Discrimination of Arabidopsis PAD4 Activities in Defense against Green Peach Aphid and Pathogens1\[W]\[OA]\[W]

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The Arabidopsis (Arabidopsis thaliana) lipase-like protein PHYTOALEXIN DEFICIENT4 (PAD4) is essential for defense against green peach aphid (GPA; Myzus persicae) and the pathogens Pseudomonas syringae and Hyaloperonospora arabidopsidis. In basal resistance to virulent strains of P. syringae and H. arabidopsidis, 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 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 salicylic acid induction deficient2 (sid2) double mutant, indicating that redundancy between EDS1 and SID2-dependent SA, previously reported for effector-triggered immunity conditioned by certain nucleotide-binding-leucine-rich repeat receptors, does not explain the 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 antibiotic and deterrence against GPA feeding, but S118 was dispensable for deterring GTA settling and promoting senescence in GPA-infested plants as well as for pathogen resistance. These results highlight distinct molecular activities of PAD4 determining particular aspects of defense against aphids and pathogens.

Plants have evolved complex defense mechanisms to counter infection by pathogens and insects. In Arabidopsis (Arabidopsis thaliana), PHYTOALEXIN DEFICIENT4 (PAD4) is an important modulator of resistance to pathogens and the green peach aphid (GPA; Myzus persicae; Glazebrook, 2005; Wiemer 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 the accumulation of the defense signaling hormone salicylic acid (SA) and the phytoalexin camalexin (Zhau 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 Toll-interleukin receptor-nucleotide-binding-leucine-rich repeat (TIR-NB-LRR) receptors (Zhou et al., 1998; Jirage et al., 1999; Feys et al., 2001; Ristèrucci et al., 2001; Rietz et al., 2011). In addition to local defenses, PAD4 drives the activation of systemic acquired resistance, which protects leaves against subsequent infection (Ristèrucci et al., 2001; Rietz et al., 2011).

PAD4 physically interacts with the sequence-related ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) protein (Feys et al., 2001, 2005; Rietz et al., 2011). This stabilizes PAD4, and the 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 the 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 eds11,262p 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 systemic acquired resistance were disrupted in eds11,262p 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 eds11,262p 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 (Rustérucci 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 the 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 a vector for several viral diseases (Kennedy et al., 1962; Matthews, 1991; Blackman and Eastop, 2000). Aphids use their slender styles to penetrate largely intercellularly to reach the sieve elements. Unlike chewing insects, this feeding strategy does not cause extensive wounding of plant tissue (Wallig, 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., 2010a). 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 SENS-ENCE ASSOCIATED GENES (SAG) genes (Pegadaraju et al., 2005, 2007). In support of a role of a senescence-like mechanism in controlling GPA infestation, PAD4-dependent constitutive SAG13 expression in the Arabidopsis suppressor of salicylic acid insensitivity2 (ssi2) mutant correlated with enhanced resistance to GPA (Louis et al., 2010a).

Studies with Arabidopsis salicylic acid induction deficient2 (ssi2) and pad3 mutant plants, which are defective in SA and camalexin synthesis, respectively, and the nonexpressor of pathogenesis-related genes1 (npr1) 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 plants (Pegadaraju et al., 2007). Moreover, constitutive overexpression (OE) of PAD4 from the cauliflower mosaic virus 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 wild-type 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., 2010a). Similarly, elevated SAG13 expression and antibiosis activity against GPA in the ssi2 mutant was not accompanied by increased antixenosis activity against GPA. Also, PAD4 expression, which is up-regulated in GPA-infested wild-type Arabidopsis (Pegadaraju et al., 2005), was not constitutively elevated in the ssi2 mutant (Louis et al., 2010a), suggesting that basal PAD4 transcript levels are sufficient for antibiosis against GPA but that 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 Ser (S), Asp (D), and His (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 forms 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 the crystal structure of a fungal lipase complexed with the substrate analog n-hexylphosphate ethyl ester, which formed a covalent bond with the S residue (Bruzowski et al., 1991). In this study, we used Arabidopsis transgenic plants expressing mutant versions of PAD4 in which these residues were individually replaced by Ala (A) to test whether the conserved amino acids are important for PAD4 involvement in defense against pathogens or GPA. We found that each of the predicted catalytic residues is dispensable for resistance to Pst and Hpu. Substitution of A at S118 severely compromises antibiosis and feeding deterrence against GPA but does not interfere with PAD4-determined deterrence of insect settling or the 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 the results from Venugopal et al. (2009) for ETI, genetic redundancy between EDS1 and SID2 does not explain the dispensability 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 subfunctions 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 overexpressed from the 35S promoter, this slightly enhanced the resistance against GPA compared with wild-type Ws and eds1-1-1 plants in a no-choice assay (Fig. 1A). In the no-choice assay, 20 apterous (wingless) GPA were released on each plant and the GPA population size (adults plus nymphs) was determined 2 d post infestation (dpi). PAD4 transcript accumulation was comparable between the EDS1 OE and wild-type plants (Fig. 1B). The slight resistance-enhancing effect of constitutive EDS1 OE may be due to the ability of the constitutively overexpressed EDS1 to stabilize PAD4 protein (Feyts 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 overexpressing 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 wild-type 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 eds1-22 sid2-1 double mutants were similar to those on wild-type 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 the EDS1- and 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. 2). Several amino acids flanking S118 are also conserved between PAD4 and these eukaryotic lipases (Fig. 2B). Ser is the key catalytic residue, and a D (D178 in PAD4) and H (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 A resulted in the 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 a 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 (Osterlund et al., 1997). To determine whether S118, D178, or H229 of PAD4 has 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 pad4S118A, pad4D178A, and pad4H229A proteins driven by the native PAD4 promoter and fused N terminally to a cMyc epitope tag. The three mutant constructs and nonmutated cMyc-PAD4 (PAD4WT) expressed from the PAD4 native promoter were transformed into the pad4-5 null mutant in Arabidopsis accession Ws, and independent homozygous transgenic lines were selected for each construct. Whereas PAD4 expression was undetectable in GPA-infested pad4-5 mutant plants, the PAD4 transcript level increased over time in GPA-infested leaves of transgenic plants expressing PAD4WT (Fig. 3A), indicating that PAD4WT complements the pad4-5 mutant defect in promoting PAD4 expression in response to GPA infestation (Fig. 3A; Louis et al., 2010b). We determined whether the pad4D178A, pad4H229A, and pad4S118A 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 pad4D178A, pad4H229A, and pad4S118A transgenic plants, indicating that S118, D178, and H229 of PAD4 are not required for increased PAD4 expression. Western blots probed with α-cMyc antibodies indicated that leaves of the transgenic PAD4WT, pad4S118A, pad4D178A, and pad4H229A plants accumulated PAD4 protein (Fig. 3B). However, compared with uninfested leaves, no increases in the PAD4WT and 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 the 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 PAD4WT or pad4S118A, pad4D178A, and pad4H229A mutant constructs to determine if the 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 with the wild type (Fig. 4A). Whereas expression of the PAD4WT construct complemented the pad4-5 defect (Fig. 4A), insect numbers on the pad4D178A 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 pad4D178A lines compared with PAD4WT and nontransgenic wild-type Ws plants.
However, loss of resistance to GPA in pad4D178A was not as extreme as in pad4S118A plants (Fig. 4A). By contrast, GPA infestation was effectively controlled on pad4H229A 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 with vascular sap-enriched petiole exudates collected from Col wild-type plants that reduce insect fecundity when added to a synthetic diet, petiole exudates from the pad4-1 mutant in accession Col lack antibiosis activity (Louis et al., 2010a). 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 pad4S118A transgenic lines. Compared with insect numbers on Ws wild-type or PAD4WT petiole exudate-supplemented diets, insect numbers on the pad4S118A 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 the PAD4 promotion of antibiosis activity.

**PAD4S118 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 Tjallingi, 2000). In EPG, a wired insect that 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 the 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), the nonprobing phase when the insect stylet is not inserted.

constitutively overexpressing EDS1 (EDS1-OE) or PAD4 (PAD4-OE) from the 35S promoter. This experiment was conducted three times with similar results. B, RT-PCR analysis of PAD4 and EDS1 expression in leaves of GPA-infested (+ GPA) plants of the indicated genotypes. Uninfested (- GPA) plants provided negative controls. ACT8 expression served as a control for RT-PCR. This experiment was conducted twice with similar results. C, Aphid population size is not impacted by simultaneous deficiency of EDS1 and SID2. The no-choice assay shows GPA numbers on wild-type accessions Ws and Col, pad4-5, eds1-1, sid2-1, and eds1-1 sid2-1 mutant plants (top panel), and wild-type Col, pad4-1, eds1-22, sid2-1, and eds1-1 sid2-2 sid2-1 mutant plants in accession Col (bottom 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 dpi (n = 10). Error bars represent se. ANOVA of GPA populations on different plant genotypes was conducted using PROC GLM (SAS Institute). Means were separated using the LSD procedure. Different letters above bars indicate values that are significantly different (P < 0.05) from each other.
into the plant tissue, and the pathway phase when the stylet is inserted in the plant tissue but outside the vasculature. EPG comparison of GPA feeding behavior between wild-type and pad4 plants had demonstrated previously that GPA spend more time in the SEP on pad4 than on wild-type plants, thus suggesting that PAD4 is essential for Arabidopsis to limit GPA feeding from the sieve elements (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 wild type as to stay on the pad4 mutant, suggesting that PAD4 deters insect settling on wild-type Arabidopsis (Pegadaraju et al., 2007). Since this deterrence effect of PAD4 was most prominent at 48 h post infestation (hpi) and was not observed prior to 12 hpi, it is unlikely that the insects are more attracted to the pad4 mutant than the wild-type plants. Instead, this difference is likely due to the ability of the insects to stay longer on the pad4 mutant compared with the wild-type 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). When given a choice between the wild type and the pad4-5 mutant plants transformed with pad4S118A, pad4D178A, and pad4H229A variants. A, Time course of PAD4 and SAG13 transcript accumulation in uninfested (−GPA) and GPA-infested (+GPA) leaves of wild-type (WT) Ws, pad4-5, and pad4-5 mutant plants transformed with PAD4WT and pad4S118A, pad4D178A, or pad4H229A constructs expressed from the PAD4 promoter. ACT8 expression served as a control for RT-PCR. B, Western-blot analysis of the PAD4 protein.
PAD4 Activities in Plant Defense

Figure 4. S118 in PAD4 is required for controlling GPA infestation. A, The no-choice assay shows GPA numbers on wild-type (WT)Ws, pad4-5, and two independently derived transgenic pad4-5 mutant lines expressing the PAD4WT (p4WT), pad4S118A (p4S118A), pad4D178A (p4D178A), and pad4D229A (p4D229A) constructs from the PAD4 promoter. GPA population size was determined 2 dpi (n = 12). This experiment was conducted three times with similar results. B, GPA numbers on a synthetic diet containing petiole exudate from PAD4WT and pad4S118A plants. Diet containing petiole exudate collected from the wild-type 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 were determined 4 d later (n = 3). This experiment was conducted three times with similar results. Error bars represent se. For details on statistical analysis, see legend to Figure 1. Different letters above the bars indicate values that are significantly different (P < 0.05) from each other.

Compared with the pad4 mutant. Alternatively, compared with the wild-type 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 A substitution at S118 impacts this behavior of the insect on Arabidopsis, the insects were given the choice of PAD4WT or pad4S118A plants. Twenty adult aphids were released at the center of each pot containing one PAD4WT plant and one pad4S118A plant, and the number of adult aphids that were present on each plant was counted 2 d later to determine if the insects preferred one genotype over the other. This experiment was conducted simultaneously with the following pairs of genotypes: wild-type Ws and pad4-5 mutant, wild-type Ws and PAD4WT, wild-type Ws and pad4S118A, PAD4WT and pad4-5 mutant, and pad4S118A and pad4-5 mutants. As expected, the average number of GPA settling on pad4-5 was higher than on wild-type Ws or PAD4WT plants, and insect numbers were similar between wild-type Ws and PAD4WT (Fig. 5A; Supplemental Fig. S2A), confirming that transgenic PAD4WT complements the pad4-5 defect. Aphids did not discriminate between the PAD4WT and pad4S118A plants (Fig. 5A; Supplemental Fig. S2A). Even at earlier time points (12 and 24 hpi), no differences in insect settling on PAD4WT versus pad4S118A were noticeable (Supplemental Fig. S3, A and B). 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 PAD4S118 for effective antibiosis and controlling the time spent by insect feeding from sieve elements (Fig. 4B; Table I). This conclusion was further supported in choice assays between the pad4-5 and pad4S118A plants, in which aphids preferred settling on pad4-5 over pad4S118A plants (Fig. 5A; Supplemental Fig. S2A). Similarly, D178 and H229 were not required for PAD4 involvement in limiting the number of insects that had settled on Arabidopsis (Fig. 5B; Supplemental Fig. S2B).

S118 Is Dispensable for PAD4-Controlled Premature Leaf Senescence in Response to Aphis Attack

PAD4-conditioned restriction of GPA infestation in wild-type Arabidopsis is accompanied by premature leaf senescence characterized by chlorophyll loss and elevated expression of the SAG13 gene (Figs. 3A and 6, A and B; Pegadaraju et al., 2005; Louis et al., 2010). The loss in chlorophyll content was reduced and up-regulation of SAG13 expression was delayed in GPA-infested pad4-5 leaves compared with leaves of GPA-infested wild-type plants (Figs. 3A and 6B; Louis et al., 2010). We determined whether the A substitution at S118 in pad4S118A plants attenuated chlorophyll loss by measuring chlorophyll contents in GPA-infested leaves of pad4S118A and PAD4WT plants as well as wild-type Ws and pad4-5 plants. As shown in Figure 6B, GPA infestation caused a similar reduction in chlorophyll content in pad4S118A plants as in PAD4WT or wild-type Ws plants. Also, the temporal pattern of SAG13 expression was comparable between GPA-infested wild-type Ws, PAD4WT, and pad4S118A plants compared with the pad4-5 mutant (Fig. 3A). These results indicate that PAD4S118 is not critical for the GPA-induced up-regulation of SAG13 or premature leaf senescence. Similarly, PAD4D178 and PAD4H229 are also not critical for the up-regulation of SAG13 expression and chlorophyll loss in GPA-infested plants (Figs. 3A and 6B).

PAD4S118A Exchange Does Not Compromise Resistance to Leaf-Infecting Pathogens

We have shown above that PAD4S118 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 pad4S118A, pad4D178A, and pad4H229A transgenic lines complemented the loss of basal immunity in pad4-5 to virulent Pst strain DC3000. PAD4WT-expressing transgenic lines and wild-type Ws plants served as positive (complementing) controls and pad4-5 and eds1-1 mutants as negative controls in the bacterial infection assay. As anticipated, the expression of PAD4WT restored basal resistance to Pst DC3000, since bacterial titers in the PAD4WT transgenic lines were similar to those in wild-type Ws at 3 dpi and significantly lower than those in the pad4-5 and eds1-1 single mutants or an eds1-1 pad4-5 double mutant (Fig. 7A). Basal resistance to Pst DC3000 was also restored in plants expressing the pad4S118A, pad4D178A, or pad4H229A variant (Fig. 7A). The extent of leaf chlorosis associated with Pst DC3000 infection was similarly reduced in PAD4WT and the pad4S118A, pad4D178A, and pad4H229A plants compared with the pad4-5 and 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 PAD4S118A is also dispensable 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 of inoculated leaves to reveal host cell death and pathogen infection structures (Rustérucci et al., 2001; Rietz et al., 2011). Leaves of wild-type Ws, PAD4WT, and pad4S118A, pad4D178A, and pad4H229A produced discrete necrotic lesions that prevented Noco2 hypal extension from infection front, whereas the pad4-5 mutant displayed a typical trailing necrotic phenotype due to a partial loss of ETI (Fig. 7C; Table II; Supplemental Fig. S4; Rietz et al., 2011). The pad4D178A and pad4H229A transgenic lines were also fully resistant to Hpa Noco2 (Fig. 7C; Table II; Supplemental Fig. S4).

**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 with chewing insect or necrotrophic pathogen attack (Walling, 2000; Glazebrook, 2005; Howe and Jander, 2008). The results presented here and our previous studies (Feyes et al., 2005; Pegadaraju et al., 2005, 2007; Wiemer 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 characterized by cell death at infection sites (Rietz et al., 2011). The surrounding cells undergo a “reinforcement” phase, requiring an EDS1-PAD4 complex to promote the accumulation of SA and transcriptional amplification of defenses involving SA (Rietz 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 infection (Fig. 1C).

Our analysis of transgenic plants expressing the pad4S118A variant, in which S at position 118 in the PAD4 putative lipase catalytic triad (Fig. 2) was exchanged with A, suggests further bifurcation of PAD4 activities between defenses against pathogens and GPA. S118 is necessary for defense against GPA (Fig. 4; Table I) but not for PAD4-mediated pathogen resistance (Fig. 7; Table II; Supplemental Fig. S4). The inability of pad4S118A transgenic plants to control GPA infestation correlated with an absence of antibiotic activity in petiole exudates of pad4S118A plants compared with PAD4WT and wild-type Ws plants (Fig. 4B) and a failure to control insect feeding from sieve elements (Table I). 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 pad4S118A plant. It is
Figure 5. S118, D178, and H229 are not essential for the PAD4-determined deterrence of insect settling on Arabidopsis. In the choice tests, insects were given the choice of settling between plants of two genotypes by releasing 20 adult apterous GPA at the center of a pot containing one plant of each indicated genotype. The total numbers of adult GPA that had settled on eight plants of each genotype were determined 48 h later. Equal preference for each pair of genotypes was tested using the pooled $\chi^2$ test. Asterisks indicate values that are significantly different ($P < 0.05$) from the other genotype. This experiment was conducted three times with similar results. For mean numbers of insects per plant with error bars for visual reference, see Supplemental Figure S2. WT, Wild type.

Figure 6. S118, D178, and H229 are not essential for the PAD4-determined chlorosis in GPA-infested plants. A, Leaves of the wild-type (WT) Ws, pad4-5, and transgenic pad4-5 plants expressing the PAD4WT ($P4^{WT}$) or pad4S118A ($P4^{S118A}$), pad4D178A ($P4^{D178A}$), and pad4H229A ($P4^{H229A}$) transgenes 5 d after release of 20 GPA on each plant. Uninfested plants (−GPA) provided the negative controls. This experiment was conducted three times with similar results. B, Relative chlorophyll contents in GPA-infested leaves of plants of the indicated genotypes 5 d after release of 20 aphids on each plant. Values are relative to the chlorophyll contents in uninfested plants of the corresponding genotype ($n = 5$). Error bars represent s. 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.

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 a 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 I) and promotes the accumulation of an antibiotic activity in vascular sap (Fig. 4B). The second PAD4 activity, which does not require S118, enables the deterrence of insect settling on the plant (Fig. 5) and drives premature leaf senescence, which is associated with chlorophyll loss and the induction of SAG13 expression (Figs. 3A and 6). Notably, the second PAD4 activity is also required for the feed-forward autoregulation 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. 4B; Louis et al., 2010a). 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

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**Figure 7.** S118, D178, and H229 are not required for PAD4-mediated resistance to virulent or avirulent pathogens. A, Growth of virulent Pst DC3000 on wild-type (WT) Ws, eds1-1, pad4-5, eds1-1 pad4-5 double mutant, PAD4$^{WT}$ (P4$^{WT}$), pad4$^{S118A}$, pad4$^{D178A}$, pad4$^{H229A}$, or pad4$^{S118A}$ pad4$^{D178A}$ pad4$^{H229A}$ 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 were 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 st. Asterisks above the bars indicate values that are significantly different ($P < 0.05$; t-test) from wild-type Ws at the equivalent time point. B, Representative Pst DC3000-inoculated leaves from plants of the indicated genotypes harvested 3 dpi. The extent of chlorosis is an indication of disease severity. C, Resistance to avirulent Hpa biotype Noco2 on wild-type Ws, pad4-5, and transgenic PAD4$^{WT}$, pad4$^{S118A}$, pad4$^{D178A}$, or pad4$^{H229A}$ plants. Sixteen-day-old seedlings of the indicated genotypes were inoculated. At 6 dpi, 21 trypan blue-stained leaves per genotype were scored using the microscope for the presence of discrete hypersensitive response lesions (identified by red arrows) at infection sites or trailing necrosis (TN; identified by black arrows). Whereas extensive trailing necrosis was observed in approximately 50% of pad4-5 leaves (Table II), only the hypersensitive response was observed in the transgenic lines and Ws (representing more than 120 infection sites per line). Photographs of representative samples are shown. All infection assays were repeated at least twice with similar results.
antibiosis activity in petiole exudates. However, previous studies indicated that hypersenescence in the Arabidopsis ssi2 mutant was accompanied by enhanced antibiosis against GPA (Louis et al., 2010a). Therefore, although not essential for the 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 wild type and the \textit{pad4} mutant (Pegadaraju et al., 2007), suggesting that the presence of PAD4 does not hamper the insect’s ability to find the sieve elements. Furthermore, when given the choice between the wild-type and \textit{pad4} mutant plants, GPA did not exhibit any difference in settling on these genotypes during the first 12 h 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 wild-type versus \textit{pad4} mutant plants was exerted at a stage after the insect had begun feeding (Pegadaraju et al., 2007). The 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 I). Insects spent more time in SEP on \textit{pad4\textsubscript{S118}} plants than on plants expressing wild-type \textit{PAD4}. However, GPA did not exhibit preferential settling on transgenic \textit{pad4\textsubscript{S118}} plants compared with \textit{PAD4\textsubscript{WT}} plants expressing the wild-type

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$^{a}$Twenty inoculated leaves were examined for this line in experiment 2.

Figure 8. Model for different PAD4 molecular activities in Arabidopsis interaction with pathogens 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 hypersensitive response involving localized host cell death and the restriction of pathogen growth. A different activity of PAD4 bound to EDS1 in a complex promotes the expression of SA biosynthetic and other genes (including \textit{PAD4} itself), leading to defense amplification (e.g. transcription of the \textit{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 S118 deters insect settling and promotes leaf senescence, characterized by chlorophyll loss and increased \textit{SAG13} expression. This activity also promotes \textit{PAD4} expression in GPA-infested tissues. A different \textit{PAD4\textsubscript{S118}}-dependent activity deters insect feeding from the sieve elements and promotes the accumulation of an antibiosis factor in petiole exudates.
**PAD4** transgene, unlike with the **pad4-5** null mutant (Fig. 5A; Supplemental Fig. 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 S-D-H triad in a large family of α/β-fold hydrolases that includes lipases and esterases (Brady et al., 1990; Winkler et al., 1990). The S 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 hydrolase activity. This is strengthened by the fact that D 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** (S. Rietz and J.E. Parker, unpublished data), and the possibility that these residues serve a structural rather than an 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 into its role in diverse defense outputs.

We noted that although **PAD4** transcript abundance increased during the course of aphid infestation of wild-type 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 the 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 the synthesis of the **PAD4** protein due to translational control. Alternatively, newly produced **PAD4** protein may be turned over faster in **GPA**-infested tissues compared with the uninfested plants. Arabidopsis might compensate for an 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** (Fey et al., 2005; Rietz et al., 2011); therefore, **EDS1** OE might increase the amount of **PAD4** protein available for defense 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 wild-type and **eds1** mutant plants in no-choice assays (Fig. 1A; Pegadaraju et al., 2007).

**CONCLUSION**

Replacement of S with A 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 radish (**Raphanus sativus** 'Early Scarlet Globe') and mustard (**Brassica juncea** 'Florida Broadleaf') in a growth chamber set at 22°C and programmed for a 14-h-light/10-h-dark cycle. The same conditions were used for cultivating Arabidopsis (**Arabidopsis thaliana**). All plants were cultivated in an autoclaved compost-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** (Fey et al., 2001, 2005; Glazebrook et al., 1997), and **eds1-22** and **sid2-1** are in accession **Col** (Navrath and Métreaux, 1999; 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 (At3g48080 and At3g48080) of **EDS1** (Yang and Hua, 2004). The **eds1-22** mutant contains a T-DNA insertion in At3g48080 and hence is not a complete loss-of-function mutation (Yang and Hua, 2004). The **PAD4-**OE line (no. 1; Pegadaraju et al., 2007) expresses **PAD4** cDNA under the control of the **SS5** promoter and fused to a C-terminal strep epitope tag on a pXCSG-Strep binary vector (Witte et al., 2004) in the **pad4-5** background. The **EDS1-OE** transgenic line overexpresses **EDS1-SII** under the control of the **SS5** promoter on the pXCSG-Strep vector (Witte et al., 2004) in the **eds1-1** background. α-**EDS1** antibodies (Fey et al., 2003) were used in western blotting for monitoring **EDS1** OE. A functional cMyc epitope-tagged **PAD4** cDNA driven by 1 kb of 5′ **PAD4** promoter sequence (Fey et al., 2001) was used as a template for introducing **S118A**, **D178A**, and **H229A** mutations using the QuickChange mutagenesis kit (Stratagene). All constructs were verified by DNA sequencing before cloning into a basta-resistant binary pSIlJ515 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 described previously (Pegadaraju et al., 2005; Louis et al., 2010a). In no-choice assays, 20 adult apterous (wingless) **GPA** were released on each plant, and the total numbers of nymphal plus adult **GPA**s were counted 2 dpi. In choice tests, 20 adult apterous aphids were released at the center of the pot containing one wild-type plant and one mutant/transgenic line, and the numbers of adult **GPA** on each plant were counted 2 dpi.

**Analysis of GPA Feeding Behavior**

The electrophysiological EPG technique (van Helden and Tjallingii, 2000) was used to monitor the feeding behavior of **GPA** on **PAD4** and **pad4** OE. **PAD4** OE
plants as described previously (Pegadaraju et al., 2007). Ten replications were performed, and the mean time spent by aphids on various activities was analyzed by the nonparametric Kruskal-Wallis test (P < 0.05).

**Petiole Exudate Collection and Feeding Trials**

Petiole exudates enriched in vascular sap were collected from 80 to 120 leaves (approximately 25–30 plants) as described previously (Chaturvedi et al., 2008). Feeding trial bioassays were performed using a synthetic diet (Müller and Dadd, 1985) as described (Louis et al., 2010a). 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 GPA were determined 4 d later.

**Pathogen Infection Assays**

Infections with *Pseudomonas syringae pv tomato* strain DC3000 were conducted by dipping leaves of 4-week-old plants into a freshly prepared bacterial suspension (1 × 10^7 colony-forming units mL⁻¹) in 10 mM MgCl₂. Bacterial numbers inside leaves were counted at 0 (3 hpi) and 3 dpi, as described previously (Birker et al., 2009). Infections with *Hyaloperonospora arabidopsidis* biotype Noco2, which is avirulent on Arabidopsis accession Ws, were done by spraying 16-d-old plants with a conidiospore suspension in distilled water (4 × 10^6 spores mL⁻¹) (Feys et al., 2005). Six days after inoculation, the development of host responses and Hpa infection structures was monitored with a light microscope after lactophenol trypan blue staining of infected leaves (Aarts et al., 1998) or observing plants with a binocular microscope with UV illumination.

**RNA Extraction and Reverse Transcription-PCR Analysis**

RNA for reverse transcription (RT)-PCR analysis was extracted from Arabidopsis leaves (Pegadaraju et al., 2005). Gene-specific PCR primers used for *ACT8* (At1g29240), *PAD4* (At3g52430), *EDS1* (At1g8090), and *SAG13* (At2g29350) were as described previously (Pegadaraju et al., 2007, Louis et al., 2010a). PCR conditions used were as follows: 95°C for 5 min, followed by 30 cycles (for Fig. 3A) or 25 cycles (for Fig. 1B) of 95°C for 30 s, 55°C for 45 s, 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 described previously (Feys et al., 2001). Fifty micrograms of protein isolated from leaves of wild-type Ws, pad4-5, and pad4-5 complemented with cMyc-tagged PAD4WT driven from its native promoter, or mutated versions of cMyc-tagged pad4S118A, pad4D178A, and pad4H229A, was 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% nonfat dry milk in TBST (Tris-buffered saline [10 mM Tris and 150 mM NaCl, pH 7.5] containing 0.2% Tween 20) and then incubated with alkaline phosphatase-linked α-cMyc antibody (1:2,500; Santa Cruz Biotechnology) 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-indolyl p-nitroblue tetrazolium chloride staining.
Identification of the active site serine of hormone-sensitive lipase by site-directed mutagenesis. FEBS Lett 344: 234–238


Louis J, Leung Q, Pegadaraju V, Reese J, Shah J (2010a) PAD4-dependent antibiosis contributes to the ssu2-confounded hyper-resistance to the green peach aphid. Mol Plant Microbe Interact 23: 618–627

Mittler TE, Dadd RH (1965) Differences in the probing responses of Myzus persicae (Sulzer) elicited by different feeding solutions behind a Parafilm membrane. Entomol Exp Appl 8: 107–122


Mortensen LT, Dadd RH (1965) Differences in the probing responses of Myzus persicae (Sulzer) elicited by different feeding solutions behind a Parafilm membrane. Entomol Exp Appl 8: 107–122


