

Phloem-based resistance to green peach aphid is controlled by *Arabidopsis* *PHYTOALEXIN DEFICIENT4* without its signaling partner *ENHANCED DISEASE SUSCEPTIBILITY1*

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Summary

Green peach aphid (GPA) *Myzus persicae* (Sülzer) is a phloem-feeding insect with an exceptionally wide host range. Previously, it has been shown that *Arabidopsis thaliana* *PHYTOALEXIN DEFICIENT4* (*PAD4*), which is expressed at elevated levels in response to GPA infestation, is required for resistance to GPA in the *Arabidopsis* accession Columbia. We demonstrate here that the role of *PAD4* in the response to GPA is conserved in *Arabidopsis* accessions Wassilewskija and Landsberg *erecta*. Electrical monitoring of aphid feeding behavior revealed that *PAD4* modulates a phloem-based defense mechanism against GPA. GPA spends more time actively feeding from the sieve elements of *pad4* mutants than from wild-type plants, and less time feeding on transgenic plants in which *PAD4* is ectopically expressed. The activity of *PAD4* in limiting phloem sap uptake serves as a deterrent in host-plant choice, and restricts aphid population size. In *Arabidopsis* defense against pathogens, all known *PAD4* functions require its signaling and stabilizing partner EDS1 (*ENHANCED DISEASE SUSCEPTIBILITY1*). Bioassays with *eds1* mutants alone or in combination with *pad4* and with plants conditionally expressing *PAD4* under the control of a dexamethasone-inducible promoter reveal that *PAD4*-modulated defense against GPA does not involve EDS1. Thus, a *PAD4* mode of action that is uncoupled from EDS1 determines the extent of aphid feeding in the phloem.

Keywords: *Myzus persicae*, plant defense, insect resistance, senescence, stress.

Introduction

Aphids are phloem-feeding insects that use their stylet-like mouthparts to obtain plant sap (Pollard, 1973). Aphids limit plant productivity by manipulating resource allocation patterns in the host plant (Dixon, 1998) and vectoring plant viruses (Kennedy *et al.*, 1962; Matthews, 1991). Plants have evolved multiple mechanisms to defend themselves against aphids, including antibiotic factors restricting insect fecundity and anti-xenotic factors deterring insects from settling on the host and feeding. These defense mechanisms can be

exerted at various stages of plant–aphid interaction. They can be employed externally when the aphid probes the plant surface, in the internal tissue layers when the aphid stylet finds its way to the sieve elements, and during the sieve element phase (SEP) when the aphid taps into sieve elements. For example, glandular trichomes present on leaf surfaces produce volatile and non-volatile metabolites that affect aphid behavior and performance (Neal *et al.*, 1990), while tissue damage that occurs during stylet penetration

causes the release of thiocyanates in *Brassica* species that are toxic to some aphids (Rask *et al.*, 2000). Also, the sealing of sieve elements in response to aphid stylet penetration, presumably because of coagulation of phloem proteins and callose deposition, can have a direct impact on sap availability to the aphid (Will and van Bel, 2006). In a susceptible host, aphids are able to suppress mechanisms that trigger sealing of sieve elements, thus allowing the insect to feed from one site for a period of hours to days (Tjallingii, 2006; Will and van Bel, 2006).

Aphids secrete two types of saliva into the plant host (Miles, 1987). A gelling saliva, which is first secreted before stylet insertion and then continuously after penetration of the plant surface, envelops the stylet and seals the wound inflicted by stylet penetration (Tjallingii, 2006). This may prevent the release of host factors that promote plugging of sieve plates upon stylet insertion (Will and van Bel, 2006). In contrast, a watery saliva that is continuously secreted during feeding may interact with phloem proteins to prevent their coagulation (Will and van Bel, 2006). Salivary secretions may also contain effectors that modulate plant defense responses (Miles, 1999). For example, the action of salivary polysaccharases on plant cell walls could release oligosaccharides that elicit plant defenses. Alternatively, polyphenol oxidases and peroxidases present in watery saliva are likely to detoxify phenolic allelochemicals and hydrogen peroxide, respectively, produced by the plant host.

The polyphagous green peach aphid (GPA) *Myzus persicae* (Sülzer) (Hemiptera: Aphididae) has an exceptionally wide host range covering more than 50 families of plants (Blackman and Eastop, 2000). GPA feeding results in the activation of premature leaf senescence in *Arabidopsis thaliana*, characterized by expression of the *SENESCENCE ASSOCIATED GENES* *SAG13*, *SAG21* and *SAG27*, chlorosis and cell death (Pegadaraju *et al.*, 2005). The activation of premature leaf senescence correlates with the ability of *Arabidopsis* to limit GPA population size. GPA numbers were diminished on the hypersenescent *ssi2* and *cpr5* mutants compared to wild-type (WT) plants (Pegadaraju *et al.*, 2005). In contrast, delayed activation of premature leaf senescence was accompanied by an increase in GPA numbers on the *Arabidopsis pad4* (*phytoalexin deficient4*) mutant compared to WT (Pegadaraju *et al.*, 2005). *PAD4* controls the synthesis of defense signals including the phenolic molecule salicylic acid (SA) and the indole derivative camalexin in resistance to invasive pathogens (Bartsch *et al.*, 2006; Glazebrook *et al.*, 1997; Jirage *et al.*, 1999; Zhou *et al.*, 1998). Genetic analysis, however, has pointed to a role of *PAD4* in defense against GPA that is independent of SA and camalexin (Pegadaraju *et al.*, 2005).

PAD4 encodes a nucleo-cytoplasmic protein that has sequence similarity to lipases (Feys *et al.*, 2001, 2005; Jirage *et al.*, 1999). In *Arabidopsis*, all of the defense signaling activities of *PAD4* identified so far were in combination with

EDS1 (*ENHANCED DISEASE SUSCEPTIBILITY1*), which encodes a structurally related protein that is also distributed between the cytoplasm and nucleus (Falk *et al.*, 1999; Feys *et al.*, 2005). *EDS1* interacts with *PAD4* in yeast and plant extracts and is required for accumulation of *PAD4* protein (Feys *et al.*, 2001, 2005). Another lipase-like protein, *SAG101* (*SENESCENCE ASSOCIATED GENE101*) (He and Gan, 2002), has been identified as an additional component of the *EDS1* defense pathway that accumulates in the nucleus and interacts with the *EDS1* protein in this compartment (Feys *et al.*, 2005). *SAG101* is partially redundant with *PAD4* in *Arabidopsis* resistance to pathogens. The presence of spatially distinct *EDS1*-*PAD4* and *EDS1*-*SAG101* complexes inside the cell suggests that *EDS1* functions as an adaptor for *PAD4* and *SAG101* in a defense signal relay (Feys *et al.*, 2005). *PAD4* and *EDS1* are also needed for transmission of defense signals to cells beyond the initial sites of pathogen infection (Rustérucci *et al.*, 2001; Wiermer *et al.*, 2005).

In this study, we demonstrate that *PAD4* is necessary to control the level of GPA feeding activity in the phloem. The *PAD4*-mediated resistance involves both antibiotic and anti-xenotic factors and operates independently of both *EDS1* and *SAG101*.

Results

Ectopic expression of Arabidopsis PAD4 confers enhanced resistance to GPA

Previously, it was shown that *PAD4* transcripts accumulated in leaves of WT *Arabidopsis* as early as 3 h post-infestation (hpi) with GPA (Pegadaraju *et al.*, 2005). Also, no-choice bioassays with WT (Col-0) and *pad4-1* mutant plants revealed that *PAD4* is needed to promote the antibiosis that decreases GPA reproduction (Pegadaraju *et al.*, 2005). To investigate further the role of *PAD4* in aphid resistance, we studied the impact of ectopic expression of *PAD4* from the CaMV 35S promoter on the plant response to GPA. Several independent *35S:PAD4* transgenic lines were created in the *pad4-5* null mutant in accession Ws-0 (see Experimental procedures), and therefore the *PAD4* mRNA accumulating in these plants is derived only from the transgene. As shown in Figure 1 (a,b), ectopic expression of *PAD4* mRNA in one representative *35S:PAD4* line did not cause enhanced accumulation of *SAG13* and *SAG21* mRNAs or lead to spontaneous cell death in leaves. However, the GPA-feeding induced accumulation of *SAG13* and *SAG21* transcripts was faster in leaves of *35S:PAD4* plants than in WT. These transcripts were detected as early as 3 hpi in GPA-infested leaves of the *35S:PAD4* line, compared to 12 hpi in GPA-infested WT leaves. Cell death was also induced more rapidly in GPA-infested *35S:PAD4* leaves than in WT. Occasional clusters of dead cells were observed as early as 6 hpi (data not shown) and were abundant in *35S:PAD4* leaves at 24 hpi,

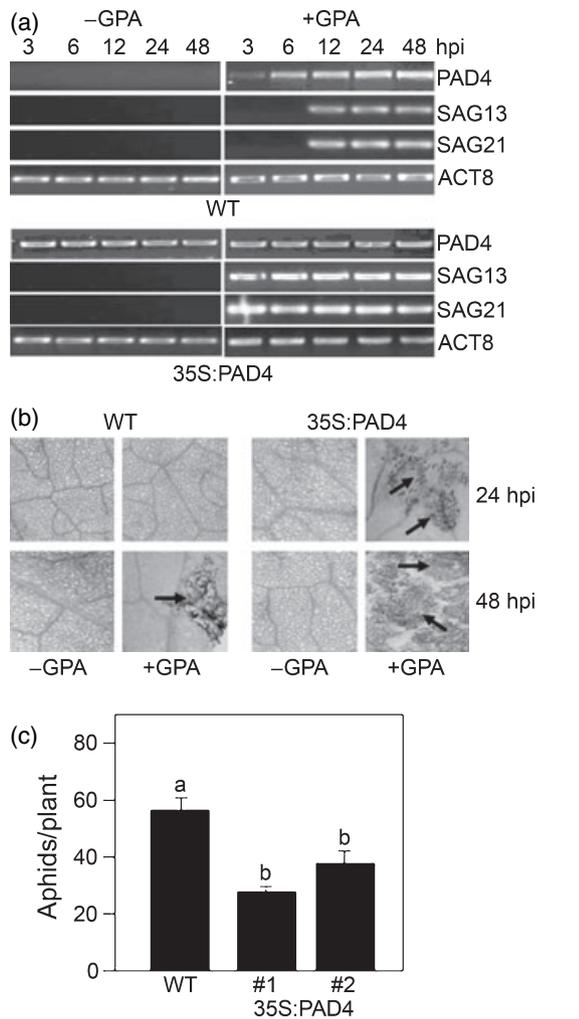


Figure 1. Ectopic expression of *PHYTOALEXIN DEFICIENT4* (*PAD4*) triggers a more rapid response and confers heightened resistance to green peach aphid (GPA) compared with wild-type.

(a) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *PAD4*, *SAG13* and *SAG21* and *ACT8* expression in leaves of wild-type (WT) accession Ws-0 (upper panel) and a transgenic *35S:PAD4* plant line 1 (lower panel) that ectopically expresses *PAD4*. RT-PCR was performed on RNA extracted from non-infested (-GPA) and GPA-infested (+GPA) plants at 3, 6, 12, 24 and 48 hpi. The *ACT8* gene served as a control for RNA quality in the RT-PCR reaction.

(b) Trypan blue staining of leaves from non-infested (-GPA) and GPA-infested (+GPA) WT and *35S:PAD4* line 1 at 24 and 48 hpi. The arrows indicate areas containing intensely stained dead cells.

(c) Comparison of GPA numbers on WT Ws-0 and two independently derived *35S:PAD4* lines in a no-choice bioassay. The numbers of GPA were counted 2 days after infestation with 20 adult apterous aphids. All values are means for 15 plants \pm SE. Different letters above the bars indicate values that are significantly different ($P < 0.05$) from each other by Student's *t*-test.

whereas cell death was first observed at 48 hpi in GPA-infested WT leaves (Figure 1b). In a no-choice bioassay, numbers of GPA were significantly lower on two independent *35S:PAD4* transgenic lines compared to WT (Figure 1c). We conclude that increased *PAD4* expression enhances antibiosis to GPA.

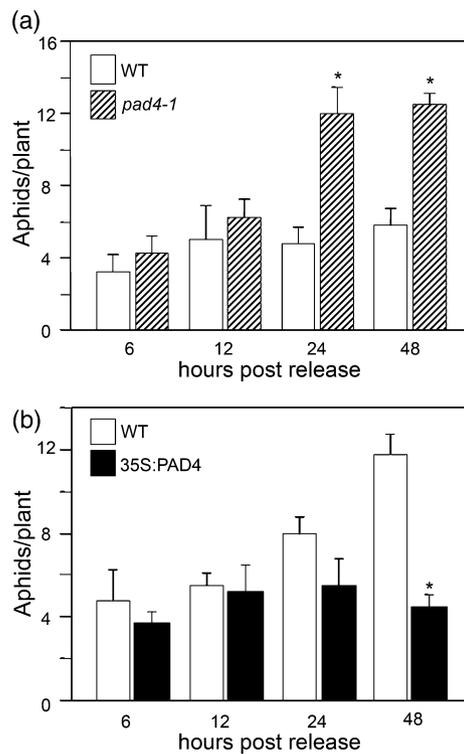


Figure 2. *PHYTOALEXIN DEFICIENT4* (*PAD4*) promotes anti-xenosis to green peach aphid (GPA).

(a) Choice test comparison of GPA preference for wild-type (WT) Col-0 versus *pad4-1* mutant plants.

(b) Choice test comparison of GPA preference for WT Ws-0 versus *35S:PAD4* plants.

GPA numbers on a minimum of four plants of each genotype were analyzed for each time point. The means were separated using paired *t*-test ($P < 0.05$). Asterisks indicate significant differences between the *pad4-1* or *35S:PAD4* plant and the corresponding WT at that time point.

PAD4 promotes anti-xenosis to GPA

To determine whether anti-xenotic (deterrent) factors are also involved in *PAD4*-mediated Arabidopsis defense against GPA, host choice by the aphid was studied. Adult apterous (wingless) insects were given the choice of feeding on WT (Col-0) or *pad4-1* mutant plants by releasing 20 insects equidistant from WT and *pad4-1* plants grown in the same pot. The number of adult insects that had settled on plants was monitored over a 48 h period. The numbers of GPA that had settled on the WT plant remained relatively constant between 12–48 h after release (Figure 2a). In contrast, the number of GPA that had settled on the *pad4-1* mutant gradually increased until 24 h after release. At 24 and 48 h after release, GPA showed a significant preference for the *pad4-1* mutant over WT ($P < 0.05$). When given a choice between the WT and *35S:PAD4* line 1, GPA preferred the WT plant ($P < 0.05$) (Figure 2b). These data indicate that *PAD4* promotes anti-xenosis in addition to antibiosis to GPA in Arabidopsis.

Table 1 Mean time (h) \pm SE spent by green peach aphid (GPA) on various activities on wild type (WT) and *phytoalexin deficient4* (*pad4-1*) plants during 8 h of recording time

Parameters	WT (Col-0)	<i>pad4-1</i>
Time to first probe	0.32 \pm 0.13	0.32 \pm 0.14
Time to first sieve element phase (SEP)	2.64 \pm 0.41	2.21 \pm 0.27
Total duration of pathway phase	4.62 \pm 0.37	3.88 \pm 0.35
Sum of SEP in a total of 8 h recording time	0.69 \pm 0.14	2.39 \pm 0.29*
Available SEP from the beginning of the first SEP until the end of recording time	5.36 \pm 0.41	5.79 \pm 0.27
Percentage of available SEP actually spent in SEP ^a	13.77 \pm 0.02	42.86 \pm 0.05*

*Significantly different from the WT accession Col-0 ($P < 0.05$).

^aAvailable SEP was used to calculate the percentage of available SEP actually spent in SEP. For example, if the GPA reaches the SEP after 4 h from the start of the experiment, and continues to the end of the recording time (8 h), the value is 100%. If the GPA reaches the SEP after 6 h from the start of the experiment and continues to the end of the recording time (8 h), the value is also 100%, but it exhibits lower total SEP.

PAD4 is required for phloem-based resistance to GPA

To study which aphid feeding stages are affected by *PAD4* expression, the electrical penetration graph (EPG) technique was utilized to compare GPA behavior between WT (Col-0) and *pad4-1* mutants, and between WT (Ws-0) and *35S:PAD4* transgenic plants. A representative EPG waveform produced by GPA probing on a WT (Col-0) plant is shown in Figure S1. Electrically recorded stylet penetration activities were categorized into various waveforms, and the mean time spent on various activities documented (Tables 1 and 2). Parameters measured include the time to first probe, total duration of pathway phase (penetrating between cells), time to reach first sieve element phase (SEP) when the stylet is located in a sieve element, sum of the duration of all SEPs in a total of 8 h recording time, and the proportion of available SEP from the beginning of the first SEP until the end of the recording. GPA spent similar amounts of time on average for the time to first probe, total duration of pathway phase and time to reach first SEP on WT (Col-0) and *pad4-1* mutants (Table 1), and on WT (Ws-0) and *35S:PAD4* plants (Table 2). The similarity of these GPA behavioral parameters on the various plant lines suggests that neither surface features nor cell-wall properties play a decisive role in *PAD4*-mediated resistance to this aphid. Measurements of other parameters such as the sum of SEP duration in a total of 8 h of recording time and the percentage of available SEP actually spent in SEP, however, revealed a significant difference between *pad4-1* and WT (Table 1). GPA spent more time in SEP on *pad4-1* plants

Table 2 Mean time (h) \pm SE spent by green peach aphid (GPA) on various activities on wild type (WT) and *35S:PHYTOALEXIN DEFICIENT4* (*PAD4*) transgenic plants during 8 h of recording time

Parameters	WT (Ws-0)	<i>35S:PAD4</i> line 1
Time to first probe	0.35 \pm 0.15	0.42 \pm 0.27
Time to first sieve element phase (SEP)	3.76 \pm 0.49	4.92 \pm 0.63
Total duration of pathway phase	4.55 \pm 0.39	4.05 \pm 0.48
Sum of SEP in a total of 8 h recording time	1.14 \pm 0.24	0.42 \pm 0.13*
Available SEP from the beginning of the first SEP until the end of recording time	4.24 \pm 0.49	3.08 \pm 0.63
Percentage of available SEP actually spent in SEP ^a	26.96 \pm 0.06	15.45 \pm 0.05*

*Significantly different from the WT accession Ws-0 ($P < 0.05$).

^aSee Table 1 for details of percentage SEP calculations.

compared to WT Col-0 ($\chi^2 = 21.65$, d.f. = 1, $P < 0.05$), reflecting more time spent feeding on the phloem sap of *pad4-1*. Moreover, the percentage of available SEP actually spent in SEP was greater on *pad4-1* than on WT ($\chi^2 = 20.65$, d.f. = 1, $P < 0.05$) suggesting that GPA consumes more photoassimilates from the *pad4-1* mutant.

Consistent with an effect of *PAD4* on aphid feeding, GPA spent significantly less time in SEP on *35S:PAD4* transgenic plants compared to WT Ws-0 (Table 2) ($\chi^2 = 7.9$, d.f. = 1, $P < 0.05$). Also, the percentage of available SEP actually spent in SEP was lower on the *35S:PAD4* transgenic plant than on WT ($\chi^2 = 5.08$, d.f. = 1, $P < 0.05$). These results support the hypothesis that Arabidopsis *PAD4* regulates phloem-based resistance against GPA. The *PAD4*-dependent anti-xenotic effect observed in the choice test (Figure 2a,b) could derive from a *PAD4*-mediated limitation on phloem sap uptake by GPA.

PAD4-conditioned resistance to GPA is independent of EDS1 and SAG101

In leaves, the activity of *PAD4* in plant defense against pathogens is within an *EDS1*-regulated pathway (Feys *et al.*, 2005). We therefore tested whether the *PAD4* activity found here that restricts GPA feeding from phloem also depends on *EDS1*. *EDS1* transcripts accumulated to a higher level in GPA-infested leaves than non-infested leaves of WT plants (Figure 3). The increase in *EDS1* expression in GPA-infested plants mirrored that of *PAD4*, with both mRNAs accumulating to higher levels than in control non-infested plants by 3 hpi. A no-choice test was performed to evaluate GPA performance on an *eds1* RNAi line in accession Col-0 in which the endogenous *EDS1* gene was stably silenced using double-stranded RNA interference technology (Feys *et al.*,

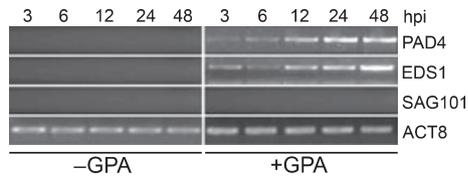


Figure 3. *EDS1* expression is induced in response to green peach aphid (GPA) infestation.

Real time-polymerase chain reaction (RT-PCR) analysis of *PAD4*, *EDS1*, *SAG101* and *ACT8* expression in leaves of non-infested (-GPA) and GPA-infested (+GPA) WT Col-0 plants. RT-PCR was performed on RNA extracted from leaves at 3, 6, 12, 24 and 48 hpi. *ACT8* served as a control for RNA quality in the RT-PCR reaction.

2005). As controls, GPA performance on WT Col-0 plants and the *pad4-1* mutant was monitored. As expected, the numbers of GPA were higher on the *pad4-1* mutant than the WT (Figure 4a). However, GPA numbers on the *eds1* RNAi line were comparable to those on WT plants, suggesting that *EDS1* is not important for plant defense against GPA.

Arabidopsis accession Col-0 expresses an *EDS1*-like gene that contributes to the total *EDS1* activity (Feys *et al.*, 2005). Hence, GPA performance was assessed on the *eds1-1* and *eds1-2* null mutants whose corresponding WT accessions Ws-0 and *Ler*, respectively, lack a functional *EDS1*-like gene. GPA performance was also assessed on the *pad4* null mutants, *pad4-5* and *pad4-2*, in the Ws-0 and *Ler* backgrounds, respectively. The numbers of GPA on the *eds1-1* (Figure 4b) and *eds1-2* (Figure 4c) mutants were comparable to those on WT, whereas the GPA numbers on the *pad4-5* and *pad4-2* mutants were higher. GPA numbers on the *pad4-5 eds1-1* double mutant were similar to those on the *pad4-5* single mutant (Figure 4b). Also, GPA feeding-induced accumulation of *SAG13* and *SAG21* transcripts and cell death were unaffected in the *eds1* RNAi line (Figure S2).

The discovery of an *EDS1*-independent activity of *PAD4* in aphid resistance was surprising as *PAD4* protein accumulation in soluble leaf extracts requires *EDS1*, and all detectable *PAD4* is associated with *EDS1* (Feys *et al.*, 2005). We tested further whether *PAD4*-mediated defense against GPA is uncoupled from *EDS1* in Arabidopsis by comparing the responses of stable transgenic *pad4-5* and *pad4-5 eds1-1* lines expressing *PAD4* (denoted d-P4) under the control of a Dex-inducible promoter (see Experimental procedures). As shown in Figure 5 (a), *PAD4* mRNA was not detectable in *pad4-5* d-P4 and *pad4-5 eds1-1* d-P4 plants pre-treated with water, nor in a *pad4-5* transgenic line containing the empty vector (d-C), 2 days after infestation with GPA. *PAD4* transcripts accumulated in GPA-infested leaves of *pad4-5* d-P4 and *pad4-5 eds1-1* d-P4 transgenic plants but not in *pad4-5* d-C leaves that were treated with Dex, confirming the Dex-inducibility of the d-P4 transgene. As expected, *PAD4* mRNA derived from the endogenous *PAD4* gene was expressed in both water- and Dex-treated GPA-infested WT and *eds1-1*

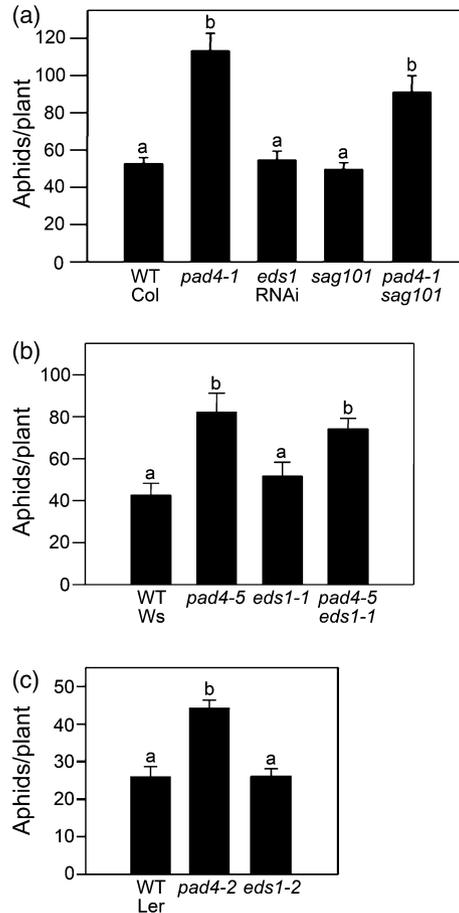


Figure 4. *EDS1* and *SAG101* are not required for Arabidopsis defense against green peach aphid (GPA).

(a) GPA numbers on wild-type (WT) Col-0, *pad4-1*, *eds1* RNAi, *sag101* and *pad4-1 sag101* plants.

(b) GPA numbers on WT Ws-0, *pad4-5*, *eds1-1* and *pad4-5 eds1-1* plants.

(c) GPA numbers on WT *Ler*, *pad4-2* and *eds1-2* plants.

The numbers of aphid in the no-choice assays were calculated 2 days after infestation of each plant with 15 GPA. All values are means from a minimum of 10 plants \pm SE. Different letters above the bars indicate values that are significantly different ($P < 0.05$) from each other by Student's *t*-test.

mutant plants but not in the non-transgenic *pad4-5* mutant. In a no-choice bioassay, GPA numbers were higher on all water-treated plants that contained the *pad4-5* mutant allele compared with water-treated WT and *eds1-1* mutant plants (Figure 5b). In contrast, numbers of GPA were lower on Dex-treated *pad4-5* d-P4 plants, demonstrating the ability of the Dex-inducible *PAD4* construct to complement *pad4-5* in aphid resistance. Significantly, Dex-induced expression of *PAD4* also resulted in lower GPA numbers on *pad4-5 eds1-1* d-P4 plants. As *eds1-1* is a null mutant allele, these results provide strong evidence that *PAD4* can function without *EDS1* in Arabidopsis defense against GPA.

Previously, *SAG101* was shown to modulate *PAD4* protein levels and possess signaling activity that is partially redundant with *PAD4* in resistance to pathogens (Feys *et al.*, 2005).

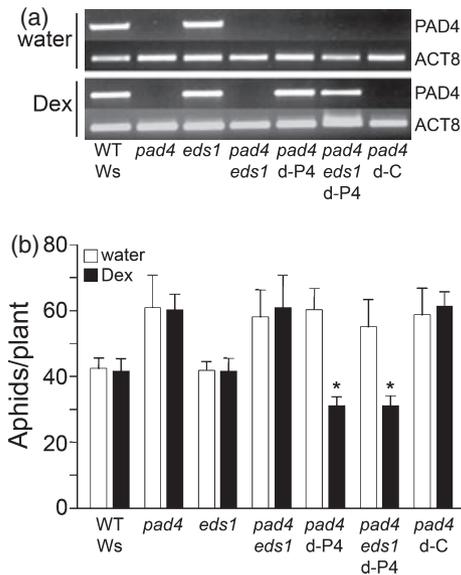


Figure 5. *EDS1* is not required for *PHYTOALEXIN DEFICIENT4* (*PAD4*)-mediated restriction of green peach aphid (GPA) infestation.

(a) Real time-polymerase chain reaction analysis of *PAD4* and *ACT8* expression in GPA-infested leaves of WT Ws-0, *pad4-5*, *eds1-1*, *pad4-5 eds1-1*, and *pad4-5 eds1-1* plants containing the Dex-inducible *PAD4* transgene d-P4, or *pad4-5* plants containing the empty vector transgene d-C. Plants were pre-treated with water or 0.05 μM dexamethasone 2 days prior to release of GPA. Leaf samples for RNA extraction were harvested 2 days after infestation of each plant with 20 GPA.

(b) Comparison of GPA numbers 2 days after infestation of plants treated as in (a). All values are the means of six plants \pm SE. Analysis of variance (ANOVA) for GPA population was conducted using PROC GLM (SAS Institute). The means were separated using Student's *t*-test ($P < 0.05$). Asterisks indicate significant differences in aphid numbers between the Dex-treated plant and its corresponding water-treated control.

A no-choice test was performed to determine whether *SAG101* also contributes to defense against GPA by comparing the numbers of GPA on WT (Col-0), *pad4-1*, *sag101*, and *pad4-1 sag101* double mutant plants at 48 hpi. As shown in Figure 4(a), GPA numbers on *sag101* were comparable to those on WT. In contrast, the numbers of GPA on *pad4-1 sag101* were significantly higher than on WT and *sag101* plants, and statistically comparable to those on *pad4-1*. *SAG101* expression was undetectable in GPA-infested WT Arabidopsis leaves (Figure 3). These results show that *SAG101*, like *EDS1*, is dispensable for the *PAD4*-dependent mechanism in resistance to GPA. Consistent with this conclusion, *SAG13* and *SAG21* transcripts accumulated to high levels in GPA-infested *sag101* mutant plants (Figure S2a), and cell death was prevalent in GPA-infested leaves of the *sag101* mutant (Figure S2b).

Discussion

We provide evidence that a *PAD4*-dependent mechanism acts within the phloem sieve elements to restrict GPA infestation of Arabidopsis plants. This *PAD4*-conditioned

resistance leads to an accelerated cell-death program (Figures 1b and S2b) (Pegadaraju *et al.*, 2005). It also limits the aphid population (Figures 1c, 4a–c and 5b), and serves as an effective deterrent to aphid feeding (Figure 2a,b and Tables 1 and 2). It is likely that these barriers to aphid feeding derive from *PAD4* activity in limiting phloem sap uptake, as monitored by EPG. This activity is a novel molecular feature of *PAD4* as it operates independently of *EDS1*, a component that is indispensable for *PAD4* function in leaves against various invasive pathogens (Wiermer *et al.*, 2005).

Despite differences in GPA performance between WT plants of Arabidopsis accessions Col-0, Ws-0 and Ler (Figure 4a–c), the role of *PAD4* in plant defense against GPA is conserved in these accessions (Figures 1c, 4a–c and 5b). Significantly, *PAD4*-modulated resistance to GPA involves both antibiosis (restricting aphid population size) and anti-xenosis (detering aphid settling and feeding). Antibiosis was evident in the no-choice assay in which the presence of *PAD4* limited the size of the GPA population (Figures 1c, 4a–c and 5b). Numbers of GPA were higher on three different *pad4* mutants than the corresponding WT plants. Conversely, GPA numbers were lower on transgenic *35S:PAD4* plants that ectopically express *PAD4* from the *35S* promoter (Figure 1c), as well as plants that conditionally expressed a *PAD4* transgene in response to Dex treatment (Figure 5b). A deterrent role for *PAD4* towards GPA settling on Arabidopsis was evident in the choice test. When given a choice between the *pad4-1* mutant and WT, adult insects preferred *pad4-1* (Figure 2a). Insects also preferred the WT plant over the *35S:PAD4* transgenic plant (Figure 2b), consistent with *PAD4* determining the extent of anti-xenosis.

Previous comparative analyses of aphid feeding behavior between resistant and susceptible plants using the EPG technique have allowed the elucidation of host mechanisms and identification of specific tissues that are important in resistance to aphids (Bernays and Funk, 2000; Kaloshian *et al.*, 2000; Klingler *et al.*, 2005; Prado and Tjallingii, 1994, 1997, 1999; Reese *et al.*, 1994; Zehnder *et al.*, 2001). Leaf surface features, cell-wall properties and composition of intercellular spaces could affect GPA performance on a plant. These factors should influence the time to first probe, and the total duration of time taken by the aphid to reach a sieve element. No significant differences were observed between the time to first probe, total duration of pathway phase, and time to reach first SEP between WT (Col-0) and *pad4-1* mutants (Table 1), or between WT (Ws-0) and *35S:PAD4* plants (Table 2). Based on this observation, we conclude that *PAD4* does not significantly alter surface features or cell-wall properties that could contribute towards resistance to GPA. As GPA spent a longer time in SEP on the *pad4-1* mutant compared to WT, and a shorter time in SEP on the *35S:PAD4* plant, we propose instead that a *PAD4*-dependent resistance factor or mechanism operates in the phloem.

The PAD4-dependent resistance could be derived from PAD4 protein expressed within the phloem sieve element or companion cells. Alternatively, PAD4 activation in other tissues by aphids could stimulate a phloem-specific program. Previously, *PAD4* was found to be necessary for transmission of mobile signals leading to cell death in the Arabidopsis *Isd1* (*lesions simulating death1*) mutant (Rust rucci et al., 2001). Also, activation of systemic acquired resistance against pathogens, which is dependent on the translocation of a factor(s) primarily through the phloem (Guedes et al., 1980; Kiefer and Slusarenko, 2003; Ross, 1966), requires functional *PAD4* (Wiermer et al., 2005; L. Jorda, A. Maldonado, C. Lamb and J. E. Parker, Max-Planck Institute for Breeding Research, unpublished data). However, the involvement of *PAD4* in long-distance signaling associated with systemic acquired resistance is part of an *EDS1*-regulated pathway (Rust rucci et al., 2001), arguing against it contributing to the resistance in the phloem described here.

We propose that a PAD4-dependent phloem-specific factor limits the total duration of phloem sap ingestion and hence the amount of sap consumed by GPA. During the phloem phase, the insect ingests sap and/or secretes saliva into the sieve element (Prado and Tjallingii, 1994; Tjallingii, 2006). Reduced phloem sap ingestion has been found to be associated with host resistance in several other aphid–plant interactions (Chen et al., 1997; van Helden and Tjallingii, 1993; Kaloshian et al., 2000; Klingler et al., 2005; Mesfin et al., 1992; Paul et al., 1996). Several processes operating individually or in concert could limit phloem sap ingestion by aphids. For example, allelochemicals present in the phloem might limit aphid feeding. *PAD4* has been shown to influence the synthesis of antimicrobial indole and phenolic compounds and other as yet structurally uncharacterized signal intermediates in Arabidopsis responses to pathogens (Bartsch et al., 2006; Glazebrook et al., 1997; Jirage et al., 1999; Zhou et al., 1998). The possibility that *PAD4* modulates the synthesis of phloem-located compounds that are directly detrimental to GPA cannot be excluded. Changes in the source–sink relationship in response to GPA feeding could also affect phloem sap composition to limit GPA feeding. Alternatively, protein coagulation at aphid feeding sites might result in the plugging of sieve elements, thereby preventing feeding (Kehr, 2006; Will and van Bel, 2006). In Arabidopsis leaves exposed to pathogens or photo-oxidative stress, *PAD4* is needed to transduce reactive oxygen species-generated signals leading to cell death (Mateo et al., 2004; Rust rucci et al., 2001). It may be that *PAD4* alters the reactive oxygen species composition of the sieve elements. Protein coagulation reactions in sieve elements are sensitive to increases in oxygen concentration (Alosi et al., 1988; Kehr, 2006). Thus, any increase in the level of reactive oxygen species in the sieve elements is likely to

induce protein clogging. Whatever the precise biochemical processes involved, *PAD4*-dependent mechanisms operating in the phloem in response to GPA feeding are effective in limiting aphid infestation.

Genetic and molecular data point to *EDS1* as the central molecule in a defense pathway against invasive pathogens that requires the partially redundant signaling functions of *PAD4* and *SAG101* (Feys et al., 2001, 2005; Lipka et al., 2005). *EDS1* mRNA was also induced with similar kinetics to *PAD4* in response to GPA infestation (Figure 3). Our finding that *EDS1* and *SAG101* are dispensable for *PAD4*-conditioned resistance to GPA in the phloem (Figures 4a–c and 5b) was therefore unexpected. Previously, it was shown that *PAD4* protein is severely depleted in *eds1* mutant leaf extracts, and that all of the detectable *PAD4* pool is associated with *EDS1* protein (Feys et al., 2005). *PAD4* protein may not require *EDS1* for its function in aphid resistance in the phloem sieve elements or associated cells, and it will be important to establish whether *PAD4* accumulates in cells associated with the phloem even in the absence of *EDS1*. Alternatively, the small amount of residual *PAD4* protein that was observed in leaf extracts of *eds1* mutant plants (Feys et al., 2005) may be sufficient and competent to exert full *PAD4* function in aphid resistance. The dispensability of *SAG101* in *PAD4*-conditioned resistance to GPA (Figure 4a) points to different molecular attributes of *PAD4* in defense signal relay, as *pad4 sag101* mutants are acutely compromised in resistance to host- and non-host-adapted pathogens (Feys et al., 2005; Lipka et al., 2005).

In conclusion, our results reveal a novel function of *PAD4* in phloem-based defense against GPA. The identification of *PAD4* as a key modulator of plant aphid resistance will aid the characterization of defense mechanisms that target sap-sucking insects.

Experimental procedures

Plant cultivation and aphid propagation

Plants and insects were cultivated at 22°C in growth chambers programmed for a 12 h light (100 $\mu\text{E m}^{-2} \text{sec}^{-1}$) and 12 h dark cycle as previously described (Pegadaraju et al., 2005). A GPA colony was propagated on a 50:50 mixture of commercially available radish (Early Scarlet Globe) and mustard (Florida Broadleaf) plants. Voucher specimens of GPA (#194) were deposited in the Kansas State University Museum of Entomological and Prairie Arthropod Research. Four-week-old soil-grown Arabidopsis plants at the rosette stage were used for all studies. All experiments were performed at least three times with similar results.

Arabidopsis mutants and transgenic lines

The *pad4-1*, *sag101-1* and *sag101-1 pad4-1* mutants and an *eds1* RNAi line in Arabidopsis accession Col-0 (Feys et al., 2005), the *pad4-2* and *eds1-2* mutants in accession Ler, and the *pad4-5* T-DNA insertion, *eds1-1* and *pad4-5 eds1-1* mutants in accession

Ws-0 have been described previously (Feys *et al.*, 2001, 2005; Glazebrook *et al.*, 1997). Multiple independent transgenic lines in the *pad4-5* background were selected that express a single copy of a *PAD4* transgene under the control of the CaMV 35S promoter with a C-terminal StreptII tag in the pAMPAT binary vector (Witte *et al.*, 2004). *35S:PAD4* lines 1 and 2 used in this study fully complemented the *pad4-5* loss of resistance to *Pseudomonas syringae* bacteria and to the oomycete pathogen *Hyaloperonospora parasitica* (data not shown). *pad4-5* transgenic lines expressing *PAD4* fused to an N-terminal c-Myc₅ epitope tag under the control of a dexamethasone-inducible promoter were created using the pTA7001 vector (Aoyama and Chua, 1997). One transgenic line (denoted d-P4) that exhibited no detectable basal but strong Dex-inducible expression of *PAD4*, and a control line expressing an empty pTA7001 vector (denoted d-C), were selected for analysis. Line d-P4 in *pad4-5* was crossed with *pad4-5 eds1-1*, and the double mutant expressing homozygous Dex:*PAD4* was selected using gene-specific primers.

No-choice and choice tests

No-choice tests were performed as previously described (Pegadaraju *et al.*, 2005). The numbers of aphids were counted 2 days after release of mature apterous (wingless) insects on each plant.

For the choice test, 20 adult apterous aphids were released on the soil in the center of a 15 cm wide pot containing one WT and one *pad4-1* mutant plant, or one WT and one *35S:PAD4* plant, approximately 4 cm from the two plants. The numbers of adult GPA on each plant were counted at various time points after release of the insects. GPA numbers on a minimum of four plants of each genotype were analyzed for each time point.

Dexamethasone treatment

Dexamethasone (Sigma-Aldrich, <http://www.sigmaaldrich.com/>) was dissolved in 100% ethanol to make a 30 mM stock, which was further diluted in water to give a 0.05 μ M solution. Four-week-old plants were sprayed to run-off with the 0.05 μ M Dex solution, or with water as control, and covered with a transparent plastic dome. Two days later, the plastic dome was removed, and 20 insects were released on each plant for no-choice assays. RNA for RT-PCR analysis was harvested 2 days after the release of insects.

Histochemistry and microscopy

Trypan blue staining of leaves was performed as previously described (Rate *et al.*, 1999).

RNA analysis

Gene-specific PCR primers for *ACT8* (At1 g49240), *SAG13* (At2 g29350) and *SAG21* (At4 g02380) were as previously described (Pegadaraju *et al.*, 2005). Primers PAD4-F (5'-ACCGAGGAACATCA-GAGGTAC-3') and PAD4-R (5'-AAATTCGCAATGTCGAGTGGC-3'), EDS1-F (5'-CACCAGATCATGGTCAGCCTC-3') and EDS1-R (5'-TTT-TGGGAAGCGTAATCCACC-3'), and SAG101-F (5'-AAGTTCTG-CATTGGGAAGC-3') and SAG101-R (5'-GAGAATGATGGGTTG-TTCTCGG-3') were used for PCR amplification of *PAD4* (At3 g52430), *EDS1* (At3 g48090) and *SAG101* (At5 g14930), respectively. RNA for RT-PCR analysis was extracted from Arabidopsis leaves as previously described (Pegadaraju *et al.*, 2005). A two-step RT-PCR was

performed on these samples. Total RNA (2 μ g) was mixed with oligo(dT) primers (Promega, <http://www.promega.com/>), and the volume was made up to a total of 15 μ l with water. This mixture was incubated at 70°C for 5 min, and then chilled on ice for 2-3 min. Then, 5 μ l of M-MLV RT buffer (Promega), 1.25 μ l dNTP mix (10nM each), 1 μ l M-MLV reverse transcriptase (Promega) and 2.75 μ l of water were added to the above mix, and cDNA synthesis allowed to proceed at 37°C for 1 h. Aliquots (1 μ l) of this cDNA were used in the subsequent PCR. PCR conditions were as follows: 95°C for 5 min, followed by 25 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 7 min.

Electrical penetration graph

The EPG technique (Reese *et al.*, 2000; Tjallingii, 1988; Walker, 2000) was used to study the feeding behavior of aphids. Drops that occur during the EPG analysis allow monitoring of stylet activities such as non-probing (when the stylet is penetrating plant tissue in a largely intercellular manner), the SEP (when the stylet is located in a sieve element) and the xylem phase (when the stylet is located in the xylem element). A thin gold wire (2-4 cm long, 10 μ m diameter) was glued to the dorsum of the aphid using conductive paint (colloidal silver, Ted Pella Inc.; <http://www.tedpella.com/>), and the other end of the wire was connected to the EPG probe. An output wire from the EPG monitor was inserted into the soil in which the plant was rooted. All plants and insects were held inside a Faraday cage during the recording at an ambient temperature of 22°C. The feeding behavior of individual aphids was monitored for 8 h. A four-channel GIGA-8 direct current amplifier (Wageningen Agricultural University, Wageningen, The Netherlands) was used for simultaneous recording from four individual aphids on four plants (two channels for the *pad4-1* or *35S:PAD4* plant and two for the WT plant). Twenty replications were performed, and waveform recordings obtained were analyzed using the EPG analysis software PROBE 3.0 (W.F. Tjallingii, Wageningen University, Wageningen, The Netherlands). The mean time spent by aphids on various activities was analyzed by the non-parametric Kruskal-Wallis' test ($P < 0.05$) (SAS/STAT Software; <http://www.sas.com/>).

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. EPG waveforms of GPA feeding on a wild-type Arabidopsis plant.

Figure S2. *EDS1* and *SAG101* are not required for the activation of *SAG* expression and cell death in GPA-infested plants.

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