to be significantly different from the other genotype in pooled chi-square tests described in Figure 5A and 5B in the main text.

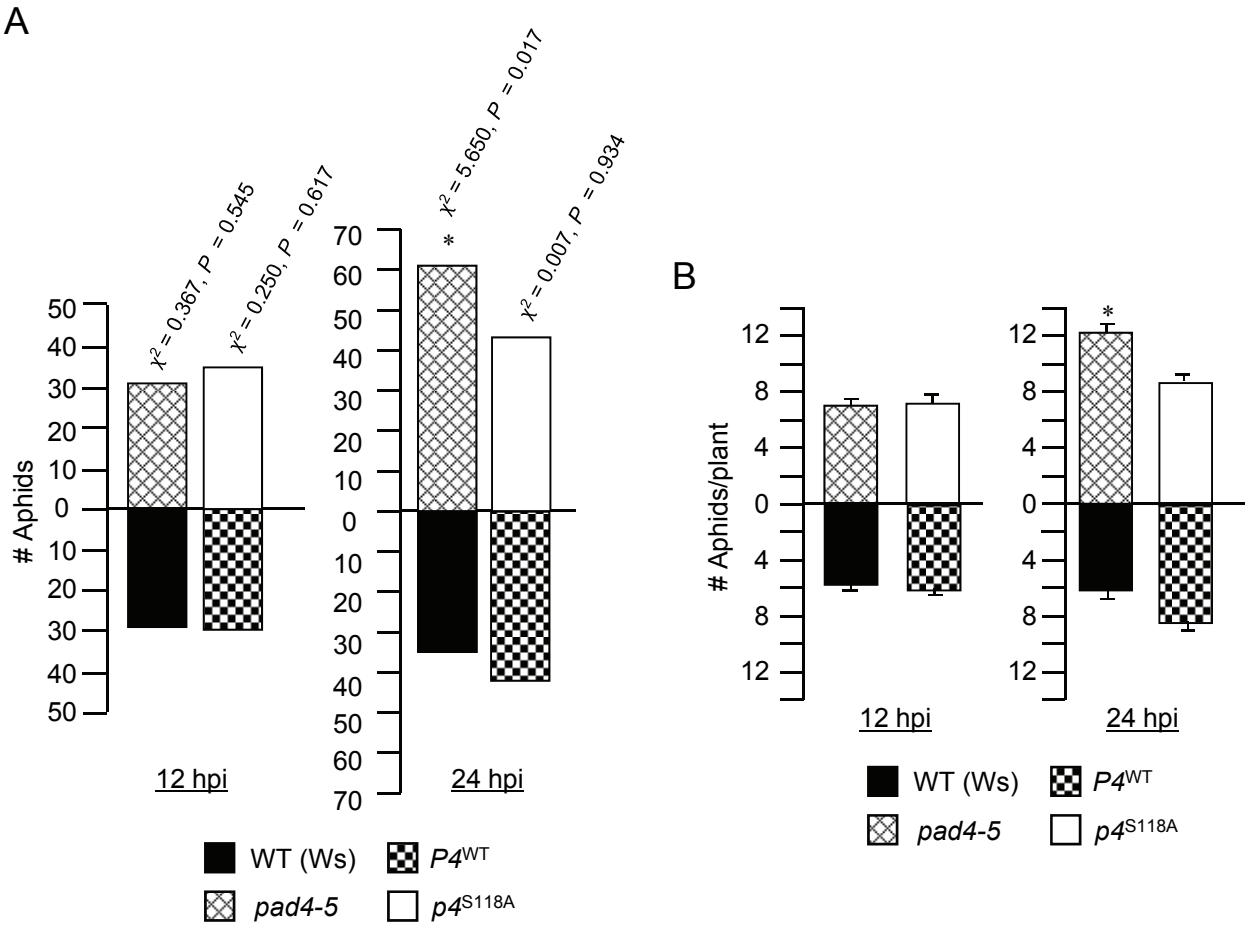

Figure S3. S118 is not essential for the *PAD4*-determined deterrence of insect settling on Arabidopsis.

A, Choice test: Comparison of GPA preference between plants of the indicated genotypes at different time points. Insects were given the choice of settling between plants of two genotypes by releasing twenty adult apterous GPA at the center of each pots containing one plant of each indicated genotypes. The total number of adult GPA that had settled on five plants of each genotype was determined after 12 and 24 hpi. Equal preference for each pair of genotypes was tested using the pooled chi-square test. An asterisk (*) indicates a value that is significantly different (P<0.05) from the other genotype.

B, The above data was plotted as mean # aphids per plant with error bars for visual reference only.

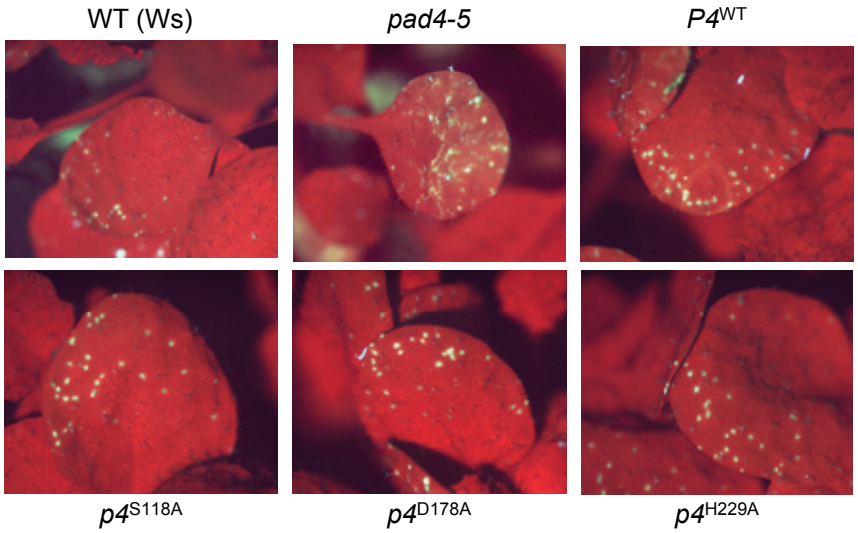

Figure S4. Leaves of *pad4*^{S118A}, *pad4*^{D178A} and *pad4*^{H229A} exchange mutant lines exhibit wild type effector-triggered immunity to *Hyaloperonospora arabidopsidis*.

Sixteen day-old plants of the indicated genotypes were inoculated with the oomycete pathogen *Hyaloperonospora arabidopsidis* biotype Noco2, which is avirulent on Arabidopsis accession Ws. The inoculated leaves were harvested 6 days post inoculation and photographed under UV light illumination. Hypersensitive response indicated by bright pin-point spots is visible in the WT-Ws, and in transgenic *pad4-5* mutant plants that express the *PAD4*^{WT} (*P4*^{WT}), *pad4*^{S118A} (*p4*^{S118A}), *pad4*^{D178A} (*p4*^{D178A}) and *pad4*^{H229A} (*p4*^{H229A}) constructs. In comparison, the non-transgenic *pad4-5* mutant leaves exhibit trailing necrosis.