BOTRYTIS-INDUCED KINASE1 Modulates Arabidopsis Resistance to Green Peach Aphids via PHYTOALEXIN DEFICIENT4

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BOTRYTIS-INDUCED KINASE1 (BIK1) plays important roles in induced defense against fungal and bacterial pathogens in Arabidopsis (Arabidopsis thaliana). Its tomato (Solanum lycopersicum) 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 hydrogen peroxide 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.

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 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 coevolution, 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 antibiotics, 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; Moran and Thompson, 2001).
Zhu-Salzman et al., 2004; De Vos et al., 2005; Thompson and Goggin, 2006; Kušniereczky 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 green peach aphids (Myzus persicae) when raised on the Arabidopsis (Arabidopsis thaliana) constitutive expression of vegetative storage protein1 mutant constantly expressing JA responses, whereas the JA-insensitive mutant coronatine-insensitive1 supports more rapid growth of aphids than wild-type 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 (Solanum lycopersicum), ET biosynthesis renders plants more susceptible to potato aphids (Macrosiphum euphorbiae; Mantelin et al., 2009). However, the Arabidopsis ET-insensitive mutant ein2 promotes performance of green peach aphids (Kettes 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 also has been implicated as a modulator of plant immune via signaling cross talk (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 hydrogen peroxide (H2O2). 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 H2O2, 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 H2O2 levels as well as HR-like cell death in rice (Oryza sativa) expressing an antisense lipoygenase (Zhou et al., 2009). 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 by promoting premature leaf senescence and cell death (Pegadaraju et al., 2005, 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-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (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 (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006). Upon binding to their cognate MAMPs, FLS2 or EFR associate with another RLK, BRASSINOSTEROID INSENSITIVE1-ASSOCIATED RECEPTOR KINASE (BAK1; Chinchilla et al., 2007). BOTRYTIS-INDUCED KINASE1 (BKI1), 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 BK1, 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 as well as to whiteflies (Bemisia tabaci) and root-knot nematodes (Meloidogyne incognita; Rossi et al., 1998; Vos et al., 1998; Nombele et al., 2003).

In this study, we examined the roles of several RLCKs, including FLS2, EFR, BAK1, and BK1, in Arabidopsis response to aphid infestation. We challenged these loss-of-function mutants with green peach aphids, 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 nonpreference (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 plants (ecotype Columbia-0; 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 one-half that on wild-type 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 the wild type. In the choice tests, approximately twice as many aphids preferred wild-type 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 due to loss of BIK1 function via complementation experiments. Transgenic plants expressing BIK1 complementary DNA (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; Supplemental Table S1), when choice tests were performed. Later, *bik1* mutants exhibited growth defect and were smaller than the wild type (Supplemental Fig. S1; Supplemental Table S1). However, the antibiotic activity was unlikely due to their small stature, as inoculating six second-instar nymphs and rearing them for 7 d on 4- to 5-week-old plants would by no means result in a population limited by space or nutrients.

Aphids Induced HR-Like Lesions in *bik1*

Despite an enhanced resistance to aphid infection, *bik1* began to show apparent lesion spots approximately 5 d after aphid infestation, while no visible lesions were observed in *fls2*, *efr*, and *bak1* mutants or in the wild type (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. Because *bik1* plants are dwarfs, the number of aphids applied was adjusted by a ratio proportional to the rosette area and intensity of ninhydrin stains (left) and with optical density at 500 nm values (right). E, Expression of *AtBIK1* represses plant resistance against aphids. No-choice tests (A) and aphid body weight (B) of indicated genotypes. For no-choice tests, six second-instar nymphs were inoculated on each plant (4-5 weeks old). Total aphid numbers were recorded 7 d later. At least 10 replications were performed for each genotype. To obtain average body weight of adult aphids, neonates were rear on the wild type (WT) or *bik1* for 10 d. Adults were then collected and were weighed as six 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 wild-type versus *bik1* pair. Sedated aphids were counted 6 h 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 the 3-week-old, uninfested wild type and mutants or in the wild type (Fig. 2A).
area. For plant symptom assessment, this ratio was applied for all genotypes exhibiting size differences relative to the wild type 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 the wild type and the other mutants by the trypan blue staining assay (Fig. 2C). By contrast, fls2, cfr, and bak1 mutants showed phenotypes similar to wild-type 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 (Fig. 2, D and E), which are also HR lesion-associated histological markers (Hunt et al., 1997; Luna et al., 2011; Williams et al., 2011). Wild-type 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; Kuśnierzyczyk et al., 2008).

Because cellular H$_2$O$_2$ accumulation precedes cell death (Hoeberichts and Woltering, 2003), earlier time points were chosen for DAB staining. Staining became apparent within 3 h upon aphid infestation in bik1 leaves but was absent from the infested wild-type leaves over the 24-h course of the experiment (Fig. 3A). When aphids were caged on specific leaves, H$_2$O$_2$ 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 H$_2$O$_2$ 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 wild-type and bik1 plants (Fig. 4A). Elevated basal SA, consistent with previous studies showing that SA is not essential for aphid defense in Arabidopsis (Pegadaraju et al., 2005). By contrast, a correlation was observed between resistance to aphids in the wild type. 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 (Fig. 5, C–E), supporting previous studies showing that SA is not essential for aphid defense in Arabidopsis (Pegadaraju et al., 2005). By 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 the wild type 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 *Pseudomonas syringae* pv *tomato* 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.
To impair ET signaling in a genetic approach was limited to choice tests. To presumably due to loss of 1-MCP function.

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-methylcyclopene (1-MCP), an inhibitor of ET action that binds to the ET receptor. In choice tests, there was no repellence initially in choice tests but appeared to play little role in cell death-mediated defense in bik1.

PAD4 is a lipase-like protein that, upon aphid feeding, promotes premature leaf senescence to suppress insect reproduction and colonization (Pegadaraju et al., 2005, 2007). Aphids induced PAD4 expression in both bik1 and the wild type (Fig. 8A). Compared with the wild-type plants, bik1 had much higher PAD4 basal expression. Consistently, a senescence marker gene, SENESCESS ASSOCIATED GENE13 (SAG13), regulated by PAD4 during aphid infestation (Weaver et al., 1998; Pegadaraju et al., 2005) shared a similar expression pattern with PAD4 (Fig. 8A). These results indicated that BIK1 suppresses PAD4 and senescence gene expression.

To learn whether potential interactions exist between BIK1 and PAD4 in cell death-mediated aphid resistance, we examined aphid performance on the bik1 pad4 double mutant. In no-choice tests, aphid numbers and body weight were both significantly higher on bik1 pad4 than on bik1 plants and were comparable to the wild type (Fig. 8B and C). Honeydew excretion showed the same trend (Fig. 8H). Likewise, in choice tests, aphids showed a strong preference for bik1 pad4 when paired with bik1 (Fig. 8D). Apparently, the antibiosis and antixenosis observed in bik1 diminished when the pad4 mutation was introduced. The pad4 mutant did not support more aphid growth than the wild-type plant, although it attracted more aphids in the choice test. Therefore, the suppression of aphid performance in bik1 was dependent on elevated basal PAD4 expression.
Consistent with insect performance, **bik1 pad4** plants displayed phenotypes similar to those of the wild type in terms of lesion formation, \( \text{H}_2\text{O}_2 \) accumulation, and cell death (Fig. 8, E–G). Inactivation of PAD4 in **bik1** blocked the cell death, indicating that PAD4 was re- quired for hypersensitivity and aphid resistance resulting from loss of BIK1 function.

Interestingly, ET emission decreased in **bik1 pad4** compared with **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 wild-type and **bik1** plants (Supplemental Fig. S3). No significant weight and size differences were detected between larvae reared on the two genotypes (Supplemental Fig. S3, A and B). In addition, fall armyworm elicited comparable \( \text{H}_2\text{O}_2 \) production on wild-type and **bik1** plants (Supplemental 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; Supplemental 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 corn earworm (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 \textit{bik1} plants relative to the wild type 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 \textit{bik1} Required Functional PAD4**

While loss of BIK1 function promoted aphid-induced lesions, no lesions were formed without aphid infestation (Figs. 2 and 3). Furthermore, the spread of the aphid-induced lesions in \textit{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 \textit{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 \textit{bik1} resistance is SA independent. Third, expression of \textit{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 \textit{bik1} resistance to aphids (Fig. 8). It should be noted that although more aphids preferred \textit{pad4} plants over the wild type in the choice tests (Fig. 8D), no obvious increase in insect reproduction was observed on \textit{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 green peach aphids on \textit{pad4} than on the wild type. 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 the wild type, which may explain the nonsignificant difference in aphid propagation on the wild type versus

**Figure 8.** Resistance to aphids and aphid-induced HR in \textit{bik1} were \textit{PAD4} dependent. A, Relative expression of \textit{PAD4} and \textit{SAG13} in wild-type (WT) and \textit{bik1} plants in the presence and absence of aphid infestation. Three-week-old plants were infested with aphids as described in “Materials and Methods.” No-choice test (B), average aphid body weight (C), and choice tests (D) were performed on genotypes indicated. 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) or after aphid infestation (bottom). H, Ninhydrin staining of honeydew after 48-h aphid feeding. Bars represent means \(\pm\) 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.** \textit{PAD4} potentially promotes ET production. ET production by wild-type (WT), \textit{bik1}, \textit{bik1 pad4}, and \textit{pad4} plants measured before or after 48-h aphid infestation as described in “Materials and Methods.” Bars represent means \(\pm\) se from at least six 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 sample’s Student’s \(t\) test (\(P < 0.05\)). FW, Fresh weight.
pad4. Furthermore, different conditions under which the ROS experiments were performed may explain the discrepancy in time needed for detection of ROS between different laboratories; 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−1 aphid-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 the aphid-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 the wild type (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 wild-type plants, DAB staining revealed only marginal differences in H2O2 production between the wild type and the pad4 mutant (Fig. 8). These data suggest that in wild-type 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 INSENSITIVITY2 (SSI2), a desaturase, resulted in hypersensitivity 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 (Supplemental Table S1). High levels of SA may be a major causal factor for the aberrant development and reduced growth of bik1 because SA depletion by sid2 and nahG largely restored the wild-type stature of bik1 plants (Fig. 5; Supplemental Fig. S1B). Furthermore, the defect in SA accumulation in pad4 could be responsible for the near wild-type plant form and leaf shape of the bik1 pad4 double mutant (Fig. 8; Supplemental Fig. S1D). 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, bik1 ein2-1 and bik1 ein3-1 double mutants suffered the same growth suppression and aberrant development as the bik1 single mutant and did not show any phenotypic recovery (Fig. 7; Supplemental Fig. SIC). Therefore, despite the essential role of ET in plant development, it is unlikely that the elevated ET level contributed to the bik1 growth abnormality.

![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.](#)
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. In addition to 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). This study showcased the cross talk 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). Because TPK1b rescues the phenotype of the Arabidopsis bik1 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 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. Thompson Institute for Plant Research, Cornell University) were cultured on cabbage (Brassica oleracea) and maintained in an environmental chamber at 23°C, 65% RH, and 12-h light/12-h dark photoperiod (63 μmol m⁻² s⁻¹). All insect treatments and bioassays were performed in this chamber.

**Arabidopsis Lines**

The previously reported Arabidopsis lines, wild-type ecotype Columbia-0, and mutants fls2 (SALK_141277), fls2 (SALK_162054), efr, bak1-1, bak1-4, bik1, sld2, nhab, bik1 sld2, bik1 nhab, ein2-1, ein2-1, 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. Tesfaye Mengiste (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-aminoacyclopropane-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 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 Affl restriction digestion. Affl cut the mutant sequence into 160- and 35-bp fragments but left the wild-type sequence intact. For ein3-1, the 222-bp PCR product remained intact in the mutant sequence but was cut by HaeIII into 190- and 32-bp fragments in the wild-type sequence. DNA fragments were resolved on 2% (w/v) agarose gel. For bik1 genotyping, a procedure developed previously was followed (Lu et al., 2010). Primer sequences are provided in Supplemental Table S2.

**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, six age-synchronized second-instar nymphs (within 24 h) were placed on 4-week-old plants. The total aphid population (adult and nymph) on each plant was counted 7 d 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 h 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 wild-type or bik1 plants and removed 24 h later to produce age-synchronized progenies. Ten days later, the new generations of adults reared on Arabidopsis genotypes were collected and were weighed as six groups of 10 aphids each.

Eggs of fall armyworm (Spodoptera frugiperda), purchased from Benson Research, were incubated in a growth chamber (27°C and 65% RH). Newly hatched larvae were transferred to 4-week-old wild-type 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 d. 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 d after aphid infestation, soaked in 0.1% (w/v) 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% (v/v) methanol for 1 h at 4°C with continuous agitation. After centrifugation at 6,000g for 1 min, the absorbance of the supernatant was measured at 500 nm (Nisbet et al., 1994). Methanol (90%) served as a blank.

**MATERIALS AND METHODS**

**Plant Growth and Aphid Rearing**

Arabidopsis (Arabidopsis thaliana) was grown in L55 potting medium (Sun Gro Horticulture) in environmental chambers at 23°C (day)/21°C (night), 65% relative humidity (RH), and 12-h light/12-h dark photoperiod with a photosynthetic photon flux density of 85 μmol m⁻² s⁻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 (Myzus persicae) or a tobacco (Nicotiana tabacum)-adapted red lineage (kind gift from Dr. Georg Jander, Boyce Thompson Institute for Plant Research, Cornell University) were cultured on cabbage (Brassica oleracea) and maintained in an environmental chamber at 23°C, 65% RH, and 12-h light/12-h dark photoperiod (63 μmol m⁻² s⁻¹). All insect treatments and bioassays were performed in this chamber.
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 the wild type, fls2, efr, bak1-3, bak1-4, bik1-1/BIK1, sid2, nahG, ein2-1, ein3-1, and pad4 (sizes comparable to the wild type), 12 on bik1, ein2-1, and ein2-1 (one-quarter the size of the wild type), and 24 on bik1 sid2, bik1 nahG, and bik1 pad4 (one-half the size of the wild type). Plants were examined daily to identify symptoms of yellowing and lesion formation. Digital images were taken of representative leaves at 6 d 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 three times.

To visualize H$_2$O$_2$ accumulation, DAB staining was performed. Leaves at 6 d post infestation, as well as control leaves, were collected and vacuum infiltrated with DAB solution (1 mg mL$^{-1}$ of DAB in pH 3.5 water) in a six-well titer plate. After an overnight incubation in the same solution in darkness, the leaves were destained in 95% (v/v) 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 lid. Leaves were destained in 95% (v/v) ethanol until they turned clear. 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% (v/v) formaldehyde, 5% (v/v) acetic acid, and 50% (v/v) 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 h or longer in the dark with 0.01% (w/v) aniline blue in 150 mM K$_2$HPO$_4$ (pH 9.5). Callose deposits were visualized with an Olympus IX-81 microscope at 10× 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 grown 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 of 2H$_6$-SA, 3.8 ng of 13C$_2$-JA, and 1 ng of 2H$_6$-ABA were added to a 5-mL glass tube with 500 μL of methanol at 55°C and extracted by vortexing three times for 10 min. The mixture was then dried, and then methylated with ethereal diazomethane. Samples were then pooled the cleared supernatants after each extraction. The pooled extracts were brought to a normal environmental atmosphere. This procedure was repeated every 12 h for 5 d to maintain the effect of JA, 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) and then treated with RNase-Free DNase (Qiagen). Equal amounts of RNA (2 μg) were used to synthesize cDNA with random hexamer primers and SuperScript II Reverse Transcriptase (Invitrogen). Quantitative reverse transcription (RT)-PCR reactions were performed using SYBR Green Master Mix (BioRad) according to the manufacturer’s protocol. Primers were designed using PerlPrimer software, and their quality was examined using Primer-BLAST (National Center for Biotechnology Information). Primer sequences are provided in Supplemental Table S1. Arabidopsis UBIQUITIN10 (AT4G05320) served as an internal control for data normalization. Quantitative RT-PCR was run on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). 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 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 χ² test was applied to the aphid choice tests (P < 0.05).

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 (AT5G45330), EFR (AT5G20480), BAK1 (AT4G33430), ERF1 (AT3G23240), PDF1.2 (AT5G44420), PR1 (AT2G14610), MYC2 (AT1G32640), SID2 (AT1G74710), EIN2 (AT5G03280), EIN3 (AT3G20770), PAD4 (AT4G23420), and SAG13 (AT4G29560).
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LITERATURE CITED


Cohn JR, Martin GB (2005) Pseudomonas syringae pv. tomato type III effectors AvrPto and AvrPtoB promote ethylene-dependent cell death in tomato. Plant J 44: 139–154


pathways by the receptor-like cytoplasmic kinase BIK1. Proc Natl Acad Sci USA 110: 12114–12119


