# Molecular Responses to Aphid Feeding in Arabidopsis in Relation to Plant Defense Pathways[1]

**Patrick J. Moran and Gary A. Thompson[2]***

Center for Insect Science and Department of Plant Sciences, University of Arizona, Tucson, Arizona 85721

Little is known about molecular responses in plants to phloem feeding by insects. The induction of genes associated with wound and pathogen response pathways was investigated following green peach aphid (*Myzus persicae*) feeding on Arabidopsis. Aphid feeding on rosette leaves induced transcription of two genes associated with salicylic acid (SA)-dependent responses to pathogens (*PR-1* and *BGL2*) 10- and 23-fold, respectively. Induction of *PR-1* and *BGL2* mRNA was reduced in *npr1* mutant plants, which are deficient in SA signaling. Application of the SA analog benzothiadiazole led to decreases in aphid reproduction on leaves of both wild-type plants and mutant plants deficient in responsiveness to SA, suggesting that wild-type SA-dependent responses do not influence resistance to aphids. Two-fold increases occurred in mRNA levels of *PDF1.2*, which encodes defensin, a peptide involved in the jasmonate (JA)-/ethylene-dependent response pathway. Transcripts encoding JA-inducible lipoxygenase (*LOX2*) and SA/JA-inducible Phe-ammonia lyase increased 1.5- to 2-fold. *PDF1.2* and *LOX2* induction by aphids did not occur in infested leaves of the JA-resistant *coi1-1* mutant. Aphid feeding induced 10-fold increases in mRNA levels of a stress-related monosaccharide symporter gene, *STP4*. Phloem feeding on Arabidopsis leads to stimulation of response pathways associated with both pathogen infection and wounding.

Phloem-feeding insects represent a special paradox in studies of plant resistance to biotic stress. The diversity and abundance of phloem-feeding insects stand in contrast with the limited amount of information available about molecular and physiological plant responses and resistance mechanisms against these herbivores. Aphids, the largest group of phloem feeders, penetrate plant tissues by probing intercellularly through epidermal and mesophyll cell layers with their stylet-like mouthparts to feed on photoassimilates translocating in the phloem sieve elements (Pollard, 1972), inflicting considerable fitness costs in many crop plants (Dixon, 1998). Aphids secrete watery saliva containing peroxidases, β-glucosidases, and other potential signal-generating enzymes into phloem sieve elements (Miles, 1999). Still uncharacterized signals arising from phloem feeding are capable of altering the expression of inducible plant physiological factors similar to those involved in defense against pathogens (van der Westhuizen et al., 1998a, 1998b; Fidantsef et al., 1999; Inbar et al., 1999). Aphids are important vectors of viral plant pathogens (Matthews, 1991), and viral disease symptom development can strongly influence aphid feeding behavior and reproduction (Purcell and Nault, 1991). The unique interactions between aphids and their host plants suggests that phloem feeding could induce subsets of responses associated with wounding and pathogen infection, and thus bridge the gap between these sources of stress.

Changes in gene expression underlying inducible responses to pathogens are known to be complex and multifaceted (Glazebrook, 1999), and early molecular level studies of responses to herbivory and mechanical wounding suggest a similar pattern of multiple, independent, but networked defense response pathways (Titarenko et al., 1997; Ryan and Pearce, 1998; Reymond et al., 2000). Recent physiological evidence indicates that signaling, responses, and resistance to pathogens and insects partially overlap (Bi et al., 1997; Stout et al., 1998; Stout et al., 1999) but antagonism can also occur (Felton et al., 1999; Preston et al., 1999). Several interconnected signaling pathways regulate responses to attack by avirulent and virulent phytopathogens in plants, including the model plant Arabidopsis (Thomma et al., 1998; Glazebrook, 1999). Pathogenesis-related (*PR)* genes encode some of the plant proteins that break down cellular constituents of pathogens or aid in signaling. Salicylic acid (SA)-dependent responses following infection by *Pseudomonas syringae* bacteria, *Peronospora parasitica* fungi, and other pathogens include increases in the expression of *PR* genes (Glazebrook, 1999). *PR-1* and an acidic, apoplastic form of β-1,3-glucanase (*BGL2*) are good *PR* gene markers of SA-dependent induction in Arabidopsis (Uknes et al., 1992; Rogers and Ausubel, 1997), and expression is correlated to systemic acquired resistance (SAR) to further pathogen infection (Ryals et al., 1992). PR proteins do not appear to play causal roles in SAR against *P. syringae* (Rogers and

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[2] Present address: Department of Applied Science, University of Arkansas, 2801 South University Avenue, Little Rock, AR 72204–1099.

* Corresponding author; email gathompson@ualr.edu; fax 501–569–8020.

Ausubel, 1997; Clarke et al., 1998; van Wees et al., 1999), but they could have activities against other sources of biotic stress. Aphid feeding has been shown to induce localized and plant-wide increases in mRNA transcription or enzyme activity of several PR proteins, including peroxidases and chitinases (Stout et al., 1998; Fidantsef et al., 1999). In wheat, PR proteins are induced to higher levels in resistant than in susceptible genotypes (van der Westhuizen et al., 1998a, 1998b). The specific resistance mechanisms of these responses are unknown. The *NPR1* regulatory gene conditions SA responsiveness in Arabidopsis (Cao et al., 1994), raising the possibility that alteration of this gene or others involved in SAR could influence plant responses to aphid herbivory.

A jasmonic acid (JA)- and ethylene-dependent pathway is induced in Arabidopsis after infection by the fungus *Alternaria brassicicola*. This pathway leads to sustained increases in mRNAs of the low-$M_r$ antimicrobial protein defensin (*PDF1.2*) and *PR* genes encoding basic proteins (Penninckx et al., 1998; Thomma et al., 1998; Pieterse and van Loon, 1999). Infection by pathogens associated with SA-dependent responses, such as *P. syringae*, ephemerally induces these JA responses (van Wees et al., 1999). Lipoxygenases, including *LOX2* in Arabidopsis, synthesize JA and are up-regulated by leaf wounding, although some forms, such as *LOX1*, are not inducible in leaves (Bell and Mullet, 1993). Aphid feeding increases lipoxygenase mRNA levels in tomato (*Lycopersicon esculentum*; Fidantsef et al., 1999), suggesting that plant sensitivity to phloem feeding could involve jasmonate synthesis and signaling activity. Herbivory by chewing larvae leads to long-lasting increases in the activities of JA-triggered defensive enzymes and proteinase inhibitors (Ryan and Pearce, 1998; Stout et al., 1998). Recent evidence suggests that SA- and JA-dependent responses are associated with mechanical wounding and chewing herbivory in Arabidopsis (Reymond et al., 2000). Wounding can induce gene expression in mutant *coi1-1* plants that are resistant to jasmonate (McConn et al., 1997; Titarenko et al., 1997). These findings suggest that even the extremely localized cellular trauma associated with aphid stylets could induce a profile of responses associated with the penetration, salivation, and ingestion processes of phloem feeding.

Transcripts encoding Phe-ammonia lyase (*PAL1*), a key enzyme in the biosynthesis of phenolics, and a monosaccharide $H^+$ symporter (*STP4*), which helps mobilize carbohydrates to wounding and infection sites, are inducible by both wounding and pathogen infection in Arabidopsis (Davis et al., 1991; Truernit et al., 1996; McConn et al., 1997). Increases in phenolic biosynthesis gene expression or enzyme activity, and accumulation of the products of these enzymes, are commonly associated with JA treatment or herbivory in many plants (Berger et al., 1996; Karban and Baldwin, 1997). The ability of aphid feeding to induce *PAL1* and *STP4* is unknown, but are involved phenolics in the formation of salivary sheaths around penetration sites (Miles, 1999) and nutrient sink formation often occurs (Dixon, 1998).

This study demonstrates induction of components of multiple plant response pathways by green peach aphids (*Myzus persicae*) feeding on leaves of Arabidopsis. The expression of *PR* genes, jasmonate-responsive genes, and more broadly sensitive genes increased to varying degrees in response to infestation. *PR* gene and lipoxygenase and defensin gene expression were compromised in the SA-resistant *npr1* and JA-resistant *coi1-1* mutants of Arabidopsis, respectively. The potential for strong *PR* gene induction to alter resistance to aphids was evaluated by measuring aphid reproduction on plants treated with the SA analog benzothiadiazole (BTH; Lawton et al., 1996). These studies used *npr1* plants, and also *eds5* and *eds9* mutant plants that exhibit reduced SAR similar to *npr1*, but show normal or near-normal increases in expression of *PR* genes after infection (Rogers and Ausubel, 1997). The results suggest similar response profiles but different inducible resistance paradigms for plant pathogens and phloem-feeding insects on Arabidopsis.

## RESULTS

### Pathogen and Wound Response Gene Induction following Aphid Infestation

*PR* genes associated with the SA-dependent response pathway were induced within 24 to 48 h after infestation by green peach aphids (Fig. 1A). Figure 1 shows hybridization results for representative lanes from RNA blots. Figure 2 shows estimates of the magnitude of gene induction in infested leaves across multiple replicate lanes and several independent experiments. *PR-1* transcripts were 6- to 10-fold higher in infested plants compared with control plants by 72 h (Fig. 2A). *BGL2* mRNA increased continuously and was approximately 23-fold higher in infested leaves after 96 h of feeding (Fig. 2A). The temporal and quantitative patterns of expression of *PR-1* and *BGL2* in response to green peach aphids on infested leaves were consistent with published data involving infection of Arabidopsis with virulent *P. syringae* bacteria or treatment with BTH (Uknes et al., 1992; Lawton et al., 1996). In contrast with pathogen or elicitor treatment, up-regulation of these genes by aphids was not systemic; increased expression of *PR-1* (Fig. 1B) and *BGL2* (data not shown) did not occur in apical uninfested leaves of infested rosettes. Wounding (repeated puncturing of the leaf with a pin) failed to induce *PR-1* and *BGL2* locally or in younger untreated leaves when leaves were harvested 2 h after 72 or 96 h of wounding (Fig. 1, A and B). Induction was also not apparent for either gene when leaves were harvested 30 min after 72 h of wounding (data not shown) in contrast with leaf-crushing treatments

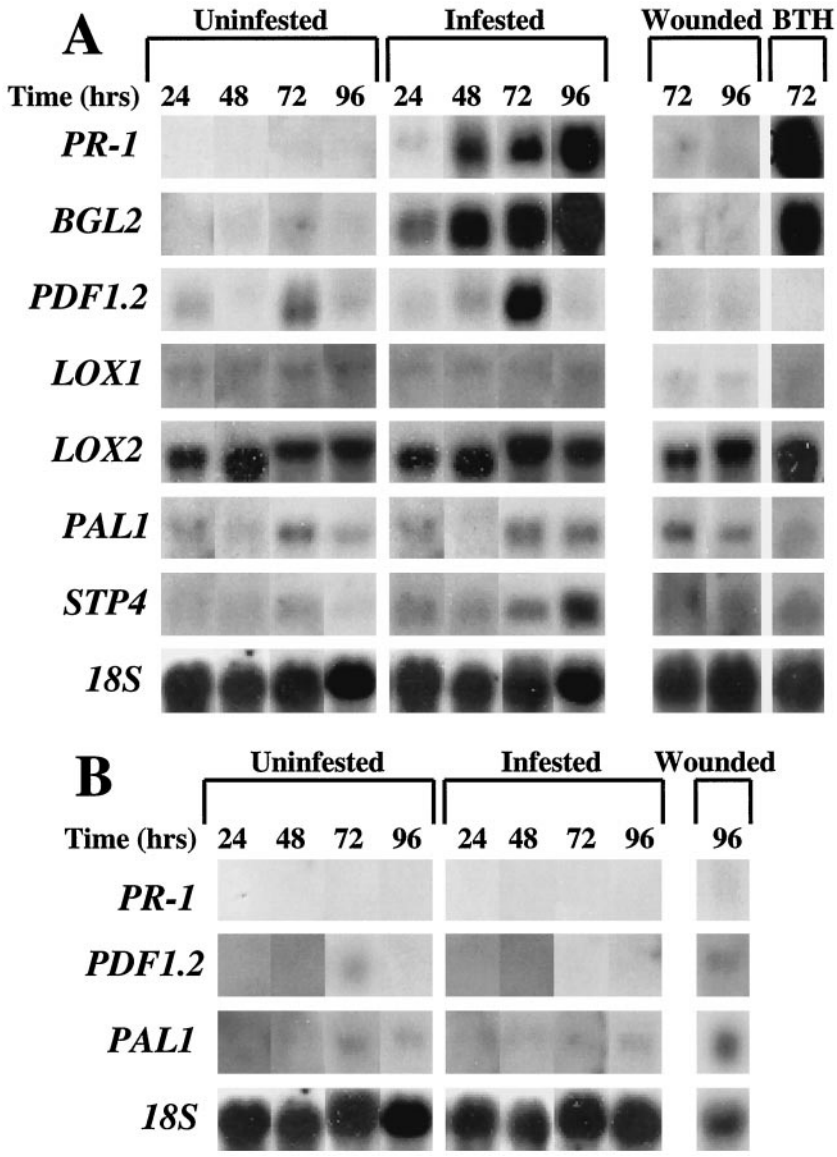


**Figure 1.** Transcript accumulation of defense response genes in rosette leaves of uninfested Arabidopsis plants or plants infested with aphids for 24 to 96 h. Separate sets of plants were subjected to mechanical leaf puncturing (Wounded) or sprayed with BTH, a chemical elicitor of SAR. A, Blots of total RNA (5–10 $\mu$g) isolated from leaves infested with aphids (local leaves). B, Blots of total RNA (5–10 $\mu$g) isolated from apical noninfested (systemic) leaves, testing for gene induction of SA- *(PR-1)*, JA- (*PDF1.2*), and SA-/JA- (*PAL1*) dependent responses. BTH induction data are not shown for systemic leaves because foliar applications involved direct contact of BTH with all leaves. All results were repeated across two to three replicate plants per time point and treatment. Induction was replicated in most cases across two to three independent experiments. Gene abbreviations: *PR-1*, acidic pathogenesis-related protein; *BGL2*, acidic, apoplastic $\beta$-1,3-glucanase; *PDF1.2*, defensin; *LOX1, LOX2*, lipoxygenase; *PAL1*, Phe-ammonia lyase; *STP4*, sugar transport protein; and *18S*, ribosomal 18S protein (RNA loading control).

that ephemerally induce both genes (Reymond et al., 2000). BTH treatment led to high *PR* gene expression (Fig. 1A) as in Lawton et al. (1996).

A probe encoding defensin in Arabidopsis (*PDF1.2*) was hybridized to RNA from infested and control plants to determine if the JA- and ethylene-dependent pathogen signaling pathway was induced by aphid feeding. *PDF1.2* expression levels in local, aphid-infested leaves were 1.5- to 2-fold higher than in controls, with maximum induction at 72 h (Figs. 1A and 2B). These changes in expression were modest and delayed relative to methyl JA and fungal infection treatment effects on defensin mRNA (Penninckx et al., 1996). *PDF1.2* expression was not induced in apical uninfested leaves of infested plants (Fig. 1B). Expression was variable in wounded tissues harvested 2 h after 72 or 96 h of treatment (Fig. 1, A and B). No localized induction was evident in a subsequent experiment in which tissues were harvested 30 min after 72 h of wounding (data not shown). BTH treatment did not alter *PDF1.2* expression (Fig. 1A).

The Arabidopsis genes encoding lipoxygenase, Phe-ammonia lyase, and sugar transport protein are inducible by both wounding and pathogen infection. Expression of *LOX1* was not induced by green peach aphid feeding at any time point by any treatment (Fig. 1A). Transcript levels of *LOX2* were approximately 1.5-fold higher after 72 and 96 h of infestation (Figs. 1A and 2B). This change was equivalent to levels associated with mechanical wounding (1.6-fold after 96 h; Fig. 1A) but appeared qualitatively lower than the effects of jasmonate, leaf crushing, or chewing herbivory on LOX2 expression (Bell and Mullet, 1993; Reymond et al., 2000). The *PAL1* and *STP4* genes were induced in infested leaves after 96 h of aphid feeding (2-fold and 10-fold, respectively; Figs. 1A and 2C). Aphid-induced changes in *PAL1* expres-

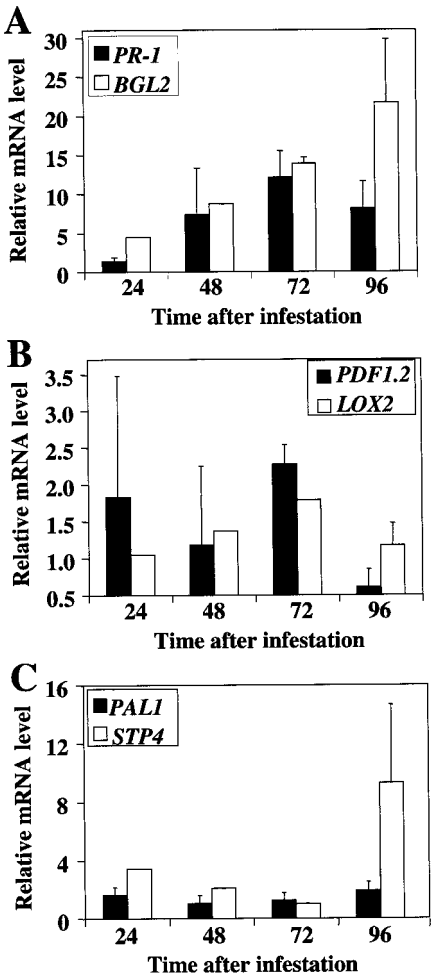


**Figure 2.** Quantification of expression levels for defense response genes in infested rosette leaves relative to control leaves after 24 to 96 h of feeding by green peach aphids. Gene abbreviations as in Figure 1. A, Expression of two SA-inducible *PR* genes, *PR-1* and *BGL2*. B, Expression of two JA-inducible response genes, *PDF1.2* and *LOX2*. C, Expression of two pathogen- and wound-inducible genes, *PAL1* and *STP4*. Error bars represent the SE of the mean infested to control ratio across two to three independent experiments. Absence of an error bar indicates that the ratio is based on one experiment. Data were normalized for variation using *18S* expression valves.

sion were lower than levels observed with artificial wounding (4-fold at 72 h; Fig. 1A) or jasmonate treatment (McConn et al., 1997). The relative increase in *STP4* expression induced by aphids were consistent with past results using other stresses (Truernit et al., 1996). Leaf puncturing did not induce quantifiable increases in *STP4* (Fig. 1A). Increases in *PAL1* (Fig. 1B), *LOX2*, and *STP4* (data not shown) mRNAs were restricted to infested leaves. Phe-ammonia lyase and sugar transport gene induction are often similarly localized to wounded, chemically treated, or infected areas (Berger et al., 1996; Truernit et al., 1996). Leaf puncturing modestly induced *PAL1* in apical untreated leaves but had no effect on *LOX2* expression. Expression levels of the three genes were not markedly influenced by BTH treatment (Fig. 1A), consistent, in the cases of *LOX2* and *PAL1*, with prior data showing a lack of SA regulation of these genes (van Wees et al., 1999).

β-1,3-glucanase mRNA levels increased 5-fold after 96 h of feeding by green peach aphids on inflorescence bolts (Fig. 3). It is interesting that no other gene in this study was induced on inflorescence stems by aphid feeding (data not shown). *PR* genes and *PAL1* are expressed in stems (Davis et al., 1991; Uknes et al., 1992), but little is known about gene induction by pathogen or wound treatment of bolt tissues of Arabidopsis.

### Attenuation of Aphid-Induced Responses in Arabidopsis Mutants

The *npr1* mutant line of Arabidopsis lacks a key positive regulatory element in the SAR pathway influencing *PR* gene induction (Cao et al., 1994). Feeding by green peach aphids on *npr1* leaves did not induce *PR-1* gene expression after 72 h (Fig. 4). *BGL2* expression was induced in the *npr1* mutant, but at a lower level than in wild-type plants. It is interesting that *PDF1.2* expression was more consistently and intensely induced in *npr1* plants (66-fold) than in wild-type plants (2.3-fold).

To evaluate the importance of the JA signaling pathway in response to aphid feeding, the jasmonate-insensitive *coi1-1* mutant (Feys et al., 1994) was examined. *PDF1.2* gene expression (elevated 6.5-fold in infested wild-type plants) was not induced in mutant plants after 72 h of feeding (Fig. 5). *LOX2* induction

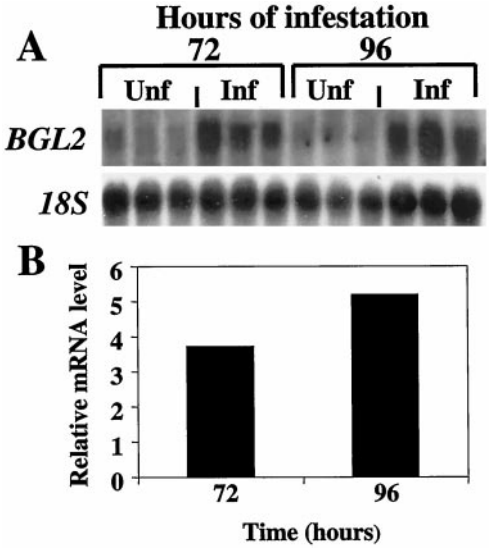


**Figure 3.** Induction of *BGL2* mRNA after 72 to 96 h of green peach aphid feeding on inflorescence bolts of Arabidopsis. A, Blot of total RNA isolated from sets of three replicate plants per time and treatment (Unf, uninfested leaves; Inf, infested leaves), hybridized to a *BGL2* cDNA probe. B, Quantification of changes in *BGL2* gene expression in infested plants, normalized for variation using *18S* expression values.

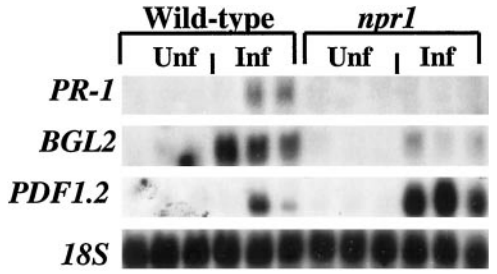


**Figure 4.** Induction by aphid feeding in the *npr1* mutant. Blots of total RNA isolated from sets of three replicate plants per genotype and treatment (Unf, uninfested leaves; Inf, infested leaves), showing expression of *PR-1*, *BGL2*, and *PDF1.2* after 72 h of green peach aphid feeding on wild-type and *npr1* mutant plants.

(1.6-fold in wild type) was also lacking in *coi1-1*. Consistent with past results for PR-1 and pathogen treatment (Penninckx et al., 1996), induction of *PR-1* by aphids (6.9-fold in wild type) was variable but not abolished in *coi1-1* plants (2.7-fold induction). *PR-1* appeared to be modestly induced by wounding in this mutant.

### Influence of Deficiencies in SAR and Prior Induction on Aphid Resistance

Activation of SA-dependent genes by aphids could be an important part of a plant defense response. Genetic and physiological manipulation of SA responses could influence aphid reproduction due to changes in host plant suitability. To determine if manipulation of SA-dependent responses affects aphid fitness, several mutants of Arabidopsis (*npr1*, *eds5*, and *eds9*) deficient in SAR were examined. BTH applications were used to verify mutant induction phenotypes over time frames parallel to those used in aphid infestation studies (72 h) and reproduction bioassays (10 d). BTH induced *PR-1* and *BGL2* mRNA within 72 h of foliar application in wild-type plants but failed to induce defensin (Fig. 6; Lawton et al., 1996; Thomma et al., 1998). *PR* gene mRNA induction was reduced in the SAR mutant *eds9* and was absent in *npr1* (Cao et al., 1994). *BGL2* appeared to still be modestly induced in *eds9* and wild-type plants after 10 d (Fig. 6). *PR-5* shows similar long-term induction by BTH in Arabidopsis (Lawton et al., 1996).

Cumulative reproduction over 1 week by cohorts of adult green peach aphids feeding on rosette and cauline leaves or inflorescence bolts of 4-week-old untreated Arabidopsis plants did not vary greatly by genotype (Fig. 7A; *F* test on variation in total counts across the four genotypes, $F_{3, 32} = 0.53$, $P = 0.67$, and $R^2 = 0.05$). Reproduction was lower on *eds5* leaf tissues than on *eds9* and *npr1* leaves in pair-wise comparisons ($F_{3, 32} = 4.76$, $P = 0.007$, and $R^2 = 0.31$), but no mutant line supported levels of reproduction different from Columbia on any tissue. Differences among mutant lines in leaves in Figure 7A were not replicated in control plants used in the BTH experiment (white bars, Fig. 7B).

BTH application followed by aphid cohort establishment resulted in slightly lower reproduction by aphids on leaves of both wild-type and SAR mutant Arabidopsis plants relative to plants receiving inert compound (Fig. 7B; two-way analyses of variance examining BTH treatment and plant genotype effects, $F_{7, 72} = 1.69$, $P = 0.13$, and $R^2 = 0.14$; BTH factor $F = 6.04$ and $P = 0.02$). BTH was not toxic when applied directly to green peach aphids in preliminary tests (see "Materials and Methods"). Reproduction was reduced by about 2-fold on BTH-treated Columbia leaves relative to controls ($F_{1, 29} = 3.82$, $P = 0.06$, and $R^2 = 0.12$). The magnitudes of reduction in the three mutant lines were similar but not statistically significant, probably because sample sizes were smaller. No effects of BTH were seen in aphid counts combined across all tissues. It is interesting that counts were 2- to 3-fold higher on inflorescence bolts than on leaves regardless of genotype or BTH treatment (compare the last two bars with the first two bars in Fig. 7B).

## DISCUSSION

The existence of an association between the generalist green peach aphid and Arabidopsis in nature is uncertain (Harrewijn, 1990), but this aphid is a frequent pest of Arabidopsis in artificial growth environments, producing multiple generations on leaves and bolts. A diverse set of genes with known roles in defense or resource allocation is induced in Arabidopsis plants infested with green peach aphids. The responses to aphids are distinct from both mechanical stress associated with wounding and elicitation by the SA analog BTH. Specific perception of phloem feeding could be occurring in Arabidopsis. Possible signals include oligosaccharides and glycoproteins resulting from aphid salivation (Hahn, 1996; Miles, 1999) and metabolites derived from endosymbiotic bacteria, which synthesize essential amino acids (Mittler, 1971). The minimal effects of elicitation on

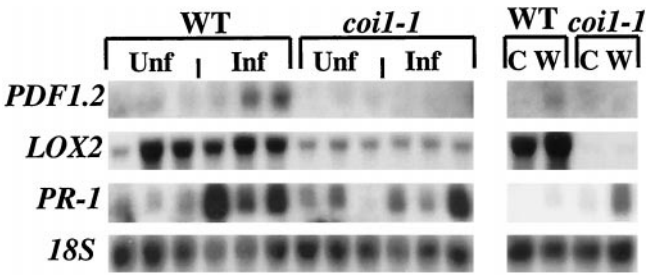


**Figure 5.** Induction by aphid feeding in the *coi1-1* mutant. Blots of total RNA isolated from sets of three replicate plants per genotype and treatment (Unf, uninfested leaves; Inf, infested leaves), showing expression of *PDF1.2*, *LOX2*, and *PR-1* after 72 h of green peach aphids feeding on wild-type (WT) and *coi1-1* mutant plants, or 72 h of mechanical wounding (C, control; W, wounded).

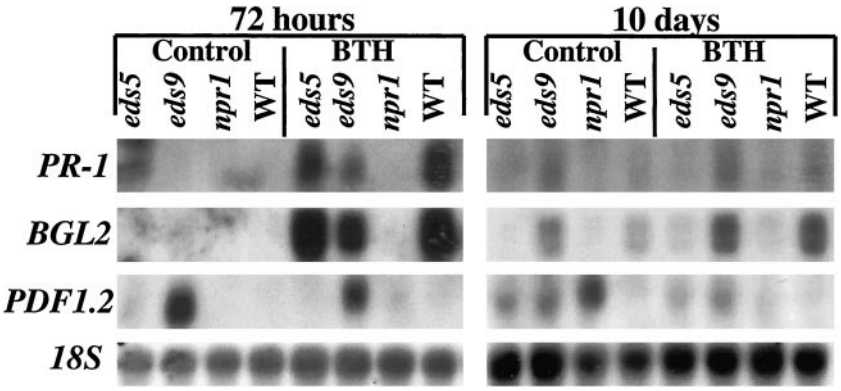


**Figure 6.** Effects of BTH on gene induction. Blots of total RNA showing expression of *PR-1*, *BGL2*, and *PDF1.2* 72 h and 10 d after foliar BTH treatment of rosette leaves of Arabidopsis wild-type (WT) and *eds5*, *eds9*, and *npr1* mutant plants.

aphid fitness on wild-type and mutant Arabidopsis point out the critical distinction between plant responsiveness to biotic stress and resistance benefits for the plant.

### Aphid Feeding Induces Multiple Plant Response Pathways

The SA-dependent response pathway in Arabidopsis leads to the induction of *PR* genes in leaves and SAR to pathogen infection. *PR-1* and *BGL2* were induced by 48 to 96 h of infestation by green peach aphids on Arabidopsis rosette leaves (Fig. 1). In local, infested leaves, the timing and intensity of induction were comparable with levels associated with virulent pathogen infection (Dong et al., 1991; Uknes et al., 1992) and BTH application (Lawton et al., 1996). Elevation of mRNA levels is not always indicative of increased protein synthesis and subsequent defensive metabolism. However, accumulation of PR-1 and acidic, apoplastic glucanase proteins, as well as defensin, occur concurrently with mRNA increases after pathogen infection (Uknes et al., 1992; Penninckx et al., 1998). Aphids influence *PR* gene expression in diverse hosts other than Arabidopsis. *P4* gene expression and peroxidase activity increase in tomato leaflets exposed to green peach or potato aphids (*Macrosiphum euphorbiae*; Stout et al., 1998; Fidantsef et al., 1999), and apoplastic PR enzyme activities increase in wheat leaves infested by Russian wheat aphids (*Diuraphis noxia*; van der Westhuizen et al., 1998a, 1998b). Phloem feeding clearly influences pathogen defense responses in intercellular spaces penetrated by aphids in the process of feeding.

In contrast with the systemic effects of *P. syringae* infection in Arabidopsis, green peach aphid-induced expression of *PR-1* and *BGL2* mRNA was limited to infested leaves. Similar localization occurs after infection of Columbia Arabidopsis plants with the nonnecrotic powdery mildew fungus *Erysiphe orontii* (Reuber et al., 1998). Aphids and mildew induce *BGL2* more strongly and consistently than *PR-1* (see wild-type lanes in Fig. 4). SA is important to local as well as plant-wide responsiveness (Glazebrook, 1999), and green peach aphid infestation likely triggers SA-dependent (Clarke et al., 1998) *PR* gene responses. The short time frames (96 h) of the aphid time course experiments contrast with the longer periods usually allowed for development of SAR (Uknes et al., 1992; Rogers and Ausubel, 1997). More than 10 d are required to see systemic gene responses to feeding

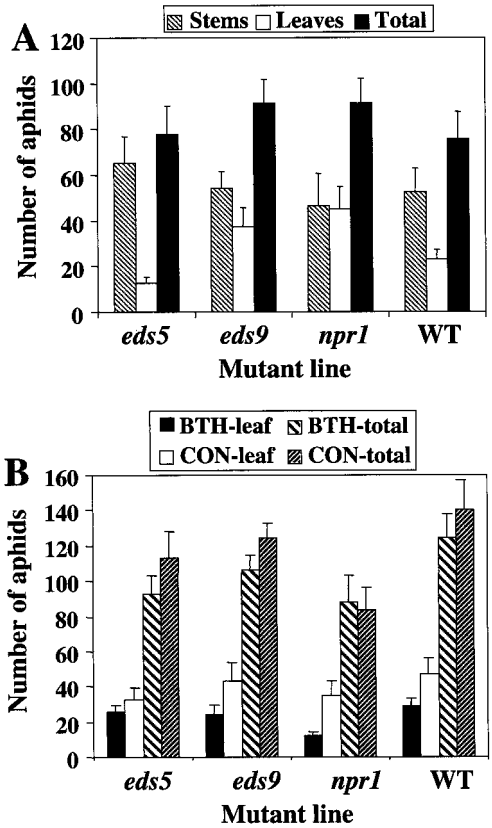


**Figure 7.** Reproduction by cohorts of two wingless adult green peach aphids on rosette and cauline leaves and inflorescence bolts of Arabidopsis wild-type (WT) and *eds5*, *eds9*, and *npr1* mutant plants. Bars indicate SE. A, Aphid populations on leaves (rosette and cauline combined) and bolts of untreated plants after 1 week of reproduction by cohorts ($n$ = 6–10 plants per mutant line). B, Numbers of aphids produced by cohorts after 1 week on leaves and combined tissues, 14 d after application of elicitor (BTH) or control formulation (CON; $n$ = 8–15 plants per mutant line per treatment).

by silverleaf whiteflies (*Bemeria tabaci*), another type of phloem-feeding insect, in leaves of squash (*Cucurbita pepo*; van de Ven et al., 2000) and tomato (Inbar et al., 1999). Rapid, plant-wide increases in PR proteins occur in aphid-infested leaves of resistant but not susceptible wheat genotypes, and necrotic lesions form at feeding sites in resistant lines (van der Westhuizen et al., 1998a). The green peach aphid does not induce a similar hypersensitive response in Arabidopsis.

Aphid elicitors did not activate JA-sensitive signaling pathways as strongly as SA pathways in Arabidopsis. Two genes associated with JA signaling, *PDF1.2* and *LOX2*, increased modestly over 96 h in leaves infested by green peach aphids relative to jasmonate-treated, infected, or (in the case of *LOX2*) wounded leaves (Fig. 1, A and B). A similar difference between SA- and JA-dependent responses induced by phloem feeding has been found in tomato plants (Fidantsef et al., 1999). Mechanical puncturing of leaves did not induce *LOX2* systemically, suggesting that the more limited wounding produced by aphids did not provide a strong stimulus to JA. *PDF1.2* regulation was independent of wounding, and expression levels were not always parallel to those of *LOX2* (Figs. 1 and 5; Penninckx et al., 1996; van Wees et al., 1999). Previous studies have shown that BTH or SA applications can suppress JA-dependent responses in Arabidopsis (van Wees et al., 1999). It is possible that induction of SA-dependent responses by aphids led to partial suppression of JA signaling, explaining the ephemeral nature of *PDF1.2* induction. However, salicylate levels were not measured in aphid-infested plants.

Induction of the *PAL1* and *STP4* genes by aphid feeding on rosette leaves (Fig. 1) could reflect localized responses to wounding and the generation of metabolic sinks. *PAL* gene expression is associated with a variety of abiotic stress sources, in addition to wounding and infection (Leyva et al., 1995; Sharma et al., 1996). Green peach aphid feeding could have stimulated localized de novo phenolic accumulation by inducing the biosynthesis of Phe-ammonia lyase, a key phenolic biosynthesis enzyme. Aphids secrete a gelling saliva containing polyphenoloxidases to sequester phenolics in the formation of the stylet sheath lining their apoplastic feeding pathways (Miles, 1999). The effect of aphid feeding on *PAL1* expression could have been a response to a localized metabolic imbalance. Similar reasoning likely explains the increase in mRNA of a monosaccharide $H^+$ symporter, *STP4*. STP proteins interact with invertases to locally increase carbohydrate import and metabolism in metabolically active wounded or infected tissues, without altering phloem loading patterns elsewhere in the plant (Buttner et al., 2000). Whether or not aphid induction of *STP4* can manipulate host plant quality is uncertain, as is the capacity of this gene for induction by SA or JA.

Aphids often moved onto inflorescence bolt tissues from leaves in resistance bioassay experiments, and final counts were usually higher on bolts, suggesting variation among tissues in host suitability. Host tissue preferences of aphids and other herbivorous insects can vary according to the ability of different tissues to induce responses to pathogen infection (Barbosa, 1991). Aphid infestation of inflorescence bolts of Arabidopsis led to mRNA induction of only the *PR* gene *BGL2* (Fig. 3). Constitutive and induced mRNA levels of *PDF1.2*, *LOX2*, and *STP4* were low in bolts, consistent with other studies (Bell and Mullet, 1993; Penninckx et al., 1996; Truernit et al., 1996). *PAL1* expression, on the other hand, was constitutively higher in bolts than in leaves (P.J. Moran and G.A. Thompson, unpublished data). *BGL2* induction could have facilitated aphid feeding in Arabidopsis, particularly in bolt tissues in which putative defense responses were lacking. Enhanced apoplastic glucanase enzyme activity near phloem sieve elements could have counteracted callose deposition, a putative host plant defense against aphids in phloem sieve elements (Shinoda, 1993).

### Plant Responses to Aphids Are Dependent on the *NPR1* and *COI1* Genes

The action of the *NPR1* gene was necessary for Arabidopsis to induce *PR* gene responses to aphids. *PR* gene induction was attenuated in the *npr1* mutant (Fig. 4). In contrast with some pathogens (Clarke et al., 1998), *NPR1* links signaling pathways and *PR* gene expression in aphid-infested leaves. The enhanced *PDF1.2* induction in *npr1* plants mirrors previous reports of induction by *A. brassicicola* (Penninckx et al., 1996) and JA treatment of other Arabidopsis mutants that accumulate less SA than wild-type plants (Gupta et al., 2000). In these mutants, jasmonate signaling was enhanced because of low SA levels. The *npr1* mutant, however, accumulates higher SA levels than wild-type plants after pathogen infection (Shah et al., 1997). Whether or not aphid induction has the same effect on SA in *npr1* is not known.

Aphid induction failed to induce *PDF1.2* and *LOX2* in the *coi1-1* mutant (Fig. 5), supporting the conclusion that elicitation of the JA/ethylene response pathway occurred in infested wild-type plants. A functional *COI1* gene is required for Arabidopsis sensitivity to aphid feeding. The inconsistent *PR* gene induction results in *coi1-1* are surprising because *PR-1* expression after *A. brassicicola* infection is normal in this mutant (Penninckx et al., 1996). Not all interactions between SA and JA are antagonistic (Glazebrook, 1999); it is possible that a lack of JA signaling in *coi1-1* influences *PR* gene up-regulation by aphid feeding. However, *coi1-1* shows wild-type levels of resistance to pathogens that induce only SA-dependent responses (Thomma et al., 1998).

### Variation in SA-Dependent Induction Does Not Influence Aphid Reproduction

Variation in *PR* gene expression capability (presence in wild-type, *eds5*, and *eds9* lines and absence in *npr1*) did not strongly influence green peach aphid fitness. The small size and short life cycle of Arabidopsis, combined with the relatively slow population development of green peach aphid (compared with bacteria), limited the length of bioassays. This combination could have obscured small differences among genotypes. Variation in aphid reproduction on untreated Arabidopsis plants (Fig. 7A) was not correlated to PR gene induction phenotypes. Applying BTH prior to cohort establishment distinguished these phenotypes over time frames that were relevant for both cohort nymph development (72 h) and reproduction by adults (10 d; Fig. 6). BTH improves host plant suitability for chewing tomato insects in both the lab and the field, at least in part because JA signaling is suppressed (Stout et al., 1999; Thaler et al., 1999). Suppression of JA by SA can occur in Arabidopsis (van Wees et al., 1999). However, prior induction by BTH did not enhance green peach aphid reproduction on wild-type, *eds5*, and *eds9* plants, and trends did not differ between these genotypes and *npr1*, which cannot inhibit jasmonate (Fig. 7B). The modest reductions in aphid reproduction observed in both mutant and wild-type BTH-treated plants suggest that diverse nutritive and defensive plant responses to BTH condition changes in resistance that vary depending on feeding strategy.

## CONCLUSIONS

Recent studies involving leaf wounding, chewing herbivory, and necrotic pathogen infection of Arabidopsis have demonstrated that plant responses regulated by seemingly divergent factors are in fact integrated and temporally controlled (van Wees et al., 1999; Reymond et al., 2000). Aphid feeding generates similar novel connections between multiple plant response pathways in Arabidopsis. SA-dependent responses to aphid feeding are similar to those associated with virulent pathogens and in contrast with the rapid, ephemeral nature of induction of *PR* genes by chewing herbivory. Phloem feeding also stimulates wound responses and could generate metabolic sinks. Aphids interact with plant pathogens through both vectoring and changes in feeding and development on infected plants (Purcell and Nault, 1991). The influence of these associations on plant gene induction is worthy of study. The breadth of plant responsiveness to aphids suggests that many novel genes and mechanisms involving plant perception, tolerance, and resistance to phloem feeding remain to be discovered.

## MATERIALS AND METHODS

### Plant and Insect Maintenance

Arabidopsis ecotype Columbia and Columbia-derived mutant plants were grown in 2.5-cm-wide square pots at 20°C and 50% relative humidity under fluorescent and incandescent light (150 $\mu$E $m^{-2}$ $s^{-1}$) with a 12-h photoperiod. Twenty-five- to 30-d-old plants were used for all studies (rosette with five–10 leaves; inflorescence bolt 2–15 cm). Mutant plants had gross morphologies similar to those of Columbia wild-type plants. *Coi1-1* mutant plants, which are male sterile, were screened from $F_2$ seed pools on Murashige and Skoog medium (5 g $L^{-1}$ Suc and 0.8% [w/v] agar content) containing 35 $\mu$M methyl jasmonate (91% [v/v] cis and 8% [v/v] trans mixture; Bedoukian Research Inc., Danbury, CT). Ten days after planting, homozygous *coi1-1* plants had undergone main root elongation (3–4 cm), whereas heterozygous and homozygous wild-type plants had short roots (<1 cm) because of JA inhibition. Mutant and wild-type plants were transferred to sterile soil and received sterile water and fertilizer solution. Green peach aphids (*Myzus persicae*) were maintained in a growth chamber on *Brassica napus* cv Ceres plants grown at 23°C and 50% relative humidity, with a 12-h photoperiod. These insects completed four juvenile (nymph) stages before molting to the adult stage and reproducing asexually. Only nymphs and apterous (wingless) adults were used in experiments.

### Gene Induction by Aphids

Sixty green peach aphids of assorted life stages were confined on rosette leaves or inflorescence bolts ("stems") of wild-type Arabidopsis plants, and on leaves of *npr1* or *coi1-1* plants. For studies of induction in leaves, two to three fully expanded and non-senescent leaves per plant were sealed at the petioles with tape that was folded out on the side facing the leaf apex, exposing the adhesive side to block aphid movement. In an alternate manner, in some experiments infested leaves were caged with 3- × 4.5-cm clear, ventilated plastic cages sealed at the base of the petiole with double-sided tape. Each leaf received 20 aphids. Leaves on control plants were sealed or caged without adding aphids. For studies of induction in inflorescence bolts, the area around the stem 3 to 4 cm from the rosette was sealed with tape and a clear plastic ring, which rested on top of the conical portion of Aracon tube bases (Lehle Seeds Inc., Round Rock, TX). Stems on control plants were sealed without adding insects.

All plants were placed inside ventilated clear Plexiglas cages (20 × 40 × 40 cm) to contain aphids. Aphids were allowed 2 h settling time to begin probing plants, and feeding time was then set to 0 h. After 24 to 96 h of feeding (three plants per treatment per time point), plants were sprayed with 1% (v/v) SDS solution, which caused aphids to remove their mouthparts (stylets) from plant tissues. Aphids were then removed and leaves (infested, local leaves and uninfested, apical [younger] leaves from the same rosettes) or bolts (with flowers and siliques removed) were excised and frozen in liquid nitrogen.

### BTH Treatment

Each wild-type or mutant plant (*eds5*, *eds9*, and *npr1*) was sprayed, using a pump aerosol sprayer, with 1 mL of an aqueous solution of 300 $\mu$M BTH [benzo (1, 2, 3) thiadiazole-7-carbothioic acid *S*-methyl ester; BION, Novartis Crop Protection, Research Triangle Park, NC; Lawton et al., 1996] in a formulation containing 50% (w/w) active ingredient. Inert ingredients in the formulation included wetting and dispersal agents, diatomaceous earth, and sodium sulfate. Control plants received formulation not containing BTH. Tissues were harvested for RNA extraction 72 h and 10 d after treatment. Mild necrosis was occasionally observed on the edges of one or two rosette leaves per plant 1 week after spraying with BTH.

### Mechanical Wounding

Aphid stylets usually penetrate the epidermal and mesophyll leaf layers intercellularly before puncturing cell walls in the phloem companion cell-sieve element tissue complex (Pollard, 1972). A wounding treatment was designed to partially simulate the mechanical stress resulting from aphid penetration. Leaves were punctured repeatedly with a size 10 (approximately 0.30-mm diameter) beading needle. One pin puncture was arbitrarily considered equal to four aphid penetration events because the treatment involved the creation of macroscopically visible wounds that were much larger than wounds made by aphids. Three rosette leaves on 30-d-old Arabidopsis plants were each punctured 5 times at 0 h, 10 times at 24 h, 15 times at 48 h, 20 times at 72 h, and 40 times at 96 h. Leaves on control plants were handled the same number of times at each time point without inflicting wounds (five plants per treatment). The puncture regime was designed to estimate the daily increase in the cumulative number of penetration events occurring during an aphid infestation lasting 96 h. Leaves were excised for RNA extraction 2 h after the 72- and 96-h wounding events. In a subsequent experiment, leaves were harvested 30 min after 72 h of wounding.

### Analysis of Gene Expression

Total RNA was extracted from leaves and stems of infested, wounded, BTH-sprayed, and control plants using the method of Carpenter and Simon (1998). Total RNA (2–10 $\mu$g) was denatured (0.9 M glyoxal, 80% [v/v] formamide, and 10 mM $NaPO_4$ buffer [pH 7.0]) at 55°C for 1 h, separated in Tris-acetic acid-EDTA gels, capillary blotted with 20× SSC buffer (1 × SSC = 0.15 M NaCl, 0.015 M $C_6H_5Na_3O_7$) onto positively charged nylon membranes (Hybond, Amersham, Arlington Heights, IL) and UV crosslinked (FB-UVXL-1000, Fisher Scientific, Pittsburgh). Labeled DNA probes ([$\alpha-^{32}$P] ATP; NEN, Boston) were made with the RadPrime random priming kit (Life Technologies, Grand Island, NY). Table I shows the sizes of the cDNA templates used to make probes (Arabidopsis Biological Resource Center clones: *BGL2*, expressed sequence tag [EST] stock 214L5T7; *LOX2,* EST stock 106C8T7; and *PDF1.2,* EST stock T04323). Prior to labeling, templates were purified from agarose gels following PCR amplification or endonuclease digestion of restriction sites in plasmid vectors. Membranes containing blotted RNA were prehybridized for 1 h and hybridized overnight (1.2 to 1.5 × $10^6$ cpm probe/mL buffer) in UltraHyb buffer (Ambion Inc., Austin, TX) at 42°C. Hybridized blots were washed with 2× SSC/0.1% (v/v) SDS at 42°C (20 min) and 0.1× SSC/0.1% (v/v) SDS at 65°C (40 min). Blots were exposed to autoradiogram film (Kodak Blue XB-1, Eastman-Kodak, Rochester, NY) or a phosphor screen (Molecular Dynamics, Sunnyvale, CA).

Sizes of hybridizing bands were determined with an RNA ladder (Ambion) and compared with published mRNA transcript sizes (see Table I). Hybridizing band intensity was quantified by densitometry of either developed film (AlphaImager 2000 system, Alpha Innotech, San Leandro, CA) or scans of exposed phosphor screens generated by a STORM System phosphorimager (model 680) and ImageQuant software (Molecular Dynamics). After no more than three cycles of hybridization followed by stripping in boiling 0.5% (v/v) SDS, variation in total RNA loading on blots was evaluated by hybridization to the maize (*Zea mays*) *18S* gene. For each gene hybridized, the average *18S*-standardized signal intensity for mRNAs from two to three aphid-infested plants was divided by the average value for similar numbers of control plants, to obtain a measure of relative gene induction by aphid feeding at each time point.

**Table I.** *Probe templates and RNA transcripts of Arabidopsis genes used to examine induction by aphid feeding*

| Gene | Protein Name | Pathway[a] | cDNA Template | mRNA Transcript | Reference |
|---|---|---|---|---|---|
| | | | *bp* | | |
| PR-1 | Acidic pathogenesis-related protein | SA | 457[b] | 757 | Uknes et al. (1992) |
| BGL2 | Acidic, apoplastic $\beta$-1,3-glucanase | SA | 1,181 | 1,181 | Uknes et al. (1992) |
| LOX1 | Lipoxygenase | JA | 900[b] | 2,801 | Melan et al. (1993) |
| LOX2 | Lipoxygenase | JA | 1,938 | 2,809 | Bell and Mullet (1993) |
| PDF1.2 | Defensin | JA + ethylene | 390 | 390 | Penninckx et al. (1996) |
| PAL1 | Phe-ammonia lyase | JA/SA | 512[b] | 2,800 | Davis et al. (1991) |
| STP4 | Sugar transport protein | Wound/pathogen[c] | 1,826 | 1,938 | Truernit et al. (1996) |

[a] Pathway refers to the sensitivity of the gene to induction by SA or JA treatment. [b] Subclone of the originally published clone. [c] Responses to JA and SA as elicitors not known.

### Aphid Reproduction Studies

Two adult apterous (non-winged) female green peach aphids were placed on 20- to 25-d-old untreated Columbia wild-type or mutant plants (one on a rosette leaf and the other on a lower portion of the inflorescence bolt). To study the effects of BTH on aphid reproduction, adults were placed on plants 24 h after spraying. After 2 d of asexual reproduction, the adult and all but one newborn aphid (nymph) were removed from each tissue type. Two weeks after establishing this cohort of two nymphs per plant, counts were made of the numbers of aphids feeding on leaf and stem tissues. Aphids feeding on the cauline leaves of the inflorescence bolt were included in counts of individuals on rosette leaves. By the end of the experiment, plants were often heavily infested (150 or more aphids per plant). Aphid counts were $\log_e$ transformed as needed to meet normality of variance requirements. Values were analyzed across mutant lines and treatments with two-way analyses of variance using SAS software (Version 7 for PC , SAS Institute, Cary, NC). Six to 10 plants were used per genotype for experiments with untreated plants. To determine the effect of BTH on aphids, seven to 15 plants per genotype per BTH or control treatment were included in a pooled analysis of several experimental trials.

To evaluate the direct toxicity of BTH to green peach aphids, two apterous adults (one on a rosette leaf, and one on the bolt) were allowed to colonize Arabidopsis wild-type plants for 5 d. Aphid counts were made directly before and 72 h after applying BTH or control compound as above to the plants, and the change in numbers of aphids was compared (five plants per treatment). No difference in changes in population sizes between BTH and control plants was observed (BTH plants [mean ± SE], 28 ± 3.96, control plants, 34 ± 6.35; $F_{1,8} = 0.83$, $P = 0.39$, and $R^2 = 0.093$). As a further test, adult aphids in a petri dish were sprayed with BTH or inert compound until moisture covered their dorsal cuticles. Two adults were allowed to colonize an untreated Columbia plant for a week and total reproduction was assessed (four plants per BTH or control aphid treatment). Reproduction did not differ between treatments ($F_{2,6} = 1.69$, $P = 0.26$, and $R^2 = 0.36$; data not shown).

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