Premature Leaf Senescence Modulated by the Arabidopsis *PHYTOALEXIN DEFICIENT4* Gene Is Associated with Defense against the Phloem-Feeding Green Peach Aphid[1][W]

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Aphids, which are phloem-feeding insects, cause extensive loss of plant productivity and are vectors of plant viruses. Aphid feeding causes changes in resource allocation in the host, resulting in an increase in flow of nutrients to the insect-infested tissue. We hypothesized that leaf senescence, which is involved in the programmed degradation of cellular components and the export of nutrients out of the senescing leaf, could be utilized by plants to limit aphid growth. Using Arabidopsis (*Arabidopsis thaliana*) and green peach aphid (GPA; *Myzus persicae* Sulzer), we found that GPA feeding induced premature chlorosis and cell death, and increased the expression of *SENESCENCE ASSOCIATED GENES* (*SAG*s), all hallmarks of leaf senescence. Hypersenescence was accompanied by enhanced resistance against GPA in the Arabidopsis *constitutive expresser of PR genes5* and *suppressor of SA insensitivity2* mutant plants. In contrast, resistance against GPA was compromised in the *phytoalexin deficient4* (*pad4*) mutant plant. The *PAD4* gene, which is expressed at elevated level in response to GPA feeding, modulates the GPA feeding-induced leaf senescence. In comparison to the wild-type plant, GPA feeding-induced chlorophyll loss, cell death, and *SAG* expression were delayed in the *pad4* mutant. Although *PAD4* is associated with camalexin synthesis and salicylic acid (SA) signaling, camalexin and SA signaling are not important for restricting GPA growth; growth of GPA on the camalexin-biosynthesis mutant, *pad3*, and the *SA deficient2* and NahG plants and the SA-signaling mutant, *nonexpresser of PR genes1*, were comparable to that on the wild-type plant. Our results suggest that *PAD4* modulates the activation of senescence in the aphid-infested leaves, which contributes to basal resistance to GPA.

Insect pests of plants fall into two main groups: the chewing insects and the piercing/sucking insects. The piercing/sucking insects pierce cells/tissues with stylets and consume copious amounts of fluids. While some piercing/sucking insects feed on mesophyll cells or epidermal and parenchyma cells, others are phloem feeders (Walling, 2000). Aphids represent a large group of phloem feeders that use their incredibly slender stylets to penetrate largely intercellularly between the epidermal and mesophyll cells to access the sieve elements for feeding (Pollard, 1973; Walling, 2000). However, on their way to sieve elements, the stylets will briefly puncture cells; these short punctures may result in both the injection of salivary secretions into the plant and the ingestion/sampling of minute amounts of plant material (Tjallingii, 1990). Once an aphid establishes its feeding site, it can use the same feeding site for hours to days. Two types of saliva are released by an aphid into the plant: a gelling saliva that sets and forms a protective sheath around the stylets and a watery digestive saliva containing several enzymes like peroxidases, pectinases, cellulases, lipases, and β-glucosidases that is released into the phloem sieve elements (Miles, 1999). Aphid feeding limits plant productivity (Dixon, 1998). In addition, aphids are vectors for several economically important plant viruses (Matthews, 1991). While some aphids have a narrow host range, the green peach aphid (GPA; *Myzus persicae* Sulzer) has a wide host range covering greater than 50 families of plants (Blackman and Eastop, 2000). Moreover, GPA is the vector for more than 100 plant viruses (Kennedy et al., 1962).

Our knowledge of plant defense against insects is based largely on studies involving chewing insects. Far less is known about plant defense mechanisms against aphids and other piercing/sucking insects (Walling, 2000). Due to their feeding behavior, unlike the chewing insects, aphids do not cause extensive wounding to the plant host, suggesting that plant

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response to phloem-feeding insects may differ from that to the chewing insects (Walling, 2000). A few studies have identified plant genes associated with defense against aphids. In tomato (*Lycopersicon esculentum*), the *Mi1.2* gene, which encodes a nucleotide-binding site, Leu-rich repeat protein, mediates gene-for-gene resistance against certain biotypes of the potato (*Solanum tuberosum*) aphid *Macrosiphum euphorbiae* (Rossi et al., 1998; Vos et al., 1998). Similarly, the apple Sd_1 gene confers resistance to two biotypes but not a third biotype of the rosy leaf-curling aphid, *Dysaphis devecta* (Roche et al., 1997), while the lettuce (*Lactuca sativa*) *Nr* gene confers resistance to a single aphid species, *Nasonovia ribisnigri* (van Helden et al., 1993). Comparable to the function of *Resistance* genes in plant response to pathogen infection (Bent, 1996; Hammond-Kosack and Jones, 1996), interaction of aphid-generated or -derived signal with a *Resistance* gene-encoded protein may presumably activate a signal transduction pathway(s) that confers expression of an appropriate defense response(s) against the aphid. Expression of the salicylic acid (SA)-inducible *Pathogenesis-Related* genes, which are involved in plant defense against pathogens (Dempsey et al., 1999; Shah and Klessig, 1999), are also induced by aphid feeding (Fidantsef et al., 1999; Moran and Thompson, 2001; Moran et al., 2002; Zhu-Salzman et al., 2004). In addition, expression of the *SA DEFICIENT2* (*SID2*) gene, which encodes an enzyme involved in SA biosynthesis, the *ENHANCED DISEASE SUSCEPTIBILITY5* (*EDS5*) gene, which is required for SA biosynthesis, and the *PHYTOALEXIN DEFICIENT4* (*PAD4*) gene, which modulates SA signaling, are induced in Arabidopsis (*Arabidopsis thaliana*) in response to GPA feeding (V. Pegadaraju and J. Shah, unpublished data). However, loss-of-function mutations in the *EDS5* gene and the Arabidopsis *NONEXPRESSER OF PR GENES1* (*NPR1*) gene, which is required for SA signaling, do not compromise resistance to GPA (Moran and Thompson, 2001), suggesting that SA accumulation and signaling may not have an important role in Arabidopsis defense against GPA. Recent studies suggest a role for oxylipin signaling in plant defense against aphids. For example, greenbug feeding transiently induced the expression of jasmonic acid (JA)-induced genes in sorghum (*Sorghum bicolor*; Zhu-Salzman et al., 2004). Similarly, the expression of JA-responsive genes was induced in Arabidopsis infested with GPA (Moran et al., 2002). Moreover, the Arabidopsis *coronatine-insensitive1* mutant, which is compromised in oxylipin signaling, supported more growth of GPA than the wild-type plant did (Ellis et al., 2002). In support of a role for JA in plant defense against aphids, the application of methyl jasmonate caused a significant reduction in greenbug infestation in sorghum (Zhu-Salzman et al., 2004).

Aphid infestation causes changes in resource allocation in the host plant; flow of nutrients to the insect-infested tissue is increased due to the creation of a strong sink in the aphid-infested organ (Mittler and Sylvester, 1961; Dixon, 1998; Girousse et al., 2005). However, flow of nutrients to the natural resource-demanding sinks, like the primary growth zones, is reduced. In fact, aphid infestation converts the uninfested natural sink tissues into source tissues (Girousse et al., 2005). From the aphid's perspective, an increase in the sink level of a tissue amounts to improved nutrient availability. Gene expression studies confirm that aphid infestation alters expression of plant genes that are potentially involved in the conversion of the feeding site into metabolic sinks. GPA feeding induced expression of the Arabidopsis *STP4* gene, which encodes a monosaccharide H^+ symporter (Moran and Thompson, 2001; Moran et al., 2002), and another gene that encodes an extracellular acidic invertase (V. Pegadaraju and J. Shah, unpublished data). STP proteins along with invertases increase the import and metabolism of carbohydrates into resource-demanding organs (Buttner et al., 2000).

Senescence is the terminal phase in leaf development that involves a programmed disassembly and degradation of cellular components (Lim et al., 2003; Thomas et al., 2003; Yoshida, 2003). The resultant products of senescence are remobilized to assimilate demanding sink organs. In Arabidopsis, leaf senescence is characterized by chlorophyll loss, elevated level of expression of the *SENESCENCE ASSOCIATED GENES* (*SAG*s), and eventually cell death (Lim et al., 2003; Yoshida, 2003). Premature senescence of the aphid-infested leaf could oppose the ability of aphids to redirect the flow of resources to the insect-infested leaves. We therefore hypothesized that leaf senescence may be utilized as a defense mechanism by plants to limit aphid growth. Here we show that GPA feeding induces premature leaf senescence in Arabidopsis. Furthermore, in comparison to the wild-type Arabidopsis plant, GPA growth is lower on mutants that exhibit hypersenescence. In contrast, a delay in the activation of GPA feeding-induced leaf senescence is accompanied by increased growth of GPA in the *pad4* mutant plant. These data implicate a role for *PAD4*-modulated leaf senescence in basal resistance to GPA.

RESULTS

GPA Feeding Activates Leaf Senescence in Arabidopsis

We first examined whether GPA feeding results in chlorophyll loss and cell death, two features of leaf senescence in Arabidopsis. In comparison to leaves from uninfested plants (Supplemental Fig. 1), as shown in Figure 1A, GPA feeding resulted in leaf chlorosis in wild-type Arabidopsis. In comparison to the uninfested wild-type plants, by 2 d postinfestation (dpi) GPA-infested leaves from the wild-type plant had lost 40% of their chlorophyll (Fig. 1B). In addition, microscopy of trypan blue-stained leaves revealed the presence of dead cells in the GPA-infested leaves from wild-type plants at 2 dpi (Fig. 1C). Senescence requires the de novo expression of genes (Gan and Amasino,

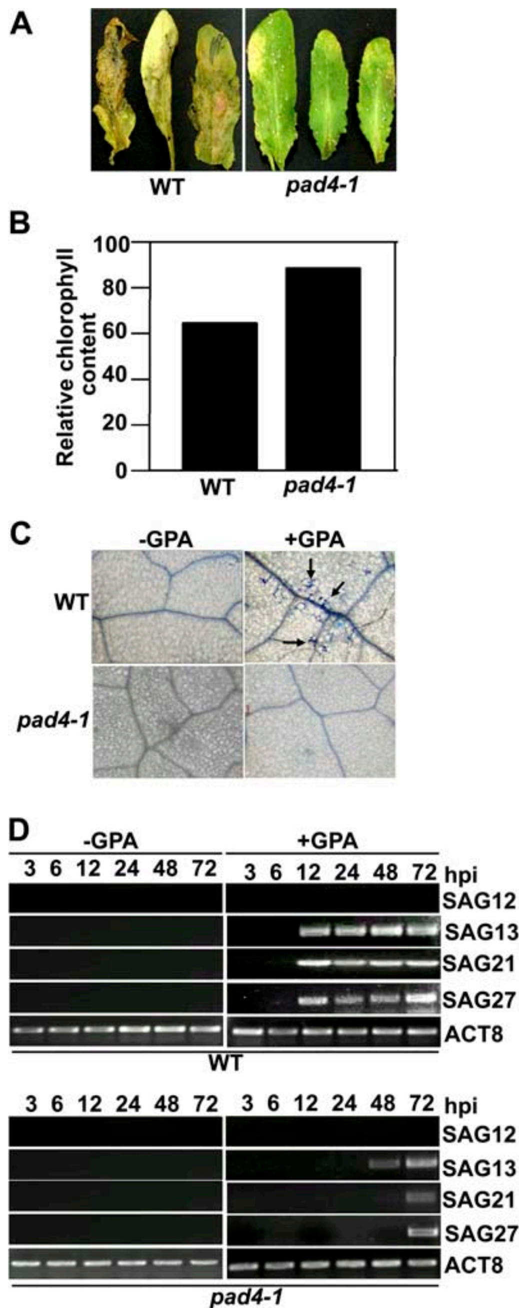

Figure 1. GPA feeding activates leaf senescence that is modulated by PAD4. A, Photograph of wild-type (WT) and *pad4-1* leaves, 7 d after release of 15 GPA per plant. B, Relative chlorophyll content in GPA-infested leaves of wild-type and *pad4-1* plants, 2 d after release of 15 insects per plant. The chlorophyll values in the GPA-infested wild-type and *pad4-1* plants are relative to that in the corresponding uninfested wild-type and *pad4-1* plants, which were assigned a value of 100. This experiment was done twice with similar results. C, Trypan blue staining of leaves from uninfested wild-type and *pad4-1* plants and from GPA-infested wild-type and *pad4-1* plants, 2 d after release of insects. The arrows point to the intensely stained dead cells. D, RT-PCR analysis of *SAG12*, *SAG13*, *SAG21*, *SAG27*, and *ACT8* expression in leaves from uninfested wild-type and *pad4-1* plants and leaves from GPA-infested wild-type and *pad4-1* plants, 3, 6, 12, 24, 48, and 72 hpi by GPA. *ACT8* expression provided a control for RT-PCR.

1997; Lim et al., 2003; Yoshida, 2003). To confirm that the chlorophyll loss and cell death observed in GPA-infested leaves is part of a plant response to aphid feeding and not a consequence of cell damage caused by insect probing/feeding, we examined expression of the *SAG* genes in the GPA-infested leaves. Expression of the *SAG13*, *SAG21*, and *SAG27* genes was induced as early as 12 h postinfestation (hpi) in GPA-infested leaves from wild-type plants (Fig. 1D). Elevated expression of the *SAG12* gene correlates with the manifestation of age-dependent senescence but not senescence induced by environmental stressors (Gan and Amasino, 1997; Lim et al., 2003; Yoshida, 2003). *SAG12* expression is first evident when the senescing organs undergo chlorosis (Weaver et al., 1998). However, in the GPA-infested leaves from the wild-type plant, *SAG12* expression was undetectable even at 72 hpi (Fig. 1D), although chlorophyll loss was evident by 48 hpi (Fig. 1B). We therefore suggest that the senescence phenomenon observed in GPA-infested leaves of wild-type plants may differ from age-dependent senescence.

Arabidopsis Hypersenescence Mutants Display Heightened Resistance to GPA

If a senescence-associated process influences Arabidopsis defense against GPA, then we expect that hypersenescence will be coupled with enhanced resistance against GPA. The Arabidopsis *constitutive expresser of PR genes5* (*cpr5*)/*hypersenescence1* mutant plant exhibits a hypersenescent phenotype; in comparison to the wild-type plant, the mutant plant has a lower content of chlorophyll, spontaneously undergoes cell death, and exhibits elevated basal levels of *SAG* gene expression (Fig. 2A; Bowling et al., 1997; Yoshida et al., 2002). We monitored aphid performance on the *cpr5* mutant plant. In comparison to the wild-type plant, GPA counts were lower on the *cpr5* mutant (Fig. 2B). Similarly, in comparison to the wild-type plant, GPA growth was reduced in the Arabidopsis *suppressor of SA insensitivity2* (*ssi2*) mutant (Fig. 2C), which like *cpr5* contains high basal levels of the *SAG13*, *SAG21*, and *SAG27* transcripts (Fig. 2A) and exhibits spontaneous cell death (Shah et al., 2001). The *cpr5* and *ssi2* mutants are dwarfs and accumulate high levels of SA (Bowling et al., 1997; Shah et al., 2001), an important signaling molecule in plant defense to pathogens. However, GPA growth was comparable on the *ssi2* single mutant and the SA-deficient *ssi2 nahG* plant (Fig. 2C), suggesting that a high level of SA is not important for the *ssi2*-conferred resistance to GPA. Aphid growth was also comparable between the wild-type plant and the Arabidopsis SA-deficient *nahG* (Fig. 2C) and *sid2*, and the SA-insensitive *npr1* mutant plants (Fig. 3A; Moran and Thompson, 2001). In addition, GPA counts on the *suppressor of npr1-1*, *constitutive 1* (*snc1*) mutant, which accumulates high levels of SA and is a dwarf (Zhang et al., 2003) like the *cpr5* and *ssi2* mutants, were comparable to those on the wild-type plant (Fig. 3B). Moreover, unlike the *cpr5*, *ssi2*, and *ssi2*

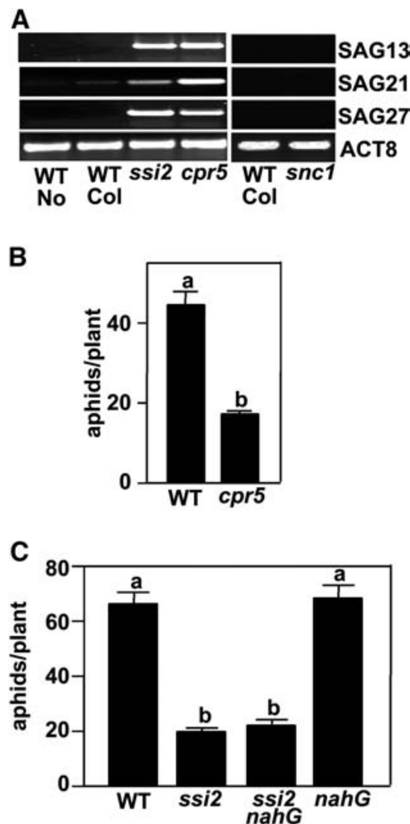

Figure 2. Hypersenescence is accompanied by enhanced resistance to GPA in the *cpr5* and *ssi2* mutant plants. A, Left section: RT-PCR analysis of *SAG13*, *SAG21*, *SAG27*, and *ACT8* expression in leaves of uninfested ecotype Nössen (WT No) and Columbia (WT Col) plants, and the *ssi2* and *cpr5* mutant plants. Right section: RT-PCR analysis of *SAG13*, *SAG21*, *SAG27*, and *ACT8* expression in leaves of uninfested wild-type Col and the *snc1* mutant plants. B, Comparison of GPA growth on the wild-type (ecotype Columbia) and *cpr5* mutant plants, 2 d after release of 15 insects per plant. C, Comparison of GPA growth on the wild-type (ecotype Nössen), and the *ssi2*, *ssi2 nahG*, and *nahG* plants, 2 d after release of 15 insects per plant. These plants are in the ecotype Nössen background. In B and C, all values are the mean of 15 plants ±SE. Different letters above the bars indicate values that are different from each other with a confidence of 95% or greater with Student's *t* test.

nahG plants, the *SAG13*, *SAG21*, and *SAG27* genes were not expressed constitutively in the *snc1* mutant (Fig. 2A; data not shown), thus supporting the hypothesis that a senescence-associated process, but not SA or dwarfing, is linked with basal resistance to the GPA.

PAD4 Modulates the GPA Feeding-Induced Leaf Senescence and Basal Resistance to GPA

In contrast to the hypersenescence mutants, *cpr5* and *ssi2*, the GPA feeding-induced senescence is delayed in the Arabidopsis *pad4-1* mutant plant. The GPA-infested *pad4-1* mutant plant stayed green for longer than the GPA-infested wild-type plant (Fig. 1A). Measurements on chlorophyll content confirmed that, in comparison to the wild-type plants, the GPA feeding-induced chlorophyll loss was compromised in the *pad4-1* mutant plant (Fig. 1B). Furthermore, the GPA feeding-induced expression of *SAG13*, *SAG21*, and *SAG27* genes was delayed in the *pad4-1* mutant (Fig. 1D). Unlike in the wild-type leaves, microscopic cell death was also not evident in the GPA-infested *pad4-1* leaves at 2 dpi (Fig. 1C). In agreement with the involvement of *PAD4* in Arabidopsis response to GPA, *PAD4* expression was activated in the GPA-infested wild-type plants as early as 3 hpi and was maintained at high levels through 48 hpi (Fig. 4A). These results suggest that *PAD4* modulates the activation of the GPA feeding-induced leaf senescence process in Arabidopsis.

To determine if the delay in activation of the GPA feeding-induced leaf senescence in *pad4-1* impacts the mutant plant's ability to control aphid growth, we compared GPA growth between the wild type and the *pad4-1* mutant plant. In a no-choice test, 2 dpi, aphid count was higher on the *pad4-1* mutant than on the wild-type plant (Fig. 4B). Similarly, in comparison to the wild-type plant, GPA count was higher on a transgenic plant (*pad4*Δ) that contained a T-DNA insertion within the *PAD4* gene (Fig. 4B), suggesting an important role for *PAD4* in Arabidopsis defense against the GPA. Consistent with the enhanced susceptibility of the *pad4-1* mutant to GPA, aphid-infested *pad4-1* plants produced 65% less seed than the aphid-infested wild-type plants (Fig. 4C). Seed yield from the uninfested *pad4-1* mutant plant was comparable to that from the

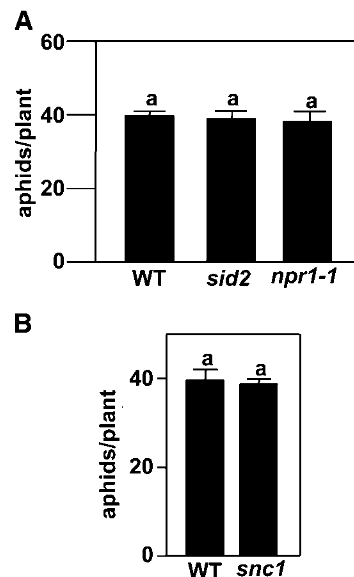

Figure 3. SA does not have an important role in basal resistance to GPA. A, Comparison of GPA numbers on wild type and the SA-deficient *sid2* and the SA-insensitive *npr1-1* mutant plants, 2 d after release of 15 insects per plant. B, Comparison of GPA numbers on wild type and the SA-hyperaccumulating *snc1* mutant plant, 2 d after release of 15 insects per plant. All values are the mean of 15 plants ±SE. Different letters above the bars indicate values that are different from each other with a confidence of 95% or greater with Student's *t* test.

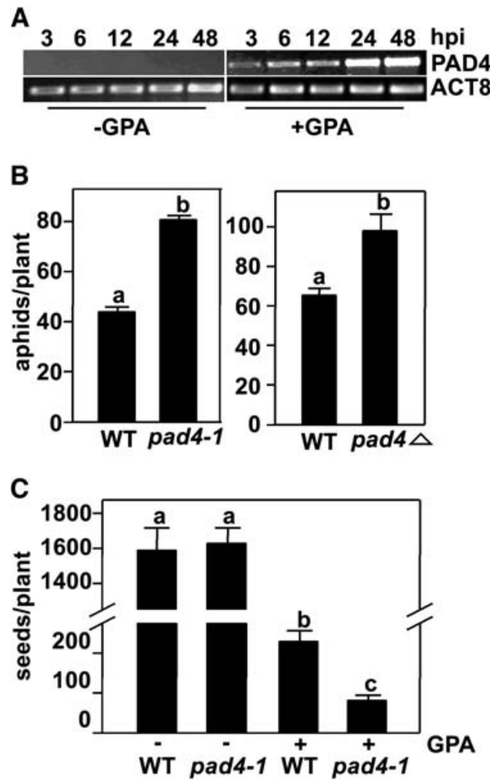

Figure 4. *PAD4* is involved in Arabidopsis defense against GPA. A, RT-PCR analysis of *PAD4* and *ACT8* expression in GPA-infested Arabidopsis leaves. RT-PCR was performed on RNA extracted 3, 6, 12, 24, and 48 hpi. RNA extracted from uninfested plants provided a negative control. *ACT8* expression provided a control for RT-PCR. B, Comparison of GPA numbers on wild type and *pad4-1* mutant (left section), and wild type and a transgenic line (*pad4*Δ) that contains a T-DNA insertion within the *PAD4* gene (right section), 2 d after release of 15 insects per plant. All values are the mean of aphid counts on 15 plants ±SE. C, Seed yield from uninfested and GPA-infested wild-type and *pad4-1* mutant plants. All values are the mean of seed yield from 10 plants ±SE. Different letters above the bars indicate values that are different from each other with a confidence of 95% or greater with Student's *t* test. This experiment was done twice with similar results.

uninfested wild-type plant, suggesting that the loss of *PAD4* affects seed yield only in aphid-infested plants.

The *PAD4* gene modulates SA signaling and synthesis of camalexin, an antimicrobial phytoalexin (Tsuji et al., 1992; Rogers et al., 1996; Zhou et al., 1998; Jirage et al.,1999). However, as shown above, SA accumulation and signaling are not critical for basal resistance to GPA. The *PAD3* gene encodes a cytochrome P450 monooxygenase, a key enzyme in camalexin biosynthesis (Zhou et al., 1999). Like *PAD4*, expression of the *PAD3* gene is induced in Arabidopsis leaves in response to GPA feeding (V. Pegadaraju and J. Shah, unpublished data). To ascertain if camalexin has a role in basal resistance to GPA, we compared aphid counts between the *pad3-1* mutant and wild-type plant. A comparable number of aphids was present on the *pad3-1* mutant and wild-type plant (Fig. 5), suggesting that camalexin is not important for basal resistance to GPA. Hence, the involvement of *PAD4* in Arabidopsis defense against GPA is most likely independent of its role in SA signaling and camalexin biosynthesis.

DISCUSSION

Our results support the hypothesis that premature leaf senescence in Arabidopsis contributes to basal resistance against GPA. We have shown that in Arabidopsis leaves GPA feeding results in chlorophyll loss; elevated expression of the *SAG13*, *SAG21*, and *SAG27* genes; and cell death (Fig. 1, A–D), all hallmarks of senescence. Hypersenescence in the *cpr5* and *ssi2* mutant plants was accompanied by enhanced resistance against GPA (Fig. 2, A–C). Furthermore, a delay in the activation of GPA feeding-induced *SAG* gene expression, chlorosis, and cell death in the *pad4* mutant plant (Fig. 1, B–D) was associated with an enhanced susceptibility to GPA (Fig. 4B). Our results suggest that the senescence induced in Arabidopsis leaves in response to GPA feeding may differ from age-dependent leaf senescence because *SAG12* expression, which is tightly associated with age-dependent senescence, was not induced in aphid-infested leaves (Fig. 1D). Similar differences from age-dependent leaf senescence have been observed in ozone-treated Arabidopsis. Ozone-induced leaf senescence was accompanied by the induction of *SAG13*, *SAG21*, and *SAG27* expression, but not *SAG12* (Miller et al., 1999). Likewise, the spontaneous cell death phenotype in the Arabidopsis *acd11* mutant was accompanied by constitutive high-level expression of the *SAG13* gene but not the *SAG12* gene (Brodersen et al., 2002). A recent study compared gene expression changes in Arabidopsis in response to a variety of biotic stressors, including GPA (De Vos et al., 2005). Evaluation of microarray data that accompanied this paper indicated that expression of *SAG21* and *SEN1*, another senescence marker, were induced in response to GPA feeding. Furthermore, similar to our observations, *SAG12* expression was not induced in response to GPA feeding in the microarray experiments accompanying the study by De Vos et al. (2005). However, unlike our observations, *SAG13* gene

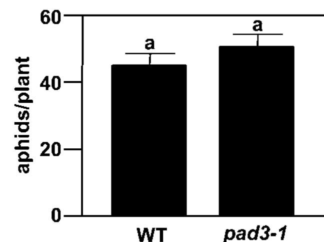

Figure 5. Camalexin is not required for basal resistance to GPA. Comparison of GPA numbers on wild type and the camalexin-deficient *pad3* mutant, 2 d after release of 15 insects per plant. All values are the mean of 15 plants ±SE. Different letters above the bars indicate values that are different from each other with a confidence of 95% or greater with Student's *t* test.

was not induced in their experiments. Ecotype differences cannot account for this disparity, since both our study and that of De Vos et al. (2005) utilized the Arabidopsis ecotype Columbia. Further experiments are needed to determine if these differences are due to differences in the biotypes of GPA used between this study and that of De Vos et al. (2005).

Flowers and developing seeds are natural sinks. In comparison to the uninfested wild-type plants, GPA feeding reduced seed set in the GPA-infested plants (Fig. 4C), confirming observations made by others (Mittler and Sylvester, 1961; Dixon, 1998; Girousse et al., 2005) that aphid feeding alters plant source-sink relationships. However, in comparison to the GPA-infested wild-type plant, seed set in GPA-infested *pad4* mutant plant was further reduced (Fig. 4C), suggesting that the wild-type plant has countermeasures to limit the ability of the aphid to alter source-sink relationships. Our results suggest that PAD4 is a component of this counter mechanism. The influence of senescence-associated processes on aphid growth has also been observed in other plants. For example, premature senescence induced by a gall aphid was shown to correlate with the reduced performance of another aphid feeding on the same leaflet of *Pistacia palaestina* trees (Inbar et al., 1995). Furthermore, in barley (*Hordeum vulgare*), resistance to Russian wheat aphid was accompanied by the activation of cell death in the resistant cultivars (Belefant-Miller et al., 1994). Similarly, premature leaf senescence also benefits plants during drought stress. Nutrient remobilization associated with drought-induced leaf senescence allows the natural sink organs like the young leaves, fruits, and flowers to benefit from the nutrients accumulated during the life span of the prematurely senescing leaf (Munné-Bosch and Alegre, 2004).

Our data suggest that *PAD4* modulates the GPA feeding-induced premature leaf senescence. Previously, the *PAD4* gene was shown to influence the manifestation of hypersensitive response-like cell death in the Arabidopsis *acd11* and *lsd1* mutants, and the *acd11*-conferred constitutive high-level expression of *SAG13* (Rustérucci et al., 2001; Brodersen et al., 2002), providing additional support for a role for *PAD4* in activation of senescence-associated processes. *PAD4* modulates camalexin synthesis and SA signaling in plant defense against pathogens (Glazebrook et al., 1997; Zhou et al., 1998; Jirage et al., 1999; Zhou et al., 1999). However, our studies of GPA performance on the camalexin biosynthesis mutant, *pad3* (Fig. 5), suggest that camalexin does not have an important role in basal resistance to GPA. An earlier study in Arabidopsis found no correlation between the activation of SA signaling and basal resistance to GPA. GPA growth was comparable between the wild-type plant and the SA-insensitive *npr1* and the SA-deficient *eds5* mutant plants (Moran and Thompson, 2001). However, both *NPR1* and *EDS5* also participate in processes that are independent of SA (Pieterse et al., 2002; Nandi et al., 2005). Our experiments with the *nahG* plant (Fig. 2C), in which SA is degraded to catechol, the SA biosynthesis mutant *sid2* (Fig. 3A), and the SA-hyperaccumulating *snc1* mutant (Fig. 3B) plants, extend Moran and Thompson's (2001) study and confirm that SA does not have an important role in basal resistance to GPA. Hence, we propose that the participation of *PAD4* in plant defense against GPA is independent of its involvement in camalexin synthesis and SA signaling. A similar association of *PAD4* in the expression of Arabidopsis genes, which is independent of PAD4s involvement in SA signaling, was observed in a microarray gene expression study (Glazebrook et al., 2003). Moreover, unlike the involvement of *PAD4* in SA signaling, which is dependent on the presence of a functional *EDS1* gene (Feys et al., 2005), *EDS1* is not important for basal resistance to GPA (V. Pegadaraju, J. Parker, and J. Shah, unpublished data), suggesting that the role of PAD4 in Arabidopsis-GPA interaction is independent of its interaction with EDS1. PAD4 protein localizes to the nucleus (Feys et al., 2005), where it may modulate the expression of genes or activity of proteins involved in the activation of this GPA-induced leaf senescence. Alternatively, since PAD4 protein can also be found in the cytosol (Feys et al., 2005), the involvement of PAD4 in Arabidopsis-GPA interaction may be due to its action in the cytosol.

Although premature senescence of aphid-infested leaves may appear as a consequence of removal of nutrients by the insect from the infested organ, our study demonstrates that *PAD4*-modulated leaf senescence in Arabidopsis contributes to defense against the generalist insect, GPA. These results have broader ramifications to agriculture because delayed senescence is one of the traits that is being selected to improve productivity in several crops (Ma and Dwyer, 1998; Ismail et al., 2000; Borrel et al., 2001; Mahalaxmi and Bidinger, 2002; Munné-Bosch and Alegre, 2004). Will delayed senescence in these crops result in lowered resistance to aphids and in parallel an increase in the spread of aphid-vectored viral diseases? Similarly, concerns have been raised about drought tolerance in plants with delayed senescence (Munné-Bosch and Alegre, 2004).

MATERIALS AND METHODS

Plant and Aphid Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were grown in soil at 22°C in a growth chamber programmed for 14-h-light (100 μE m^{-2} s^{-1}) and 10-h-dark cycle. Approximately 4-week-old Arabidopsis plants were used for all studies. A combination of commercially available radish (*Raphanus sativus*, Early scarlet globe) and mustard (*Brassica juncea* Florida broadleaf), at a 50:50 ratio, were used for the routine propagation of GPA (*Myzus persicae*) at 22°C in a growth chamber programmed for 14-h-light (100 μE m^{-2} s^{-1}) and 10-h-dark cycle. All experiments reported in this article were performed at least three times with similar results, unless noted otherwise.

Arabidopsis Mutants

The *pad3-1* (Zhou et al., 1999), *pad4-1* (Glazebrook et al., 1997), *cpr5* (Bowling et al., 1997), *snc1* (Zhang et al., 2003), *npr1-1* (Cao et al., 1994), and

sid2-2 (Wildermuth et al., 2001) mutants used in this study are in the ecotype Columbia background. The *ssi2*, *ssi2 nahG*, and *nahG* plants are in the ecotype Nössen background (Shah et al., 1999, 2001). The *pad4*Δ T-DNA insertion line (SALK_089936) that was identified from the Salk collection is in the ecotype Columbia background (http://signal.salk.edu/).

No-Choice Test

A no-choice test was used to assay aphid growth on wild-type and mutant plants. Approximately 4-week-old Arabidopsis plants were used in the bioassay with a clonally propagated GPA population. For the no-choice test each Arabidopsis plant received 15 mature apterous aphids at the center of the rosette and the plants were incubated at 22°C as described above. Two days later, the plants were harvested and number of aphids residing on each plant was counted. Student's *t* tests were performed using SigmaPlot version 5.0 (SPSS).

Histochemistry and Microscopy

Leaf samples for trypan blue staining were processed and analyzed as described previously (Rate et al., 1999).

Chlorophyll Extraction and Estimation

Leaves were ground in a mortar with a pestle in the presence of liquid nitrogen. Chlorophyll was extracted with an extraction buffer consisting of an 85:15 (v/v) mix of acetone:Tris-HCl (1 M; pH 8.0 in water). The absorbance of the extract was recorded at 664 and 647 nm against an extraction buffer control and the chlorophyll content calculated as described previously (Lichtenthaler, 1987).

DNA and RNA Analysis

DNA for the PCR analysis was extracted from leaves as described previously (Konieczny and Ausubel, 1993). A transgenic Arabidopsis line (Salk_089936), which contains a T-DNA insertion within the *PAD4* gene, was identified in the Salk collection (http://signal.salk.edu/). Multiplex-PCR analysis was performed on the segregating plant material to identify plants homozygous for the T-DNA insertion. The PAD4-F (5′-GCTCTCCTC-TGCTGGAAACC-3′), PAD4-R (5′-TTTTCTCGCCTCATCCAACCA-3′), and T-DNA left border primer (5′-GCGTGGACCGCTTGCTGCAAC-3′) were used for the multiplex PCR. PCR was performed with the following conditions: 95°C for 5 min followed by 30 cycles of 95°C for 0.5 min, 65°C for 0.5 min, and 72°C for 2 min, with final extension at 72°C for 5 min. The PCR products were resolved on 1.2% agarose gel, stained with ethidium bromide, and visualized with a BioDoc-It system (UVP).

For RNA extraction, leaf material from uninfested and GPA-infested plants was harvested and quick-frozen in liquid nitrogen. RNA was extracted by a guanidine-phenol method (Chomczynski and Sachhi, 1987). The isolated RNA was purified using the RNeasy Mini kit (Qiagen), spectrophotometrically quantified at 260 nm and subsequently used in the reverse transcription (RT)-PCR reactions. RT-PCR analysis was performed with the Superscript One-Step RT-PCR kit (Invitrogen). The RT reaction was carried out at 50°C for 30 min in a 20-μL reaction with 100 ng of the total RNA as template as recommended by the manufacturer. PCR conditions for the *ACT8* (At1g49240), *SAG12* (At5g45890), *SAG13* (At2g29350), *SAG21* (At4g02380), *SAG27* (At2g44300), and *PAD4* (At3g52430) were as follows: 95°C for 5 min followed by 25 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 1 min with a final extension at 72°C for 5 min. The ACT8-F (5′-ATGAAGATTAAG-GTCGTGGCA-3′) and ACT8-R (5′-TCCGAGTTTGAAGAGGCTAC-3′), SAG12-F (5′-TCTCGTCCACTCGACAATGAA-3′) and SAG12-R (5′-AGC-TTTCATGGCAAGACCACA-3′), SAG13-F (5′-CAAGATGGAGTCTTGGAG-GCA-3′) and SAG13-R (5′-GGAAAAAACCGTTAACAGTGGA-3′), SAG21-F (5′-CCAATGCTATCTTCCGACGTG-3′) and SAG21-R (5′-GAACCGGTTTC-GGGTCTGTAA-3′), SAG27-F (5′-TCCTGGCCCTGAAGTAGAAA-3′) and SAG27-R (5′-GTCCCGCAAGAACCTGTCC-3′), and PAD4-F (5′-GCTCTC-CTCTGCTCGGAAACC-3′) and PAD4-R (5′-TTTTCTCGCCTCATCCAA-CCA-3′) gene-specific primers were used for PCR amplification of *ACT8*, *SAG12*, *SAG13*, *SAG21*, *SAG27*, and *PAD4*, respectively.

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