Running title: ArBIK1 represses plant resistance against aphid

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BOTRYTIS-INDUCED KINASE1 modulates *Arabidopsis* resistance to green peach aphids via PHYTOALEXIN DEFICIENT4

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**One-sentence summary**: *Arabidopsis* BOTRYTIS-INDUCED KINASE1 negatively regulates plant resistance against green peach aphid through PHYTOALEXIN DEFICIENT4-dependent hypersensitive response.
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ABSTRACT

*BOTRYTIS*-INDUCED KINASE1 (BIK1) plays important roles in induced defense against fungal and bacterial pathogens in *Arabidopsis thaliana*. Its tomato homolog is required for host plant resistance to a chewing insect herbivore. However, it remains unknown whether BIK1 functions in plant defense against aphids, a group of insects with a specialized phloem sap-feeding style. In this study, the potential role of BIK1 was investigated in *Arabidopsis* infested with the green peach aphid, *Myzus persicae*. In contrast to the previously reported positive role of intact BIK1 in defense response, loss of BIK1 function adversely impacted aphid settling, feeding and reproduction. Relative to wild-type plants, *bik1* displayed higher aphid-induced H₂O₂ accumulation and more severe lesions, resembling a hypersensitive response (HR) against pathogens. These symptoms were limited to the infested leaves. The *bik1* mutant showed elevated basal as well as induced salicylic acid and ethylene accumulation. Intriguingly, elevated salicylic acid levels did not contribute to the HR-like symptoms or to the heightened aphid resistance associated with the *bik1* mutant. Elevated ethylene levels in *bik1* accounted for an initial, short-term repellence. Introducing a loss-of-function mutation in the aphid resistance and senescence-promoting gene *PHYTOALEXIN DEFICIENT4 (PAD4)* into the *bik1* background blocked both aphid resistance and HR-like symptoms, indicating *bik1*-mediated resistance to aphids is PAD4-dependent. Taken together, *Arabidopsis* BIK1 confers susceptibility to aphid infestation through its suppression of PAD4 expression. Furthermore, the results underscore the role of reactive oxygen species and cell death in plant defense against phloem sap-feeding insects.
INTRODUCTION

Aphids are specialized to feed and survive on phloem sap of their host plants. In contrast to chewing insects that cause extensive plant tissue damage, aphids have evolved to manipulate resource allocation within the host plant by converting the feeding site into a sink to deplete photoassimilates (Girousse et al., 2005). Their highly modified stylets navigate through plant tissues predominantly intercellularly before reaching phloem, causing very limited host cell damage. During probing and feeding, aphids secrete gelling and watery saliva (Tjallingii, 2006). Gelling saliva forms the sheath enveloping the stylet along the pathway leading to the vascular bundle. The sheath limits damage to plant cells and avoids triggering extracellular defenses. Watery saliva is thought not only to prevent clogging of phloem sieve elements and the food canal in aphid stylets due to protein coagulation, but also to modulate host cellular processes and mitigate host defense (Tjallingii, 2006; Will and van Bel, 2006; Will et al., 2007). Aphids make use of their stealthy feeding strategies and intimate associations with their hosts to disguise themselves and overcome plant defense, reminiscent of the deceptive strategies frequently employed by pathogens (Kaloshian, 2004; Walling, 2008).

During the long history of co-evolution, plants have developed sophisticated means to protect themselves against assaults from various herbivorous insects. Most plants are equipped with constitutive and induced defense mechanisms including physical barriers, such as trichomes and cell walls, and chemical defense, such as secondary metabolites. Despite the deceptive feeding style of aphids, the brief intracellular punctures along the stylet passage and secretions from salivation nevertheless can trigger responses in host plants (Tjallingii, 2006; Will and van Bel, 2006; De Vos and Jander, 2009; Bos et al., 2010). Plant defense responses can be classified as antibiosis, which curtails insect survival and reproduction, and/or antixenosis, which deters insect settling and herbivory. Transcriptomic studies suggest that phloem sap feeders modulate known defense signaling pathways, oxidative stress response, senescence, and plant metabolism and structure (Moran and Thompson, 2001; Zhu-Salzman et al., 2004; De Vos et al., 2005; Thompson and Goggin, 2006; Kusnierczyk et al., 2008). Plant response to aphids involves genes regulated by the major plant hormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA), and genes encoding transcriptional regulators. Exogenous JA application
enhances plant resistance to aphids (Ellis et al., 2002; Zhu-Salzman et al., 2004; Cooper and Goggin, 2005). Furthermore, reduced population expansion was observed in *M. persicae* when raised on the *Arabidopsis constitutive expression of vegetative storage protein 1 (cev1)* mutant constantly expressing JA responses, whereas the JA-insensitive mutant *coronatine-insensitive 1 (coil)* supports more rapid growth of aphids than WT plants (Ellis et al., 2002; Mewis et al., 2005). Aphid infestation has been shown to trigger ET production (Mantelin et al., 2009). Elevated ET levels have been both positively and negatively correlated with plant resistance to aphids (Thompson and Goggin, 2006). In tomato, ET biosynthesis renders plants more susceptible to potato aphids (* Macrosiphum euphorbiae*) (Mantelin et al., 2009). However, the *Arabidopsis* ethylene-insensitive mutant *ein2* promotes performance of green peach aphids (*M. persicae*) (Kettles et al., 2013), indicating that ET plays a defensive role in *Arabidopsis*. Aphid feeding activates the SA signaling pathway in a number of plant species (Moran and Thompson, 2001; Moran et al., 2002; Zhu-Salzman et al., 2004). SA-mediated resistance to aphids has been observed on some occasions (Mohase and van der Westhuizen, 2002; Kaloshian, 2004), but SA does not seem to play a defensive role in *Arabidopsis* against aphids (Pegadaraju et al., 2005). ABA has also been implicated as a modulator of plant immunity via signaling crosstalk (Fujita et al., 2006; Koornneef and Pieterse, 2008). Mutations in ABA biosynthesis and signaling have significant impacts on aphid population growth (Kerchev et al., 2013). Comparison of plant gene expression profiles reveals that aphid feeding and pathogen infection induce both similarly and differentially regulated gene sets (Barah et al., 2013).

The localized cell death elicited by microbial pathogens known as the hypersensitive response (HR) is considered a defense mechanism used by plants to prevent further spread of infection (Torres et al., 2006). A hallmark of hypersensitivity in many plants is local production of reactive oxygen species (ROS), such as H$_2$O$_2$. HR-like symptoms, manifested as localized chlorotic and necrotic lesion spots, can also be detected in plants attacked by various insect herbivores. Strong HR-like symptoms, including rapid and prolonged accumulation of H$_2$O$_2$, were detected in lines of wheat (*Triticum aestivum*) resistant to Hessian fly (*Mayetiola destructor*), but not in the susceptible line (Liu et al., 2010). Enhanced resistance against phloem sap-sucking brown planthopper (*Nilaparvata lugens*) is accompanied by increased H$_2$O$_2$ levels as well as HR-like cell death in rice (*Oryza sativa*) expressing an antisense lipoxygenase (Zhou et
Oxidative stress induced by insect herbivory is considered a component of soybean (*Glycine max*) resistance to invading corn earworm (*Helicoverpa zea*) (Bi and Felton, 1995). *Arabidopsis* PHYTOALEXIN DEFICIENT4 (PAD4), a lipase-like protein essential for defense against microbial pathogens (Jirage et al., 1999), has been demonstrated to enhance plant resistance to green peach aphid (*Myzus persicae*) by promoting premature leaf senescence and cell death (Pegadaraju et al., 2005; Pegadaraju et al., 2007). Functional dissection further revealed that the molecular mechanism of PAD4 resistance against aphids is distinct from that against pathogens (Louis et al., 2012).

Basal disease resistance, the first line of plant defense response is elicited upon detection of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) by specific transmembrane pattern-recognition receptors and is collectively termed PAMP-triggered immunity (Boller and Felix, 2009; Monaghan and Zipfel, 2012). Among the best characterized *Arabidopsis* PAMP/MAMP receptors are receptor-like kinases (RLKs) such as FLAGELLIN-SENSITIVE2 (FLS2) that recognizes bacterial flagellin, and EF-TU RECEPTOR (EFR) that recognizes bacterial elongation factor EF-Tu (Gomez-Gomez and Boller, 2000; Zipfel et al., 2006). Upon binding to their cognate MAMPs, FLS2 or EFR associate with another RLK, BRI1-ASSOCIATED RECEPTOR KINASE (BAK1) (Chinchilla et al., 2007). *Botrytis*-INDUCED KINASE1 (BIK1), a receptor-like cytoplasmic kinase (RLCK) is directly phosphorylated by BAK1 and associates with FLS2/BAK1 complex in modulating PAMP-mediated signaling (Lu et al., 2010; Zhang et al., 2010; Liu et al., 2013). Most recently, BAK1 is shown to be required for aphid elicitor-mediated ROS induction and plant innate immunity to aphids (Prince et al., 2014). Likewise, TOMATO PROTEIN KINASE1b (TPK1b), the tomato homolog of BIK1, plays an important role in plant resistance to a chewing insect herbivore (Abuqamar et al., 2008). The second layer of plant defense response is mediated by plant disease resistance (R) proteins, which recognize specific avirulence proteins from pathogens. R gene-mediated resistance to aphids has been reported although the corresponding avirulence proteins from aphids remain unknown (Kaloshian, 2004). The tomato R gene *Mi-1* confers resistance to some biotypes of potato aphids (*Macrosiphum euphorbiae*), as well as to whiteflies (*Bemisia tabaci*) and root-knot nematodes (*Meloidogyne incognita*) (Rossi et al., 1998; Vos et al., 1998; Nombela et al., 2003).
In this study, we examined the roles of several RL(C)Ks, including FLS2, EFR, BAK1, and BIK1, in *Arabidopsis* response to aphid infestation. We challenged these loss-of-function mutants with *M. persicae*, a phloem sap-feeding generalist, to evaluate aphid performance and plant response. bik1 plants displayed heightened antibiosis and antixenosis toward aphids, which was correlated with pronounced aphid-induced HR-like cell death. Further exploration of potential interactions between BIK1 and known defense pathways revealed that BIK1 modulated plant response to aphid infestation through its control of PAD4 expression.

**RESULTS**

*bik1* exhibited increased resistance to green peach aphids

Plant defense response upon aphid infestation is often reflected by reduced offspring production (antibiosis) in a no-choice test with reduced feeding and body weight, or by non-preference (antixenosis) in a choice test. To determine whether the several known RL(C)Ks, which play important roles in PAMP-triggered immunity, extend their function to aphid-associated defense response, we evaluated aphid performance on the loss-of-function mutants (Fig. 1). Aphids infesting *fls2, efr* and *bak1* mutants had fecundities comparable to that on the wild-type (WT) Col-0 plants (Fig. 1A). Likewise, no particular preference was detected among them (Fig. 1C), suggesting that these RLKs may not play a major role in plant defense against aphids. Interestingly, on *bik1*, the amount of aphid progeny was on average about half that on WT plants (Fig. 1A). In agreement with this no-choice test result, aphids on *bik1* excreted less honeydew (Fig. 1D), indicative of less food intake, and had less body weight (Fig. 1B) than those reared on WT. In the choice tests, approximately twice as many aphids preferred WT versus *bik1* plants (Fig. 1C). Thus, BIK1 was a negative regulator of plant resistance to aphids. In addition, we confirmed that the heightened resistance in *bik1* is indeed due to loss of BIK1 function via complementation experiments. Transgenic plants expressing *BIK1* cDNA in *bik1* mutant recovered the susceptibility to aphids in both choice and no-choice tests (Fig. 1E), verifying that the observed aphid resistance in *bik1* was due to loss of *BIK1* function.
Notably, *bik1* mutant showed comparable size and biomass during the first 3 weeks of growth (Fig. 1C; Table S1), when choice tests were performed. Later, *bik1* mutant exhibited growth defect and were smaller than WT (Fig. S1; Table S1). However, the antibiotic activity was unlikely due to their small stature, as inoculating six 2nd instar nymphs and rearing them for 7 days on 4 to 5-week-old plants would by no means result in a population limited by space or nutrients.

**Aphids induced hypersensitive response (HR)-like lesions in *bik1***

Despite an enhanced resistance to aphid infection, *bik1* began to show apparent lesion spots approximately 5 days after aphid infestation, while no visible lesions were observed in *fls2*, *efr* and *bak1* mutants or in WT (Fig. 2A). With continued aphid infestation, all infested plants, regardless of the genotype, eventually displayed stunted growth, yellowing and necrosis with lesions spreading to the entire leaf and the whole plants. Notably, *bik1* is not a lesion mimic mutant as no spontaneous lesions were observed without aphid infestation. Since *bik1* plants are dwarfs, the number of aphids applied was adjusted by a ratio proportional to the rosette area. For plant symptom assessment, this ratio was applied for all genotypes exhibiting size differences relative to WT, to exclude potential misjudgment due to size discrepancies.

We further examined whether the aphid-induced lesion formation in the *bik1* mutant resembles the features with an HR process that is often correlated with plant resistance against microbial pathogens (Lamb and Dixon, 1997; Heath, 2000). Using 3,3'-diaminobenzidine (DAB) staining, we observed that leaves of aphid-infested *bik1* plants had much higher H$_2$O$_2$ accumulation than any other genotypes examined (Fig 2B). Likewise, more severe cell death was shown in aphid-infested *bik1* leaves compared with WT and the other mutants by the trypan blue staining assay (Fig. 2C). In contrast, *fls2*, *efr* and *bak1* mutants showed phenotypes similar to WT plants in either H$_2$O$_2$ or cell death assays. Furthermore, we detected accumulation of autofluorescent phenolic compounds and deposition of callose at necrotic spots in aphid-infested *bik1* plants (Figs. 2D, 2E), which are also HR lesion-associated histological markers (Hunt et al., 1997; Luna et al., 2011; Williams et al., 2011). WT levels of H$_2$O$_2$ and lesions upon aphid
infection were restored in the bik1 BIK1 complementation line (Fig. 2). Taken together, the data indicate that aphid-induced lesions in bik1 were an HR-like response.

Although BIK1 is highly induced by pathogens (Veronese et al., 2006), we did not detect a significant change in BIK1 expression upon aphid infestation (Fig. 2F). This is further supported by published microarray data (Couldridge et al., 2007; Kusnierczyk et al., 2007; Kusnierczyk et al., 2008).

Since cellular H2O2 accumulation precedes cell death (Hoeberichts and Woltering, 2003), earlier time points were chosen for DAB staining. Staining became apparent within 3 hours upon aphid infestation in bik1 leaves, but was absent from the infested WT leaves over the 24 hour course of the experiment (Fig. 3A). When aphids were caged on specific leaves, H2O2 could only be detected in infested local leaves, not in uninfested systemic leaves (Fig. 3B), supporting our conclusion that the lesion formation in bik1 is an HR rather than a constitutive plant damage phenotype. Correlation between plant symptoms and aphid performance suggests that elevated H2O2 accumulation and cell death in bik1 could be the defense mechanism compromising aphid fitness. BIK1 thus functions to counteract aphid-induced ROS production and cell death, distinct from its role in PAMP pathways.

**Aphids altered phytohormone contents and gene expression in bik1**

Aphid-induced plant defense and cell death pathways are often regulated by certain plant hormones (De Vos et al., 2005). To determine whether the resistance to aphids conferred by loss of BIK1 function involved defense-related plant hormones, we measured SA, JA, ET and ABA levels in the presence and absence of aphid feeding in both WT and bik1 plants (Fig. 4A). Elevated basal SA (consistent with Veronese et al. (2006)) and ET levels were detected in bik1, while JA and ABA contents were comparable in both genotypes. SA and ET levels increased in both WT and bik1 upon aphid infestation, and the levels of both hormones were higher in bik1 than in WT (Fig. 4A). No significant changes in JA and ABA were observed after aphid feeding. Basal expression levels of the SA-signaling marker gene PR-1, and the ET/JA marker genes ERF1 and PDF1.2 were greater in bik1 compared to WT (Fig. 4B). Aphid infestation
upregulated expression of these genes in both WT and mutant plants. In comparison, basal expression of the JA-regulated transcription factor MYC2 was similar in both genotypes and was not altered by aphid infestation in either genotype (Fig. 4B). These data imply that BIK1 may function as a negative regulator of SA and ET accumulation both in the presence and absence of aphid infestation, thereby suppressing expression of their responsive genes.

**Resistance to aphids conferred by loss of BIK1 function was SA-independent**

To assess the role that SA may play in bik1 resistance to aphids, bik1 sid2 and bik1 nahG plants were used for choice and no-choice tests (Fig. 5). Loss of SALICYLIC ACID INDUCTION DEFICIENT2 (SID2) function blocks SA biosynthesis (Wildermuth et al., 2001), and nahG plants express salicylate hydroxylase that degrades SA to catechol (Delaney et al., 1994). In no-choice tests, the aphid numbers on bik1 sid2 or bik1 nahG plants paralleled those on bik1, and numbers on SA-deficient sid2 or nahG did not significantly differ from the WT (Fig. 5A). Similar results were obtained in choice tests (Fig. 5B), as well as from honeydew excretion assays (Fig. 5F). Apparently, reducing the SA level did not weaken aphid resistance in bik1, nor did it influence aphid response in WT. Therefore, elevated SA accumulation was not required for bik1 resistance to the aphid, in contrast to its requirement for bik1’s resistance to a virulent strain of *Pseudomonas syringae* (Veronese et al., 2006).

To examine how SA impacted the aphid-triggered HR-like lesion formation, H$_2$O$_2$ production and cell death in bik1, DAB and trypan blue staining were conducted on the SA-deficient plants. No correlations were observed between the SA status and lesion formation, H$_2$O$_2$ production or cell death phenotypes (Figs. 5C, 5D, 5E), a result supporting previous studies showing that SA is not essential for aphid defense in *Arabidopsis* (Pegadaraju et al., 2005). In contrast, a correlation was observed between resistance to aphids and H$_2$O$_2$ production as well as cell death occurrence. Notably, in terms of the plant size and morphology, bik1 sid2 and bik1 nahG were closer to WT than to bik1, yet they exhibited levels of H$_2$O$_2$ production, cell death and aphid resistance comparable to bik1. Therefore, dwarfism was unlikely the cause of enhanced resistance to aphids in bik1. Heightened endogenous SA has been reported previously to confer bik1 with resistance to the bacterial pathogen *Pst*DC3000 (Veronese et al., 2006).
Results from our study revealed differential function of SA in BIK1-mediated plant responses to bacterial pathogens versus phloem sap-feeding aphids.

Elevated ET signaling in bik1 increased aphid repellence during early stages of infestation

Like SA, ET is known to play a key role in cell death and plant response to pathogens and insects (Dong et al., 2004; Cohn and Martin, 2005; Bouchez et al., 2007). To examine whether elevated ET has a role in aphid resistance in bik1, we pretreated plants with 1-methylcyclopropene (1-MCP), an inhibitor of ET action that binds to the ET receptor. In choice tests, there was no significant difference in the number of aphids on 1-MCP-treated bik1 and WT plants 6 hr after aphid inoculation (Fig. 6), suggesting that 1-MCP may have compromised resistance in bik1. As time went on, however, 1-MCP-treated bik1 gradually regained their aphid repellence, presumably due to loss of 1-MCP function.

Since the 1-MCP effect was temporary, this pharmacological approach was limited to choice tests. To further investigate whether increased ET contributes to bik1 resistance to aphids, a genetic approach was used to impair ET signaling in bik1 and longer-term no-choice tests were performed. The bik1 mutant was crossed with two ET-insensitive mutants, ethylene insensitive 2-1 (ein2-1) and ein3-1 (Guo and Ecker, 2004; Broekaert et al., 2006). EIN2 (a transducer of ethylene signaling) and EIN3 (a primary ET-responsive transcription factor) are essential components of the ET signaling pathway. In no-choice tests, the bik1 ein2-1 double mutant showed resistance comparable to bik1 (Fig. 7A), suggesting that ET was not important in suppressing aphid reproduction in bik1, in agreement with honeydew secretion data (Fig. 7F). However in choice tests, blocking ET signaling in bik1 (i.e. bik1 ein2-1) increased plant attractiveness to aphids (Fig. 7B), implying that elevated ET in bik1 contributed to its aphid repellence. Interestingly, when compared with bik1, bik1 ein2-1 was preferred more by aphids early on. As experiments continued, the difference in the number of aphids on each genotype became non-significant. Thus, the overall effect of ET on bik1-mediated aphid resistance appeared to be only temporary and rather subtle.
The \textit{bik1 ein2-1} double mutant maintained the small stature of the \textit{bik1} single mutant (Fig. S1C). Feeding response in the \textit{bik1 ein2-1} double mutant, i.e. lesion formation, H$_2$O$_2$ production and cell death upon aphid infestation, resembled those of \textit{bik1} (Figs. 7C, 7D, 7E). Similar results were obtained with \textit{bik1 ein3-1} plants (Fig. S2). Taken together, ET signaling in \textit{bik1} was mainly involved in aphid deterrence initially in choice tests, but appeared to play little role in cell death-mediated defense in \textit{bik1}.

**Aphid resistance and HR-like cell death in \textit{bik1} is \textit{PAD4}-dependent**

\textit{PAD4} is a lipase-like protein that, upon aphid feeding, promotes premature leaf senescence to suppress insect reproduction and colonization (Pegadaraju et al., 2005; Pegadaraju et al., 2007). Aphids induced \textit{PAD4} expression in both \textit{bik1} and WT (Fig. 8A). Compared to the WT plants, \textit{bik1} had much higher \textit{PAD4} basal expression. Consistently, a senescence marker gene, \textit{SENEGENCE ASSOCIATED GENE 13 (SAG13)} regulated by \textit{PAD4} during aphid infestation (Weaver et al., 1998; Pegadaraju et al., 2005) shared a similar expression pattern with \textit{PAD4} (Fig. 8A). These results indicated that \textit{BIK1} suppresses \textit{PAD4} and senescence gene expression.

To learn whether potential interactions exist between \textit{BIK1} and \textit{PAD4} in cell death-mediated aphid resistance, we examined aphid performance on the \textit{bik1 pad4} double mutant. In no-choice tests, aphid numbers and body weight were both significantly higher on \textit{bik1 pad4} than on \textit{bik1} plants, and were comparable to WT (Figs. 8B, 8C). Honeydew excretion showed the same trend (Fig. 8H). Likewise, in choice tests, aphids showed a strong preference for \textit{bik1 pad4} when paired with \textit{bik1} (Fig. 8D). Apparently, the antibiosis and antixenosis observed in \textit{bik1} diminished when the \textit{pad4} mutation was introduced. The \textit{pad4} mutant did not support more aphid growth than the WT plant, although it attracted more aphids in the choice test. Therefore, the suppression of aphid performance in \textit{bik1} was dependent on elevated basal \textit{PAD4} expression.

Consistent with insect performance, \textit{bik1 pad4} plants displayed phenotypes similar to those of WT in terms of lesion formation, H$_2$O$_2$ accumulation and cell death (Figs. 8E, 8F, 8G).
Inactivation of PAD4 in bik1 blocked the cell death, indicating that PAD4 was required for hypersensitivity and aphid resistance resulting from loss of BIK1 function.

Interestingly, ET emission decreased in bik1 pad4 compared to bik1, both in the presence and absence of aphids (Fig. 9). This observation suggested that PAD4 may positively regulate ET accumulation.

**Loss of BIK1 function did not confer resistance to chewing insects**

Unlike aphids, chewing insects massively damage the host cells during infestation. To assess the role of BIK1 in Arabidopsis defense against chewing insects, we performed bioassays using fall armyworm (Spodoptera frugiperda) neonate larvae placed on 4-week-old WT and bik1 plants (Fig. S3). No significant weight and size differences were detected between larvae reared on the two genotypes (Figs. S3A, B). In addition, fall armyworm elicited comparable H₂O₂ production on WT and bik1 plants (Fig. S3C). The data suggested that BIK1 has distinct roles in Arabidopsis response to two groups of insects that differ in their feeding behaviors. This observation is also different from a previous study showing that TPK1b, the tomato homolog of BIK1, enhances host plant resistance against tobacco hornworm (Manduca sexta) (Abuqamar et al., 2008).

**DISCUSSION**

Plants in the natural environment are constantly challenged by insect herbivory and pathogen infection. As a result, they have developed a plethora of sophisticated means to cope with diverse biotic stresses. Given the common features between plant responses to phloem sap-feeders and pathogens, we studied several PAMP/MAMP signal receptors for involvement in plant response to aphids using their loss-of-function lines. While FLS2, BAK1 and EFR did not seem to be associated with response to aphid infestation, BIK1 acted as a negative regulator of the defense response against aphids. This is in contrast to its positive role in resistance to fungal necrotrophs (Veronese et al., 2006) and flagellin-mediated immune responses (Lu et al., 2010).
Thus, the PAMP-recognition components did not seem to have a parallel role in perceiving or transmitting signals from invading aphids.

**HR-like cell death could be pivotal for aphid resistance in bik1 plants**

The bik1 mutant exhibited heightened resistance to aphids as well as enhanced local H$_2$O$_2$ production and necrotic cell death upon aphid infestation (Figs. 1 and 2). As in plant-microbe interactions, cell death could be either considered a plant defense factor, or viewed as an effect of aphid manipulation of host nutritional quality (Goggin, 2007). Although bik1 plants displayed severe lesion formation, this aphid-induced symptom correlated with impeded aphid colonization, growth and reproduction. Thus, rather than a damage symptom, H$_2$O$_2$ accumulation and cell death represent a major defense mechanism in bik1 to enhance resistance to aphids. These features were limited to aphid-infested bik1 leaves (Fig. 3) and unrelated to dwarfism (Fig. 5; Fig. S1B). Furthermore, SA, JA, ET and ABA did not have major involvement.

Oxidative stress induced by insect feeding is believed to be an important component of plant resistance to invading insects. Detoxification of ROS may decrease antioxidant levels and increase toxic oxidation products in plants as shown in soybean following herbivory by *Helicoverpa zea* (Bi and Felton, 1995). In addition, increased H$_2$O$_2$ and other oxidative products in plants also directly damage the insect midgut and affect growth. Consumption of artificial diets containing even relatively low concentrations of H$_2$O$_2$ caused high mortality of insects (Liu et al., 2010). At high concentrations, ROS can react with almost all cellular macromolecules, including proteins, lipids and DNAs (Van Breusegem and Dat, 2006). Accordingly, the elevated ROS generated in bik1 may result in decreased quantity and quality of nutrients and antioxidants, causing damage to aphid tissues and ultimately reducing their fitness. Furthermore, it is plausible that H$_2$O$_2$-potentiated HR in infected and adjacent cells could limit photoassimilate flow to the feeding sites, although it is questionable how effective such an approach can be, given that aphids can move away from their feeding sites before a sufficient defense response is mounted. Nevertheless, poor aphid performance on bik1 plants relative to WT supported the hypothesis that rapid and potent HR-like cell death placed limitations on aphid infestation.
ROS production, cell death and defense against aphids in *bik1* required functional PAD4

While loss of BIK1 function promoted aphid-induced lesions, no lesions were formed without aphid infestation (Figs. 2, 3). Furthermore, the spread of the aphid-induced lesions in *bik1* required continued aphid feeding (Data not shown). These data suggest that BIK1 does not directly repress but rather indirectly modulates a cell death pathway through an aphid-responsive component. We postulated that BIK1 may exert its negative regulation via PAD4, a lipase-like protein, for the following reasons: First, PAD4 regulates the activation of premature leaf senescence, i.e. a cell death-mediated resistance mechanism against aphids (Pegadaraju et al., 2005), consistent with the tight correlation between HR lesions and resistance we observed in *bik1*. Second, although PAD4 is involved in SA signaling, SA is not important for the defense against aphids conferred by PAD4, agreeing with our conclusion that *bik1* resistance is SA-independent. Third, expression of *PAD4* is induced in response to aphid feeding (Pegadaraju et al., 2005), potentially furnishing an aphid-triggered control point downstream of BIK1. Experimental results demonstrated that PAD4 was required for *bik1* resistance to aphids (Fig. 8). It should be noted that although more aphids preferred *pad4* plants over WT in the choice tests (Fig. 8D), no obvious increase in insect reproduction was observed on *pad4* in the no-choice tests (Fig. 8B). This is in contrast to the observations of Pegadaraju et al. (2005), who reported significantly higher population growth of *M. persicae* on *pad4* than on WT. Differences in plant growth conditions or in insect strain, age and quantity used by the two laboratories could account for the different results. We witnessed relatively mild lesion formation in WT, which may explain the non-significant difference in aphid propagation on WT versus *pad4*. Furthermore, different conditions under which the ROS experiments were performed may explain the discrepancy in time needed for detection of ROS between different labs; in the current *in vivo* study, oral secretion was delivered via the aphid’s fine mouthpart and was only in contact with a very limited number of plant cells, probably making ROS hard to detect in the early stage. Prince et al (2014), on the other hand, used leaf disks submerged in 5 mg/mL GPA-derived extract. It is possible that exposing the entire leaf tissue to a relatively high concentration of aphid elicitors permitted early ROS response. Alternatively, the early response could be triggered by factors in GPA-derived extract that normally would not come into direct contact with the host cells.
We propose that BIK1 modulates cell death and resistance to aphids through its control of PAD4 (Fig. 10). Removal of PAD4 function was sufficient to eliminate the strong HR-like cell death of bik1 and restore its susceptibility to aphids. Ectopic expression of PAD4 triggered more rapid cell death in aphid-infested leaves and stronger resistance to aphids than in WT (Pegadaraju et al., 2007). Inactivation of BIK1 repression in a sense resembles overexpression of PAD4. On the other hand, although aphid feeding induced PAD4 expression and localized cell death in WT plants, DAB staining revealed only marginal differences in H$_2$O$_2$ production between the WT and the pad4 mutant (Fig. 8). These data suggest that in WT plants, BIK1 suppression most likely is the dominant control factor for cell death, prevailing over the stimulus from aphid feeding. It should be pointed out that high basal PAD4 expression alone, i.e. in the bik1 mutant without aphid feeding, was insufficient to result in cell death. Contrasting results of DAB staining of the bik1 mutant with and without aphid treatment appeared to support this assumption. It is possible that PAD4-mediated cell death is initiated and propagated by aphid oral secretion-triggered signaling cascades, which are predominantly repressed by BIK1.

It should be noted that bik1 is not the only mutant conferring PAD4-dependent aphid resistance. Loss of function of SUPPRESSOR OF SALICYLIC ACID INSENSITIVITY (SSI2), a desaturase, resulted in hyper-resistance to aphids, and the resistance required PAD4 as well (Louis et al., 2012). As with bik1, ssi2 resistance diminished in the ssi2 pad4 double mutant. But unlike the bik1 mutant that expressed high basal PAD4 transcript, the ssi2 mutant did not show elevated PAD4 expression in the absence of aphid feeding. Thus, the role of PAD4 in aphid resistance could be regulated by distinct pathways; while bik1 may exert its resistance through releasing the suppression of PAD4 by BIK1, the interaction with SSI2 could be indirect.

**Pleiotropic effects of BIK1**

It is rather counterintuitive, at first glance, that a gene like BIK1 that confers plant susceptibility to invaders exists. A logical explanation could be that it plays an indispensable role in other processes, and/or is involved in multiple pathways in the plant where a balance has to be achieved through cross-talk. Constitutive defense is often associated with fitness costs, e.g.
altered leaf morphology, stunted growth and decreased fertility (Heil and Baldwin, 2002). Evidently, BIK1 is necessary for normal plant growth (Veronese et al., 2006) and seed production (Table S1). High levels of SA may be a major causal factor for the aberrant development and reduced growth of bikl since SA depletion by sid2 and nahG largely restored the WT stature of bikl plants (Fig. 5; Fig. S1B). Furthermore, the defect in SA accumulation in pad4 could be responsible for the near WT plant form and leaf shape of the bikl pad4 double mutant (Fig. 5; Fig. S1D). Indeed, many lesion mimic mutants display altered plant morphology due to production of elevated levels of SA and its constitutive interaction with other pathways (Lorrain et al., 2003). Therefore, it is very likely that BIK1 regulates normal plant growth in part by controlling SA levels. Conversely, bikl ein2-1 and bikl ein3-1 double mutants suffered the same growth suppression and aberrant development as the bikl single mutant, and did not show any phenotypic recovery (Fig. 7; Fig. S1C). Therefore, despite the essential role of ET in plant development, it is unlikely that the elevated ET level contributed to the bikl growth abnormality.

Notably, although BIK1 enhanced susceptibility to aphids, its presence did not block induction of effective aphid resistance genes but reduced their basal expression (Fig. 8). Perhaps, without BIK1 the penalty in general plant fitness imposed by maintaining a defense system in a no-pest environment outweighs an immediately available defense when plants are facing aphid attack. Besides plant development, BIK1 confers resistance to necrotrophic pathogens (Veronese et al., 2006) and is involved in activation of PAMP-triggered signaling pathways (Lu et al., 2010). Our current study showcased the crosstalk among signaling pathways involved in plant development and defense against insects versus pathogens.

In contrast to our results showing that BIK1 negatively regulated resistance to a phloem sap feeder and had no effect on a chewing insect, studies on the BIK1 homolog in tomato, TPK1b, indicate that TPK1b positively regulates plant resistance against herbivory of tobacco hornworm, also a chewing insect (Abuqamar et al., 2008). Since TPK1b rescues the phenotype of the Arabidopsis bikl mutant, i.e. restoring its resistance to Botrytis, TPK1b and BIK1 are thought to perform similar functions in their respective species. The differential, even opposing functions exhibited by BIK1 and TPK1 suggests that the involvement of BIK1 in plant defense
against insects could be shaped by specific insects through their distinct feeding styles and unique interactions with their host plants formed over the long history of coevolution.

Our study has drawn an important link between ROS production/cell death and plant resistance to aphids. However, uncoupling cell death from insect resistance has also been reported in studies with *Medicago truncatula* (Klingler et al., 2009). In these studies, it is clearly demonstrated that HR lesions are not required for resistance to the pea aphid (*Acyrthosiphon pisum*). In plant-pathogen interactions where the HR is often considered a major form of resistance, it has been shown that the *Arabidopsis* defense no death (*dnd*) mutant exhibits enhanced resistance against pathogen infection in the virtual absence of HR cell death (Yu et al., 1998). Further investigation is needed to establish whether the hypersensitivity is the basis for aphid resistance in *bik1* plants. It also remains to be elucidated whether HR lesions directly cause plant defense or if they are the consequence of defensive biochemical reactions activated by aphids.

**MATERIALS AND METHODS**

**Plant growth and aphid rearing**

*Arabidopsis thaliana* was grown in LP5 potting medium (Sun Gro Horticulture, Bellevue, WA) in environmental chambers at 23°C (day) /21°C (night), 65% relative humidity (RH) and 12L/12D photoperiod with a photosynthetic photon flux density of 85 µMoles m⁻²s⁻¹. For plant damage evaluation, histochemical assays and aphid no-choice tests, 4 to 5-week-old plants were used. For plant gene expression analyses and hormone measurements, as well as for aphid choice tests, 3 to 4-week-old plants were used.

Phloem sap-feeding green peach aphids *M. persicae* (a tobacco-adapted red lineage, kind gift from Dr. Georg Jander, Boyce Thompson Institute for Plant Research, Cornell University, NY) were cultured on cabbage (*Brassica oleracea*) and maintained in an environmental chamber at 21°C, 65% RH, and 12L/12D photoperiod (63 µMoles m⁻²s⁻¹). All insect treatments and bioassays were performed in this chamber.
Arabidopsis lines

The previously reported Arabidopsis lines, wild-type Col-0 and mutants fls2 (SALK_141277), fls2 (SALK_062054), efr, bak1-3, bak1-4, bik1, sid2, nahG, bik1 sid2, bik1 nahG, ein2-1, ein3-1, pad4, bik1 pad4 and the bik1 complementation line bik1+BIK1 used in this study (Jirage et al., 1999; Veronese et al., 2006; Lu et al., 2010; Laluk et al., 2011; Lin et al., 2013) were kindly provided by Dr. T. Mengiste at Purdue University or obtained from the Arabidopsis Biological Resource Center, Ohio State University. To generate bik1 ein2-1 and bik1 ein3-1 double mutants, we crossed bik1 with ein2-1 and ein3-1 using bik1 as the female parental line. The F2 seeds were germinated in the dark on Murashige and Skoog agar medium containing 50 µM 1-aminocyclopropane-1-carboxylic acid. The seedlings that lacked a triple response were selected and transferred to soil. The presence of ein2-1 and ein3-1 was confirmed by the derived cleaved amplified polymorphic sequence (dCAPS) method as previous described, with modification (Nandi et al., 2003; Binder et al., 2007; Bouchez et al., 2007; Chen et al., 2009). For ein2-1 genotyping, a 195 bp fragment flanking the point mutation was amplified by PCR, followed by purification and AflII restriction digestion. AflII cut the mutant sequence into 160 bp and 35 bp fragments but left the WT sequence intact. For ein3-1, the 222 bp PCR product remained intact in the mutant sequence but was cut by HaeIII into 190 bp and 32 bp fragments in the WT sequence. DNA fragments were resolved on 2% agarose gel. For bik1 genotyping, a procedure developed previously was followed (Lu et al., 2010).

Insect bioassays

Aphid no-choice and choice tests were performed to assess the antibiotic and antixenotic resistance of different Arabidopsis genotypes. For the no-choice tests, 6 age-synchronized second instar nymphs (within 24 hr) were placed on 4-week-old plants. The total aphid population (adult and nymph) on each plant was counted 7 days after infestation. Each genotype had at least 10 replicates. For the choice tests, 35 adults were released at an equal distance between two plants of different genotypes. The number of adult aphids settled on each plant was
recorded 6 and 24 hours after releasing. At least 10 pairs of plants were used in each comparison. All experiments were repeated at least three times and a representative data set was presented.

To obtain the average adult aphid body weight, adult aphids were transferred to WT or bik1 plants and removed 24 hrs later to produce age-synchronized progenies. Ten days later, the new generations of adults reared on Arabidopsis genotypes were collected and were weighed as 6 groups of 10 aphids each.

Eggs of fall armyworm, purchased from Benzon Research Inc (Carlisle, PA), were incubated in a growth chamber (27°C and 65% RH). Newly hatched larvae were transferred to 4-week-old WT or bik1 plants. Plants were replaced once a week to ensure sufficient food supply. Larvae reared on Arabidopsis genotypes were weighed after feeding for 16 or 22 days. At least 30 larvae were measured for each genotype.

Ninhydrin staining and quantification of aphid honeydew

Honeydew production served as an indicator of insect feeding activity. To determine honeydew secretion, Whatman filter papers, protected by a plastic membrane to avoid absorbance of water from soil, were placed under Arabidopsis plants of various genotypes infested by 30 adult aphids. These filter papers were collected 1, 2 and 3 days after aphid infestation, soaked in 0.1% ninhydrin in acetone, and dried in a 65°C oven for 30 min. Honeydew stained by ninhydrin was shown as purple spots (Kim and Jander, 2007).

To quantify the honeydew stains, the filter papers were cut into pieces and stains were extracted into 1 mL of 90% methanol for 1 h at 4°C with continuous agitation. After centrifugation at 6,000 g for 1 min, the absorbance of the supernatant was measured at 500 nm (Nisbet et al., 1994). Methanol (90%) served as a blank.

Plant damage and histochemical assays
Four to five-week-old *Arabidopsis* plants were infested with adult aphids taking into consideration the variation of the rosette size of each genotype. Accordingly, 48 aphids were placed on WT, *fls2*, *efr*, *bak1-3*, *bak1-4*, *bik1+BIK1*, *sid2*, *nahG*, *ein2-1*, *ein3-1* and *pad4* (sizes comparable to WT), 12 on *bik1*, *bik1 ein2-1* and *bik1 ein3-1* (one quarter the size of WT), and 24 on *bik1 sid2*, *bik1 nahG* and *bik1 pad4* (one half size of WT). Plants were examined daily to identify symptoms of yellowing and lesion formation. Digital images were taken of representative leaves at 6-days post aphid infestation. Leaves obtained in the same manner were subjected to histochemical assay (see below). For every experiment, eight plants or more of each genotype were used. All experiments were repeated at least 3 times.

To visualize H$_2$O$_2$ accumulation, 3,3'-diaminobenzidine (DAB) staining was performed. Leaves at 6-days post infestation, as well as control leaves, were collected and vacuum-infiltrated with DAB solution (1 mg/ml DAB, in pH 3.5 water) in a 6-well titer plate. After an overnight incubation in the same solution in darkness, the leaves were destained in 95% ethanol until they turned clear. Images were then captured with a digital camera.

To determine local and systemic ROS accumulation, aphids were placed in clear plastic cups (4 cm diameter, 4 cm height) with mesh cloth replacing the bottoms for ventilation. Twenty insects were used for WT, and 10 for *bik1*. The cage was fitted around the leaf petiole between the cap and the cup, and sealed with cotton to avoid wounding as well as aphid escape, restricting the aphids onto one 4-week-old *Arabidopsis* leaf for the desired time (Kim and Jander, 2007). Caged leaves without aphids served as controls. After treatments, the cages were removed and leaves were excised for DAB staining.

Trypan blue staining was performed to visualize cell death. Trypan blue was dissolved in lactophenol solution (phenol: lactic acid: glycerol: water [1: 1: 1: 1]) at a concentration of 0.125 mg/mL. Leaves prepared as above were boiled in this staining solution for 1 min. After cooling, leaf samples were destained in 95% ethanol, and photographed with an Olympus SZX2-ILLK microscope (Olympus Corporation, Tokyo, Japan).
The accumulation of autofluorescent compounds and deposition of callose are features of HR lesions (Hunt et al., 1997). Lesions on *Arabidopsis* leaves were examined 6 days after aphid infestation using the Olympus microscope under bright field or UV excitation with a green fluorescent protein (GFP) filter. Images of lesions and autofluorescence emitted from the same lesion sites were recorded (Stewart et al., 2009).

Aniline blue staining (Clay et al., 2009) was performed to detect callose deposition. *Arabidopsis* leaves were fixed in buffer containing 10% formaldehyde, 5% acetic acid, and 50% ethanol at 37°C overnight. Slightly translucent leaves were then washed in 95% ethanol several times until clear, rinsed twice in water, and then stained for 4 hr or longer in the dark with 0.01% aniline blue in 150 mM K$_2$HPO$_4$ (pH 9.5). Callose deposits were visualized with an Olympus IX-81 microscope at 10x magnification under UV illumination with a broadband DAPI filter set.

**JA, SA and ABA measurements**

For SA, JA and ABA measurements, 3-week-old plants were infested with aphids (30 per plant). Two days later, treated or control plants were ground to a fine powder in liquid nitrogen. For each sample replicate, ground tissue (60 mg) and a mixture of stable isotope-labeled hormones including 10 ng $^2$H$_4$-SA, 3.8 ng $^{13}$C$_2$-JA, and 1 ng of $^2$H$_6$-ABA were added to a 5 mL glass tube with 500 μL of methanol at 55°C, and extracted by vortexing three times during a 10 min incubation. The tissue was re-extracted with 500 μL methanol, and then once with 500 μL of 80% ethanol warmed to 55°C, centrifuging and pooling the cleared supernatants after each extraction. The pooled extracts were dried and the residue was resuspended in 800 μL of chloroform and partitioned against 1 mL of H$_2$O adjusted to pH 9.0 with NH$_4$OH. The aqueous fraction was recovered, adjusted to pH 5.0 with acetic acid and partitioned against 1 mL of ethyl acetate. The organic fraction was transferred to a Reactivial, dried, and then methylated with ethereal diazomethane. Samples were then analyzed on an Agilent 7890A/7693A/5975C XL GC-MS equipped with a 0.25 mm x 30 M DB-5MS column (0.25μ film) using pulsed splitless injection. Helium was used as the carrier gas at 0.75 mL/min. The inlet was maintained at 250°C and the oven was ramped from 45°C (2.25 min initial hold) to 250°C at 40°C per minute, held at 250°C for 3 min, and then ramped to 290°C at 40°C per min. The ion source temperature
was maintained at 230°C and the quadrupole was heated to 150°C. The ion source was operated in electron impact mode and both scan and selected ion data were acquired. Two ions were monitored for each hormone, and the larger fragment was used for peak area quantification (SA-120,124,152,156; JA-193,195,224,226; ABA-162,166,190,194 m/z).

**ET measurement and 1-MCP treatment**

Three-week old *Arabidopsis* were infested with aphids (30 per plant) for 2 days. Shoots were excised, weighed and kept in 10 mL-syringes with 3-way stopcocks to seal them. One hour later, 1 mL of headspace gas was injected into a Photovac 10SPlus gas chromatograph (Photovac, Markham, Ontario, Canada). At least 6 individual plants were averaged for each treatment. Each experiment was repeated at least three times. ET was quantified by integration of peak area, relative to an authentic standard (Finlayson et al., 2007).

1-methylcyclopropene (1-MCP) gas was generated by dissolving a solid formulation of a proprietary 1-MCP α-cyclodextrin complex (AgroFresh) in 0.1N NaOH in a flask fitted with a septum. The mass of the 1-MCP α-cyclodextrin complex used was calculated to produce 1000 ppm 1-MCP gas in the headspace of the flask. An aliquot of the concentrated 1-MCP gas was then injected into a desiccator to give a final calculated concentration of 1 ppm. Plants in the desiccator thus were subjected to 1-MCP treatment. After 1 hr exposure to 1-MCP, plants were brought to a normal environmental atmosphere. This procedure was repeated every 12 hr for 5 days to maintain the effect of 1-MCP, followed by aphid choice tests. Control plants were handled in the same manner without 1-MCP gas.

**Quantitative RT-PCR**

Plant samples were harvested, frozen and ground in liquid nitrogen to a fine powder. Total RNA was extracted with TRIZol Reagent (Invitrogen, Carlsbad, CA) and then treated with RNase-free DNase (QIAGEN, Valencia, CA). Equal amounts of RNA (2 µg) were used to synthesize cDNA with random hexamer primers and SuperScript™ II Reverse Transcriptase (Invitrogen). Quantitative RT-PCR (qRT-PCR) reactions were performed using SYBR Green
Mastermix (BioRad, Hercules, CA) according to the manufacturer’s protocol. Primers were
designed using PerlPrimer software (Marshall OJ, 2004), and their quality was examined using
NCBI Primer Blast. *Arabidopsis UBQ10* (AT4G05320) served as an internal control for data
normalization. qRT-PCR was run on an ABI Prism 7900HT Sequence Detection System
(Applied Biosystems, Foster City, CA). Controls using untranscribed RNA confirmed that there
was no genomic DNA contamination. Dissociation curve analyses were applied to check
amplification specificity. The mean fold change in gene expression was calculated as described
previously (Zhu-Salzman et al., 2003).

**Statistical analysis**

SPSS 16.0 software (SPSS Inc, Chicago IL) was used for analyses of all data. The no-
choice tests of aphid performance among genotypes were analyzed by one-way ANOVA.
Tukey’s multiple range test analysis was used for pairwise comparisons of the difference
between treatments for mean separation ($P < 0.05$). The Chi-square test was applied to the aphid
choice tests ($P < 0.05$).

**Accession numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or
GenBank/EMBL databases under the following accession numbers: BIK1 (AT2G39660), FLS2
(AT5G46330), EFR (AT5G20480), BAK1 (AT4G33430), ERF1 (AT3G23240), PDF1.2
(AT5G44420), PR1 (AT2G14610), MYC2 (AT1G32640), SID2 (AT1G74710), EIN2
(AT5G03280), EIN3 (AT3G20770), PAD4 (AT3G52430), and SAG13 (AT2G29350).

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Signaling from Multiple Plant Immune Receptors and Are Targeted by a *Pseudomonas syringae* Effector. Cell Host & Microbe 7: 290-301


FIGURE LEGENDS

Figure 1. Loss of BIK1 function confers resistance to green peach aphids. (A) No-choice tests and (B) aphid body weight of indicated genotypes. For no-choice tests, 6 second instar nymphs were inoculated on each plant (4-5 weeks old). Total aphid numbers were recorded 7 days later. At least 10 replications were performed for each genotype. To obtain average body weight of adult aphids, neonates were reared on WT or bik1 for 10 days. Adults were then collected and were weighed as 6 groups of 10 aphids each. (C) Choice tests. Three-week old plants were used. At this developmental stage, no apparent size differences were observed between genotypes including the WT vs. bik1 pair. Settled aphids were counted 6 hr after releasing 35 adults in between two plants of the tested genotypes. Each test was comprised of 10 replicates. Inset image of the shoot phenotypes of 3-week old, uninfested WT and bik1. (D) Aphids on bik1 excreted less honeydew than those reared on WT. Quantity of honeydew secretion was correlated with the area and intensity of ninhydrin stains (left) and with OD500 values (right). (E) Expression of BIK1 cDNA confers WT levels of aphid susceptibility to bik1. One-way ANOVA was applied to no-choice tests and the Chi-square test was used to analyze data derived from choice tests. Body weight and honeydew secretion data were analyzed by independent samples t-test. Bars represent means ± standard error (SE). Statistical significance for treatment effects is marked *(P < 0.05), **(P < 0.01) or ***(P < 0.001). Means with different letters were significantly different (P < 0.05).

Figure 2. Aphid infestation induces a heightened hypersensitive response in bik1. Representative leaf images of (A) lesion formation, (B) DAB staining (H2O2 indicator) and (C) trypan blue staining (cell death indicator) prior to (top panel) or 6 days after (bottom panel) aphid infestation of genotypes indicated. (D) Autofluorescence of aphid-induced lesion spots under UV excitation with green fluorescent protein filter set (right). The same fields of view are shown under visible light (left). (E) Callose deposition at lesion sites. Left: control leaves; right: callose deposition after aphid treatment. Arrows point to lesion sites. (F) Relative expression of BIK1 in WT plants in the presence and absence of aphid infestation. Three-week-old plants were infested with aphids as described in Materials and Methods. Data were analyzed by independent samples t-test. Means with different letters were significantly different (P < 0.05).
**Figure 3.** *bik1* exhibits earlier and stronger ROS accumulation in locally infested leaves compared with WT.

(A) DAB staining (H$_2$O$_2$ indicator) of aphid-infested leaves collected at 3, 6, 12 and 24 hrs post infestation. Four-week-old *Arabidopsis* plants were infested with aphids using the caged-leaf method as described in Materials and Methods. Caged (24 hr) but uninfested leaves served as a control. Scale bars =1.0 cm. (B) DAB staining of local, infested and systemic, uninfested leaves of the same plant at 24 hrs post infestation. All leaves were caged. Controls were caged leaves from uninfested plants. Experiments were repeated 3 times.

**Figure 4.** *bik1* shows higher basal and induced levels of SA and ET and elevated expression of their marker genes during aphid infestation than WT.

(A) SA, JA, ABA and ET levels in WT and *bik1* before and after aphid infestation. Three-week-old plants were infested with aphids for 48 hrs. Four replicates were used for each genotype. Hormone measurements were performed as described in Materials and Methods. Data were analyzed by independent samples t-test ($P < 0.05$). Different lowercase letters indicate significant differences between genotypes within the same treatment. Different uppercase letters indicate significant differences between treatments within the same genotype. (B) Relative expression of SA, JA and ET marker genes, *PR1*, *MYC2*, *ERF1* and *PDF1.2* in response to aphid feeding at 0 and 48 hr time points. Data were analyzed by one-way ANOVA. Tukey’s multiple range test analysis was used for pairwise comparisons of the difference between treatments for mean separation ($P < 0.05$).

**Figure 5.** SA is not required for resistance to aphids and is not responsible for heightened hypersensitive response in *bik1*.

(A) No-choice and (B) Choice tests on genotypes indicated. (C, D, E) Representative leaf images of 4 to 5-week-old plants (C), DAB staining (D, H$_2$O$_2$ indicator) and trypan blue staining (E, cell death indicator) before (top panel) or after aphid infestation (bottom panel). (F) Ninhydrin staining of honeydew after 48 hr aphid feeding. All experiments were performed as described in in Materials and Methods. Bars represent means ± SE. Statistical significance for
treatment effects is marked * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$). Means with different letters were significantly different ($P < 0.05$).

**Figure 6.** 1-MCP temporarily attenuates bik1 deterrence of aphids. Choice tests between 3-week-old WT and bik1 plants in the presence and absence of 1-MCP. Settled aphids were recorded 6 and 12 hr after aphid infestation. Application of 1-MCP began 5 days prior to choice tests, and was reapplied every 12 hrs to prevent the loss of its effectiveness. Control plants were subjected to the same manipulation without 1-MCP. Statistical significance for treatment effects is marked * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

**Figure 7.** Elevated ET increases bik1 repellence against aphids but shows no effect on aphid reproduction or on aphid-induced plant hypersensitive response. (A) No-choice and (B) Choice tests on genotypes and at time points as indicated. (C, D, E) Representative leaf images of 4 to 5-week-old plants (C), DAB staining (D, H$_2$O$_2$ indicator) and trypan blue staining (E, cell death indicator) before (top panel) or after aphid infestation (bottom panel). (F) Ninhydin staining of honeydew after 48 hr aphid feeding. All experiments were performed as described in in Materials and Methods. Bars represent means ± SE. Statistical significance for treatment effects is marked * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$). Means with different letters were significantly different ($P < 0.05$).

**Figure 8.** Resistance to aphids and aphid-induced hypersensitive response in bik1 were PAD4-dependent. (A) Relative expression of PAD4 and SAG13 in WT and bik1 plants in the presence and absence of aphid infestation. Three-week-old plants were infested with aphids as described in Materials and Methods. (B) No-choice test, (C) average aphid body weight, and (D) choice tests were performed on genotypes indicated. (E, F, G) Representative leaf images of 4 to 5-week-old plants (E), DAB staining (F, H$_2$O$_2$ indicator) and trypan blue staining (G, cell death indicator) before (top panel) or after aphid infestation (bottom panel). (H) Ninhydin staining of honeydew after 48 hr aphid feeding. Bars represent means ± SE. Statistical significance for treatment effects is marked *($P < 0.05$), **($P < 0.01$) or ***($P < 0.001$). Means with different letters were significantly different ($P < 0.05$).
Figure 9. PAD4 potentially promotes ET production.

ET production by WT, bik1, bik1 pad4 and pad4 plants measured before or after 48 hr aphid infestation as described in Materials and Methods. Bars represent means ± SE from at least 6 individual plants. Different lowercase letters indicate significant differences between genotypes by one-way ANOVA and Tukey’s multiple range test ($P < 0.05$). Different uppercase letters indicate significant differences between treatments by an independent samples t-test ($P < 0.05$).

Figure 10. Model depicting Arabidopsis resistance to aphids conferred by bik1 mutant.

PAD4 is a positive regulator of aphid-induced plant antibiotic and antixenotic responses. PAD4-regulated defense, potentially resulting from ROS-mediated cell death, is suppressed by BIK1. Based on the intensity of DAB staining, the BIK1 suppression is presumably much stronger than the aphid induction, illustrated by thicker lines in the graph. BIK1 also suppresses SA and ET accumulation. SA has no direct influence on resistance to aphids. ET increased host repellence early on, possibly prior to significant ROS production.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Mature shoot phenotypes of various Arabidopsis genotypes used in the study.

Shoot phenotypes of (A) 4 to 5-week-old WT, fls2 (SALK_141277), fls2 (SALK_062054), efr, bak1-3, bak1-4, bik1 and bik1+BIK1 Arabidopsis, and of mutants used in plant damage assays in evaluation of effects of (B) SA, (C) ET, and (D) PAD4 on bik1-mediated hypersensitivity and resistance to aphids.

Figure S2. The effect of ein3-1 mutation on bik1-mediated resistance against aphids.

(A) No-choice and (B) Choice tests on genotypes and at time points as indicated. (C, D, E) Representative leaf images of 4 to 5-week-old plants (C), DAB staining (D), and trypan blue staining (E) before (top panel) or after aphid infestation (bottom panel). All experiments were performed as described in in Materials and Methods. Bars represent means ± SE. Statistical
significance for treatment effects is marked * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).
Means with different letters were significantly different ($P < 0.05$).

**Figure S3.** Loss of BIK1 function did not confer *Arabidopsis* resistance to fall armyworm.
(A) Comparison of larval body weight after 16 d or 22 d feeding on WT or *bik1* plants ($n = 30$).
Different letters indicate significant differences between samples ($P < 0.05$). (B) Images of representative larvae feeding on each genotype. (C) Images of DAB-stained WT and *bik1* plants after aphid and fall armyworm feeding.

**SUPPLEMENTAL MATERIALS**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Mature shoot phenotypes of various *Arabidopsis* genotypes used in the study.

**Supplemental Figure 2.** The effect of *ein3-1* mutation on *bik1*-mediated resistance against aphids.

**Supplemental Figure 3.** Loss of BIK1 function did not confer *Arabidopsis* resistance to fall armyworm.

**Supplemental Table 1.** Loss of BIK1 function negatively affects growth and reproduction traits in *Arabidopsis*

**Supplemental Table 2.** Primers used in this study.
Figure 1. Loss of BIK1 function confers resistance to green peach aphids. (A) No-choice tests and (B) aphid body weight of indicated genotypes. For no-choice tests, 6 second instar nymphs were inoculated on each plant (4-5 weeks old). Total aphid numbers were recorded 7 days later. At least 10 replications were performed for each genotype. To obtain average body weight of adult aphids, neonates were reared on WT or bik1 for 10 days. Adults
were then collected and were weighed as 6 groups of 10 aphids each. (C) Choice tests. Three-week old plants were used. At this developmental stage, no apparent size differences were observed between genotypes including the WT vs. bik1 pair. Settled aphids were counted 6 hr after releasing 35 adults in between two plants of the tested genotypes. Each test was comprised of 10 replicates. Inset image of the shoot phenotypes of 3-week old, uninfested WT and bik1. (D) Aphids on bik1 excreted less honeydew than those reared on WT. Quantity of honeydew secretion was correlated with the area and intensity of ninhydrin stains (left) and with OD<sub>500</sub> values (right). (E) Expression of BIK1 cDNA confers WT levels of aphid susceptibility to bik1. One-way ANOVA was applied to no-choice tests and the Chi-square test was used to analyze data derived from choice tests. Body weight and honeydew secretion data were analyzed by independent samples t-test. Bars represent means ± standard error (SE). Statistical significance for treatment effects is marked *(P < 0.05), **(P < 0.01) or ***(P < 0.001). Means with different letters were significantly different *(P < 0.05).
Figure 2. Aphid infestation induces a heightened hypersensitive response in bik1.
Representative leaf images of (A) lesion formation, (B) DAB staining (H$_2$O$_2$ indicator) and (C) trypan blue staining (cell death indicator) prior to (top panel) or 6 days after (bottom panel) aphid infestation of genotypes indicated. (D) Autofluorescence of aphid-induced lesion spots under
UV excitation with green fluorescent protein filter set (right). The same fields of view are shown under visible light (left). (E) Callose deposition at lesion sites. Left: control leaves; right: callose deposition after aphid treatment. Arrows point to lesion sites. (F) Relative expression of BIK1 in WT plants in the presence and absence of aphid infestation. Three-week-old plants were infested with aphids as described in Materials and Methods. Data were analyzed by independent samples t-test. Means with different letters were significantly different ($P < 0.05$).
**Figure 3.** *bik1* exhibits earlier and stronger ROS accumulation in locally infested leaves compared with WT.

(A) DAB staining (H$_2$O$_2$ indicator) of aphid-infested leaves collected at 3, 6, 12 and 24 hrs post infestation. Four-week-old *Arabidopsis* plants were infested with aphids using the caged-leaf method as described in Materials and Methods. Caged (24 hr) but uninfested leaves served as a control. Scale bars =1.0 cm. (B) DAB staining of local, infested and systemic, uninfested leaves of the same plant at 24 hrs post infestation. All leaves were caged. Controls were caged leaves from uninfested plants. Experiments were repeated 3 times.
**Figure 4.** *bik1* shows higher basal and induced levels of SA and ET and elevated expression of their marker genes during aphid infestation than WT.

**(A)** SA, JA, ABA and ET levels in WT and *bik1* before and after aphid infestation. Three-week-old plants were infested with aphids for 48 hrs. Four replicates were used for each genotype. Hormone measurements were performed as described in Materials and Methods. Data were analyzed by independent samples t-test ($P < 0.05$). Different lowercase letters indicate significant differences between genotypes within the same treatment. Different uppercase letters indicate significant differences between treatments within the same genotype. **(B)** Relative expression of SA, JA and ET marker genes, *PR1, MYC2, ERF1* and *PDF1.2* in response to aphid feeding at 0 and 48 hr time points. Data were analyzed by one-way ANOVA. Tukey’s multiple range test analysis was used for pairwise comparisons of the difference between treatments for mean separation ($P < 0.05$).
Figure 5. SA is not required for resistance to aphids and is not responsible for heightened hypersensitive response in bik1.

(A) No-choice and (B) Choice tests on genotypes indicated. (C, D, E) Representative leaf images of 4 to 5-week-old plants (C), DAB staining (D, H$_2$O$_2$ indicator) and trypan blue staining (E, cell death indicator) before (top panel) or after aphid infestation (bottom panel). (F) Ninhydrin staining of honeydew after 48 hr aphid feeding. All experiments were performed as described in in Materials and Methods. Bars represent means ± SE. Statistical significance for treatment effects is marked * (P < 0.05), ** (P < 0.01) or *** (P < 0.001). Means with different letters were significantly different (P < 0.05).
Figure 6. 1-MCP temporarily attenuates *bik1* deterrence of aphids.
Choice tests between 3-week-old WT and *bik1* plants in the presence and absence of 1-MCP. Settled aphids were recorded 6 and 12 hr after aphid infestation. Application of 1-MCP began 5 days prior to choice tests, and was reapplied every 12 hrs to prevent the loss of its effectiveness. Control plants were subjected to the same manipulation without 1-MCP. Statistical significance for treatment effects is marked * (P < 0.05), ** (P < 0.01) or *** (P < 0.001).
Figure 7. Elevated ET increases *bik1* repellence against aphids but shows no effect on aphid reproduction or on aphid-induced plant hypersensitive response.

(A) No-choice and (B) Choice tests on genotypes and at time points as indicated. (C, D, E) Representative leaf images of 4 to 5-week-old plants (C), DAB staining (D, H$_2$O$_2$ indicator) and trypan blue staining (E, cell death indicator) before (top panel) or after aphid infestation (bottom panel). (F) Ninhydrin staining of honeydew after 48 hr aphid feeding. All experiments were performed as described in Materials and Methods. Bars represent means ± SE. Statistical significance for treatment effects is marked * (P < 0.05), ** (P < 0.01) or *** (P < 0.001). Means with different letters were significantly different (P < 0.05).
Figure 8. Resistance to aphids and aphid-induced hypersensitive response in bik1 were PAD4-dependent.

(A) Relative expression of PAD4 and SAG13 in WT and bik1 plants in the presence and absence of aphid infestation. Three-week-old plants were infested with aphids as described in Materials and Methods. (B) No-choice test, (C) average aphid body weight, and (D) choice tests were performed on genotypes indicated. (E, F, G) Representative leaf images of 4 to 5-week-old plants (E), DAB staining (F, H2O2 indicator) and trypan blue staining (G, cell death indicator) before (top panel) or after aphid infestation (bottom panel). (H) Ninhydrin staining of honeydew after 48 hr aphid feeding. Bars represent means ± SE. Statistical significance for treatment effects is marked *(P < 0.05), **(P < 0.01) or ***(P < 0.001). Means with different letters were significantly different *(P < 0.05).
**Figure 9.** PAD4 potentially promotes ET production.

ET production by WT, *bik1*, *bik1 pad4* and *pad4* plants measured before or after 48 hr aphid infestation as described in Materials and Methods. Bars represent means ± SE from at least 6 individual plants. Different lowercase letters indicate significant differences between genotypes by one-way ANOVA and Tukey’s multiple range test (*P* < 0.05). Different uppercase letters indicate significant differences between treatments by an independent samples t-test (*P* < 0.05).
PAD4 is a positive regulator of aphid-induced plant antibiotic and antixenotic responses. PAD4-regulated defense, potentially resulting from ROS-mediated cell death, is suppressed by BIK1. Based on the intensity of DAB staining, the BIK1 suppression is presumably much stronger than the aphid induction, illustrated by thicker lines in the graph. BIK1 also suppresses SA and ET accumulation. SA has no direct influence on resistance to aphids. ET increased host repellence early on, possibly prior to significant ROS production.