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The leucine-rich repeat receptor-like kinase BAK1 and the cytochrome P450 PAD3 contribute to innate immunity to aphids in Arabidopsis

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One sentence summary

Aphid-derived protein elicitors trigger distinct plant innate immune responses that are dependent on BAK1 and PAD3.
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Abstract

The importance of pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI) against microbial pathogens has been recently demonstrated. However, it is currently unclear if this layer of immunity mediated by surface-localized pattern recognition receptors (PRRs) also plays a role in basal resistance to insects, such as aphids. Here we show that PTI is an important component of plant innate immunity to insects. Extract of the green peach aphid (GPA) *Myzus persicae* triggers responses characteristic of PTI in Arabidopsis. Two separate eliciting GPA-derived fractions trigger induced-resistance to GPA that is dependent on the leucine-rich repeat receptor like kinase (LRR-RLK) BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1/ SOMATIC-EMBRYOGENESIS RECEPTOR-LIKE KINASE3 (BAK1/AtSERK3), which is a key regulator of several LRR-containing PRRs. BAK1 is required for GPA elicitor-mediated induction of reactive oxygen species (ROS) and callose deposition. Arabidopsis *bak1* mutant plants are also compromised in immunity to *Acyrthosiphon pisum* (pea aphid) for which Arabidopsis is normally a non-host. Aphid-derived elicitors induce expression of PHYTOALEXIN DEFICIENT 3 (PAD3), a key cytochrome P450 involved in the biosynthesis of camalexin, which is a major Arabidopsis phytoalexin that is toxic to GPA. PAD3 is also required for induced-resistance to GPA, independently of BAK1 and ROS production. Our results reveal that plant innate immunity to insects may involve early perception of elicitors by cell surface-localized PRRs leading to subsequent downstream immune signaling.
Introduction

Close to a million insect species have so far been described, and nearly half of them feed on plants (Wu and Baldwin, 2010). Within these plant feeding insects, most feed on a few related plant species with only 10% feeding upon multiple plant families (Schoonhoven et al., 2005). Plant defense to insects include several layers (Bos and Hogenhout, 2011; Hogenhout and Bos, 2011). Physical barriers, volatile cues and composition of secondary metabolites of plants are important components that determine insect host choice (Howe and Jander, 2008; Bruce and Pickett, 2011). In addition, plants induce a variety of plant defense responses upon perception of herbivore oral secretions (OS), saliva and eggs (De Vos and Jander, 2009; Bruessow et al., 2010; Ma et al., 2010; Wu and Baldwin, 2010). These responses may provide full protection against the majority of insect herbivores, and insects that are able to colonize specific plant species likely produce effectors in their saliva or during egg laying that suppress these induced defense responses (Bos and Hogenhout, 2011; Hogenhout and Bos, 2011; Pitino and Hogenhout, 2013).

Aphids are sap-feeding insects of the order Hemiptera and are among the most destructive pests in agriculture, particularly in temperate regions (Blackman and Eastop, 2000). More than 4000 aphid species in 10 families are known (Dixon, 1998). Most aphid species are specialists and use one or a few closely related plant species within one family as host for feeding and reproduction. Examples are Acyrthosiphon pisum (pea aphid), Brevicoryne brassicae (cabbage aphid) and Sitobion avenae (English grain aphid) that colonize plant species within the legumes (family Fabaceae), brassicas (Brassicaceae) and grasses (Gramineae), respectively. The green peach aphid (GPA) Myzus persicae is one of few aphid species with a broad host range and can colonize hundreds of plants species in over 40 plant families, including brassicas (Blackman and Eastop, 2000). Aphids possess mouthparts composed of stylets that navigate to the plant vascular system, predominantly the phloem, for long-term feeding. However, before establishing a long-term feeding site these insects display a host selection behavior by probing the upper leaf cell layers with their stylets, a behavior seen on host and non-host plants of the aphid (Nam and Hardie, 2012). When the plant is judged unsuitable the aphid takes off to find an alternative plant host. It is not yet clear what happens in the initial stages of insect interactions with plants.

It is known that plants sense microbial organisms (including bacteria, fungi and oomycetes)
through perception of conserved molecules, named microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) via pattern recognition receptors (PRRs) to induce the first stage of plant immunity, termed PAMP-triggered immunity (PTI). PTI is effective against the majority of plant pathogens. Bacterial and fungal PAMPs characterized so far include bacterial flagellin (or its derived peptide flg22), bacterial elongation factor (EF)-Tu (or its derived peptide elf18), bacterial lipopolysaccharides and bacterial cold shock protein, chitin oligosaccharides and the oomycete elicitor INF1 (Boller and Felix, 2009).

Plant PRRs are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs). Most leucine-rich repeat (LRR)-type PRRs associate with and rely for their function on the small regulatory LRR-RLK BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1/SOMATIC-EMBRYOGENESIS RECEPTOR-LIKE KINASE3 (BAK1/SERK3) (Monaghan and Zipfel, 2012). For example, in Arabidopsis, flg22 and elf18 bind to the LRR-RLKs FLAGELLIN SENSITIVE 2 (FLS2) and EF-TU RECEPTOR (EFR), respectively, leading to a quasi-instant association with BAK1 (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006; Chinchilla et al., 2007; Heese et al., 2007; Schulze et al., 2010; Roux et al., 2011; Sun et al., 2013). BAK1 is required for optimal downstream immune signaling events, such as mitogen-activated protein kinases (MAPK) activation, reactive oxygen species (ROS) bursts, callose depositions, induction of immune genes and induced-resistance. Similarly, BAK1 is a positive regulator of innate immune responses triggered by the Arabidopsis LRR-RLKs PEPR1 and PEPR2 that bind the Arabidopsis-derived damage-associated molecular pattern AtPep1 (Krol et al., 2010; Postel et al., 2010; Roux et al., 2011), and by the tomato LRR-RLP Ve1 that recognizes Ave1 derived from Verticillium (Fradin et al., 2009; de Jonge et al., 2012). Consistent with the role of BAK1 downstream of numerous PRRs, BAK1 is required for full immunity to a number of bacterial, fungal, oomycete and viral pathogens (Heese et al., 2007; Kemmerling et al., 2007; Fradin et al., 2009; Chaparro-Garcia et al., 2011; Roux et al., 2011; Kørner et al., 2013).

Notably, it has been recently shown that the ortholog of BAK1 in *Nicotiana attenuata* regulates the induction of jasmonic acid (JA) accumulation upon herbivory (Yang et al., 2011). However, immunity to insects was not affected when BAK1 was silenced and the observed effect on JA accumulation may be due to an indirect effect on brassinosteroid (BR) responses for which BAK1 is also an important positive regulator (Li et al., 2002; Nam and Li, 2002). Therefore, it is currently unclear if BAK1 is involved in the early recognition of
insect-derived elicitors leading to immunity.

We discovered that the key regulatory LRR-RLK BAK1 participates in plant defense to an insect herbivore. We found that extracts of GPA/M. persicae trigger plant defense responses in Arabidopsis that are characteristic of PTI. Arabidopsis bak1 mutant plants are compromised in defense to GPA, which colonizes Arabidopsis, and to pea aphid (A. pisum) for which Arabidopsis is a non-host. BAK1 is required for ROS bursts, callose deposition and induced-resistance in Arabidopsis upon perception of aphid-derived elicitors. One of the defense genes induced by GPA-derived extracts encodes PHYTOALEXIN DEFICIENT 3 (PAD3), a cytochrome P450 that catalyzes the conversion of dihydrocamalexin acid to camalexin, which is a major Arabidopsis phytoalexin that is toxic to GPA (Kettles et al., 2013). PAD3 expression is required for Arabidopsis induced-resistance to GPA, independently of BAK1 and ROS. Our results provide evidence that innate immunity to insect herbivores may rely on the early perception of elicitors by cell surface-localized PRR.

Results

We first investigated if GPA-derived elicitors trigger cellular responses characteristic of PTI responses, including the induction of PTI marker genes, ROS bursts and callose depositions (Boller and Felix, 2009). Aphids secrete saliva into the plant whilst probing and feeding, however the plant is not only exposed to aphid saliva but also aphid mouthparts and honeydew. In addition, aphid saliva collected from feeding membranes differs in composition depending on the medium into which it is secreted (Cherqui and Tjallingii, 2000; Cooper et al., 2010). Studies of aphid saliva have identified proteins that were not detected in the salivary gland (Carolan et al., 2011), did not possess secretion signals (Harmel et al., 2008), or originated from bacterial endosymbionts (Filichkin et al., 1997). Therefore the composition of aphid saliva is complex and unlikely to be entirely represented by collecting secretions from feeding membranes. Aphid honeydew contains proteins from the aphid plus its endosymbiotic bacteria and gut flora, including known PAMPs (Sabri et al., 2013). In light of this, we opted to expose the plant to whole aphid extracts rather than aphid saliva only.

Treatment of Arabidopsis leaves with a GPA-derived extract up-regulates transcript levels of genes encoding FLG22-INDUCED RECEPTOR-LIKE KINASE 1 (FRK1), CYTOCHROME P450, FAMILY 81, SUBFAMILY F, POLYPEPTIDE 2 (CYP81F2) and
PAD3/CYP71B15 (Fig. 1A), which are markers for early immune signaling, indolic glucosinolate production and camalexin biosynthesis, respectively (Zhou et al., 1999; Asai et al., 2002; Bednarek et al., 2009). These genes have been previously shown to be induced by both protein and carbohydrate elicitors (Gust et al., 2007; Denoux et al., 2008). The levels of gene inductions to GPA-derived extract and flg22 were similar, except for pad3, which was more up-regulated in GPA-derived extract than in flg22-treated leaves (Fig. 1A). Callose deposition is a commonly observed plant response to elicitors, the timing of which depends on the elicitor used (Luna et al., 2010). We assayed callose deposition 24 hours after elicitor treatment and observed increased numbers of callose deposits in Arabidopsis leaves treated with GPA-derived extract compared to a buffer control, although not quite as high as in flg22-treated leaves (Fig. 1B). Similarly, a ROS burst was observed in Arabidopsis leaves treated with GPA-derived extract (Fig. 1D). This ROS burst was however delayed compared to that of the flg22 treatment; the ROS burst to flg22 occurred within 10 to 20 minutes (Fig. 1C), whilst that to GPA-derived extract occurred after one hour. At this time the flg22-induced ROS levels were returning to base level (Fig. 1D). Nonetheless, these data show that GPA-derived extract contains one or several elicitors that trigger PTI-like plant responses.

We next investigated whether PTI-like responses triggered by GPA-derived extract required components involved in PTI. Flg22-triggered ROS burst is dependent on the NADPH-oxidase AtRbohD (Nühse et al., 2007; Zhang et al., 2007). We previously found that an aphid candidate effector Mp10 suppresses the flg22-mediated ROS burst (Bos et al., 2010), a response that also requires BAK1 (Chinchilla et al., 2007; Heese et al., 2007). Because BAK1 is an essential regulator of many PTI responses characterized so far (Monaghan and Zipfel, 2012), we also investigated if BAK1 was required for the PTI-like responses to GPA-derived extract. The GPA-derived extract-triggered ROS burst was reduced in the semi-dominant bak1-5 mutant and was completely absent in AtRbohD (Fig. 2A). Flg22-triggered callose deposition requires biosynthesis of 4-methoxylated indole glucosinolates, mediated by CYP81F2 (Clay et al., 2009), and are diminished in mutants of PEN2, which encodes a myrosinase involved in glucosinolate metabolism (Lipka et al., 2005; Bednarek et al., 2009; Clay et al., 2009; Luna et al., 2010). As GPA-derived extract induces CYP81F2 expression (Fig. 1A) we investigated whether PEN2 and BAK1 were required for GPA triggered callose depositions. The number of callose deposits was significantly reduced in bak1-5 and pen2-1 mutants compared to Col-0 after treatment with GPA-derived extract (Fig. 2B). Together
these data provide evidence that PTI-like responses to GPA-derived extract require components involved in PTI responses.

As very little is known about plant cell surface perception of insect-derived elicitors we further investigated the role of BAK1 in immunity to aphids. Besides its role in PTI signaling, BAK1 is also involved in BR responses (Li et al., 2002; Nam and Li, 2002), light signaling (Whippo and Hangarter, 2005) and cell death control (He et al., 2007; Kemmerling et al., 2007). Null bak1 mutants are compromised in all of these areas. The ethyl methane sulphonate mutant bak1-5 has a substitution in the cytoplasmic kinase domain that leads to compromised innate immune signaling, but is not impaired in BR or cell death control (Schwessinger et al., 2011), allowing its use to investigate the relevance of BAK1 in resistance to pathogens with different lifestyles (Roux et al., 2011). We investigated GPA performance on bak1-5, the null mutant bak1-4 (He et al., 2007) and a null mutant of BAK1-LIKE1/SERK4 (BKK1/SERK4) (bkk1-1), which is the closest paralog of BAK1 and similarly controls PTI, BR and cell death responses (He et al., 2007; Roux et al., 2011). GPA reproduction on wild-type Col-0 and bak1-5 plants were more similar than the reproduction rates of this aphid on bak1-4 and bkk1-1 plants (Fig. S1). This suggests that the pleiotropic phenotypes, such as deregulated cell death, of the null mutants affect aphid performance (He et al., 2007; Kemmerling et al., 2007). These results are consistent with the response of the obligate biotrophic oomycetes Hyaloperonospora arabidopsidis, which showed decreased reproduction on bak1-4 plants but no increase in reproduction on bak1-5 plants for three Hyaloperonospora arabidopsidis isolates (Roux et al., 2011). Therefore, we continued our investigation with the Arabidopsis bak1-5 mutant alone.

Treatment with exogenous PAMPs enhances plant resistance to pathogens, this is also known as induced-resistance (Zipfel et al., 2004; Balmer et al., 2013). De Vos and Jander (2009) previously observed that GPA saliva proteins between 3 to 10 (3-10) kD in molecular mass elicit induced-resistance to GPA in Arabidopsis (De Vos and Jander, 2009). To investigate if BAK1 is involved in this response, wild type Col-0 plants were treated with GPA-derived extract and GPA reproduction on these leaves was then assessed over a period of 10 days. Induced-resistance was triggered by whole GPA-derived extract (Fig. 3A), the GPA-derived 3-10 kD fraction (Fig. 3B) and the 3-10 kD GPA saliva fraction (Fig. S2). Induced-resistance was dramatically reduced in the bak1-5 mutant (Fig. 3A, 3B and S2). These demonstrate that aphid elicitors present in whole GPA-derived extract and
saliva are recognized in a BAK1-dependent manner, leading to immunity to GPA.

Next, we investigated if PAD3 is involved in Arabidopsis induced-resistance to GPA. The cytochrome P450 PAD3 catalyzes the conversion of dihydrocamalexic acid to camalexin, the major Arabidopsis phytoalexin, and acts downstream of CYP79B2 and CYP79B3 enzymes in the glucosinolate biosynthetic pathway (Zhao et al., 2002; Schuhegger et al., 2006). We previously demonstrated that camalexin is toxic to GPA (Kettles et al., 2013). Moreover, PAD3 expression is induced upon perception of aphid elicitors (Fig. 1A), GPA saliva (De Vos and Jander, 2009) and GPA feeding (De Vos et al., 2005; Kettles et al., 2013).

We found that Arabidopsis pad3 and cyp79b2/cyp79b3 mutants do not show induced-resistance to GPA upon treatment of plants with GPA-derived extract (Fig. 3C). To determine whether the PAD3-dependent induced-resistance requires BAK1 and apoplastic ROS production we measured PAD3 induction in bak1-5 and AtrbohD plants in response to GPA-derived extract. PAD3 expression was reduced in bak1-5 and AtrbohD in response to flg22 but not GPA-derived extract (Fig. 3D), suggesting that PAD3-dependent induced-resistance to GPA-derived extract is independent of BAK1 and apoplastic ROS production.

Therefore, Arabidopsis induced-resistance to GPA is dependent on BAK1 and PAD3 through separate signaling pathways.

We sought to characterize further the biochemical properties of the GPA-derived elicitors. The ROS burst and induced-resistance responses disappeared when GPA-derived extract was boiled (Fig. 4A and 4B). The proteinase-K-treated GPA-derived extract did not generate an induced-resistance response to GPA (Fig. 4B), even though proteinase K itself induced a ROS burst in Arabidopsis Col-0 that started at about one hour after treatment and disappeared upon boiling of proteinase K (Fig. S3A and B). The 3-10 kD fraction induced a ROS burst, whilst fractions that are smaller than (<) 3 kD and larger than (> 10 kD did not (Fig. 4C). Induced-resistance to GPA was, however, observed for both the 3-10 kD and >10 kD fractions, but not for the <3 kD fraction (Fig. 4D). Altogether these results indicate the presence of at least two eliciting fractions in GPA-derived extract, which are likely to contain heat-sensitive proteins or peptides.

Arabidopsis bak1-5 mutant plants produce significantly less ROS in response to the GPA-derived 3-10 kD extract (Fig. 5A). BAK1 is a co-receptor that associate with several LRR-
RLK-type PRRs, such as FLS2, EFR and PEPR1/2 (Chinchilla et al., 2007; Heese et al., 2007; Postel et al., 2010; Roux et al., 2011), which perceive bacterial flagellin, bacterial EF-Tu and the damage-associated molecular patterns AtPeps, respectively (Gómez-Gómez and Boller, 2000; Yamaguchi et al., 2006; Zipfel et al., 2006; Yamaguchi et al., 2010). However, Arabidopsis mutant lines in these PRRs did not show reduced ROS bursts to the 3-10 kD extract (Figs. 5B and 5C). Whilst the LysM-RLK CERK1 does not require BAK1 for signaling, this receptor is involved in the perception of chitin (Miya et al., 2007; Wan et al., 2008), which is abundant in the aphid cytoskeleton, including the aphid mouthparts that are in contact with the plant during feeding. Nonetheless, the response to GPA-derived extract was not reduced in an Arabidopsis fls2 efr cerk1 triple mutant (Fig. 5B). Thus, aphid elicitor-induced ROS burst is dependent on BAK1 and a thus-far unknown PRR.

We also investigated whether BAK1 was involved in the induced-resistance to the >10 kD eliciting fraction. Induced-resistance was observed on Col-0 Arabidopsis plants, but not on the bak1-5 mutant plants for the 3-10 kD and >10 kD fractions (Fig. 5D). Therefore BAK1 is involved in the signaling pathways to both of these eliciting fractions.

Elicitors perceived by PRRs are often conserved among groups of pathogens (Medzhitov and Janeway Jr, 1997). To investigate if this is also the case for aphids, we examined the expression levels of the PTI marker genes FRK1, CYP81F2 and PAD3 in Arabidopsis plants treated with extracts of various aphid species (A. pisum, B. brassicae and S. avenae). The expression of these genes were induced to similar levels after treatment with aphid-derived extracts from the three other species tested, although the induction of FRK1 and CYP81F2 was not statistically significant upon treatment with S. avenae-derived extract (Fig. 6A). These results provide evidence that aphid-derived elicitors perceived by Arabidopsis are potentially conserved amongst different aphid genera/species.

The A. pisum (pea aphid) host range is mostly restricted to plants of the legume family; these insects do not like to feed on brassicas, such as Arabidopsis. Because PRRs regulate the first active line of plant defense response and are proposed to be involved in non-host resistance in plant species distantly related to the natural host (Schulze-Lefert and Panstruga, 2011), we investigated if A. pisum survives better on Arabidopsis bak1-5 mutant plants. About 50% of the pea aphids on Arabidopsis Col-0 are still alive between 3 and 4 days (Fig. 6B). Remarkably, at this time the survival rates of pea aphids were significantly higher, about 75%, on the Arabidopsis bak1-5 mutant plants (Fig. 6C). Thus, non-host
resistance of Arabidopsis to the pea aphid appears compromised in the bak1-5 background, further reflecting an important contribution of BAK1 (and by extension PRR-mediated immunity) to plant immunity against aphids.

Discussion

Our research provides an increased understanding of plant perception of insects, by showing that BAK1 is required for the ROS burst and induced-resistance triggered by GPA-derived elicitors. GPA-derived elicitors trigger plant immunity characteristic of PTI, including the induction of PTI marker genes, AtRbohD-dependent ROS burst, PEN2 dependent callose deposition, and induced-resistance. The GPA-derived eliciting fractions are likely to contain heat-sensitive peptides of 3-10 kD and >10 kD in which the 3-10 kD fraction induces the ROS burst and both 3-10 kD and >10 kD fractions elicit induced-resistance to GPA. Induced-resistance is dependent on PAD3, the expression of which is induced upon Arabidopsis perception of aphid-derived elicitors and is independent of BAK1 and ROS. Finally, the legume-specialist pea aphid survives better on the Arabidopsis bak1-5 mutant than on wild-type Col-0 plants.

Our results are in agreement with those of De Vos and Jander (De Vos and Jander, 2009), who found that the 3-10 kD GPA saliva fraction generates induced-resistance, which is lost upon boiling and proteinase K treatments of the fraction (De Vos and Jander, 2009). In addition, Arabidopsis colonization by another aphid species, the cabbage aphid (B. brassicae), triggers a ROS burst and the expression of PAD3, CYP81F2 and FRK1 genes (Kusnierczyk et al., 2008; Barah et al., 2013). These findings and our observation that multiple aphids induce PAD3, CYP81F2 and FRK1 expression (Fig. 5A) suggest that the eliciting components are conserved among aphids. Our study shows evidence that there are at least two eliciting fractions derived from aphids; the GPA 3-10 kD fraction that triggers a ROS burst and induced-resistance, and the >10 kD fraction that does not induce ROS burst but nonetheless triggers induced-resistance. The eliciting activities of both fractions require BAK1 and are lost upon boiling and proteinase K treatments indicating that the elicitors are likely proteins with enzymatic activities. It is possible that the two eliciting fractions contain different concentrations of the same elicitor due to incomplete separation by the molecular weight cut-off columns. Therefore the elicitor may be in sufficient quantity to trigger a ROS burst in the 3-10 kD fraction but not the >10 kD fraction. It is important to note that the elicitors perceived by Arabidopsis are either derived directly from aphids or
from their endosymbionts. However, the possibility remains that elicitors in GPA-derived extract may not normally come into contact with plants. Further investigation is required to identify the elicitors and their origin. This will then allow the availability of the GPA-derived elicitors to be perceived by the plant during the plant-aphid interaction to be assessed.

The ROS burst triggered by flg22 is an early transient response, which starts very soon after addition of the PAMP and finishes within 30 minutes. In contrast, the ROS burst triggered by the GPA-derived 3-10 kD fraction occurs much later, starting more than an hour after addition of the extract. Its duration is also longer compared to flg22, as the burst takes nearly 9 hours to reach basal level again. These kinetics are consistent with a potential enzymatic activities of the GPA-derived elicitors. However, the kinetics of plant immune responses triggered by distinct elicitors can be highly variable. For example, *P. infestans* elicitin INF1 triggers a BAK1-dependent ROS burst in *N. benthamiana* that is also much longer than that of flg22 (Chaparro-Garcia et al., 2011). Whilst there is a delay in the GPA-derived elicitor ROS burst compared to that of flg22 there is no delay in GPA-derived gene expression of *PAD3, CYP81F2* and *FRK1*. We show that *PAD3* expression to GPA-derived elicitors does not require ROS (Fig. 3D). *CYP81F2* and *FRK1* are MAPK activated genes (Boudsocq et al., 2010) and MAPK activation in PTI does not require ROS (Ranf et al., 2011; Segonzac et al., 2011). Consistent with this, *FRK1* expression upon flg22 treatment is not reduced in *AtrbohD* (Macho et al., 2012).

GPA elicitation is specific, as proteinase K triggers a ROS burst in Arabidopsis that is lost upon boiling, but this ROS burst does not generate induced-resistance to GPA. Arabidopsis can generate induced-resistance to GPA without a measurable ROS burst as evidenced by the induced-resistance triggered by the >10 kD GPA fraction. Nonetheless, the ROS burst plays a role in Arabidopsis innate immunity to GPA given that Arabidopsis mutants in RbohD, which is required for PTI- and ETI-triggered ROS burst (Torres et al., 2002; Zhang et al., 2007) are more susceptible to GPA (Miller et al., 2009). Thus, aphid-derived elicitors are likely to trigger different immune pathways in plants, some of which involve ROS bursts and others that do not. All these pathways together contribute to an effective immunity against aphids.

BAK1 is required for the establishment of PTI by ligand-induced heteromerization with surface-localized PRRs. Characterized PRRs that require BAK1 for signaling include FLS2,
EFR and PEPR1/PEPR2 (Chinchilla et al., 2007; Heese et al., 2007; Postel et al., 2010; Roux et al., 2011). However, Arabidopsis mutants for FLS2, EFR, PEPR1 and PEPR2 are not affected in ROS bursts to the 3-10 kD GPA fraction. Therefore, elicitors in the 3-10 kD GPA fraction are likely to interact with thus-far unknown Arabidopsis PRRs, which form ligand-induced heteromers with BAK1 for triggering a ROS burst upon perception of aphid-derived elicitors.

The involvement of BAK1 in plant-herbivore interactions was previously investigated in *Nicotiana attenuata* (Yang et al., 2011). Plants are likely to perceive insect elicitors, often referred to as herbivory-associated molecular patterns in insect OS and egg-associated molecular patterns in egg fluid (Wu and Baldwin, 2010; Gouhier-Darimont et al., 2013). Application of OS into wounds activates two MAPKs, salicylic acid (SA)-induced protein kinase and wound-induced protein kinase, which are required for the accumulation of jasmonic acid, JA-Ile and ethylene (ET), phytohormones that are important for mediating plant immunity to insects (Wu and Baldwin, 2010). The LECTIN-RECEPTOR KINASES (LecRKs), LecRK1 and LecRK-I.8, act upstream or downstream of phytohormone signaling events (Gilardoni et al., 2011; Gouhier-Darimont et al., 2013). Whilst silencing of BAK1 in *N. attenuata* leads to attenuated JA and JA-Ile levels in wounded and OS-treated plants, activities of the two MAPKs were not impaired (Yang et al., 2011). This indicated that BR signaling but not innate immunity may be compromised in these BAK1-silenced plants (Yang et al., 2011). The Arabidopsis *bak1-5* mutant used in our study is severely compromised in PTI signaling, but is not impaired in BR signaling and cell death control (Schwessinger et al., 2011). In addition, the saliva induced-resistance to GPA in Arabidopsis is not dependent on JA, SA and ET signaling (De Vos and Jander, 2009). This is in agreement with a study of Arabidopsis responses to the necrotrophic fungus *Botrytis cinerea* showing that plant-derived oligogalacturonides induce a resistance that is not dependent on JA, SA and ET (Ferrari et al., 2007). Similarly to aphids, the induction of resistance to *B. cinerea* requires PAD3 (Ferrari et al., 2007). Thus, BAK1 contributes most likely to innate immunity to GPA in a manner that is independent of BR, JA, SA, and ET signaling in Arabidopsis.

Arabidopsis is a non-host to the pea aphid *A. pisum*. We observed that these aphids nonetheless attempt to feed on Arabidopsis leaves, but do not adopt a settled feeding behavior and often walk to the top of the leaf cages where they die within 6 days. Notably,
pea aphids survive longer on Arabidopsis bak1-5 plants compared to Col-0 indicating that they may obtain more nutrition from the mutant plant or receive fewer toxic compounds. Whilst BAK1 has a role in plant immune signaling upon pea aphid perception, the observation that pea aphids do not fully survive on Arabidopsis bak1-5 plants suggests that other BAK1-independent receptor complexes and/or additional downstream components also contribute to the triggering of plant immunity to aphids. Studying of pea aphid-Arabidopsis interactions will be useful for the identification of such components. Aphids that use brassicas, including Arabidopsis, as hosts, such as GPA and the cabbage aphid, are likely to possess specific effectors that suppress the PTI-like plant immune responses. We identified about 50 candidate effectors in GPA (Bos et al., 2010) and found that three promote GPA colonization on Arabidopsis, whereas the pea aphid homologs of these three effectors do not promote GPA colonization on this plant (Pitino and Hogenhout, 2013). It remains to be investigated if the GPA effectors but not pea aphid effectors suppress PTI-like plant defenses.

In summary, we identified an upstream (BAK1) and downstream (camalexin) component of two independent pathways in plant innate immunity to aphids. This is in agreement with earlier findings that camalexin is involved in plant defense to aphids (Kusnierczyk et al., 2008; Kettles et al., 2013). Aphids are likely to suppress innate immunity in order to colonize plants. This is in agreement with the identification of a GPA effector that suppress PTI (Bos et al., 2010) and aphid effectors that promote colonization of the plant (Atamian et al., 2013; Pitino and Hogenhout, 2013).

Materials and methods

Aphids

Myzus persicae/green peach aphid (GPA) (RR3 genotype O) (Bos et al., 2010) were reared on Chinese cabbage (Brassica rapa, subspecies chinensis) and Acrthosiphon pisum/pea aphid were reared on broad bean (Vicia faba) in 52 cm x 52 cm x 50 cm cages. Brevicoryne brassicae/cabbage aphid were reared on Chinese cabbage and Sitobion avenae/English grain aphid were reared on oat (Avena sativa) in 24 cm x 54 cm x 47 cm cages. All species were reared in controlled-environment conditions with a 14 h day (90 μmol m^-2 sec^-1 at 18°C) and a 10 h night (15°C) photoperiod.
Plant growth conditions

All plants were germinated and grown in Scotts Levington F2 compost (Scotts, Ipswich, UK). Arabidopsis seeds were vernalized for one week at 5-6°C and then grown in a controlled environment room (CER) with a 10 h day (90 μmol m^-2 sec^-1) and a 14 h night photoperiod and at a constant temperature of 22°C.

All Arabidopsis mutants used in this study were generated in Columbia (Col-0) ecotype background, except pen2-1 which is in the glabrous1 background. The bak1-5, bak1-4, bkk1-1, efr-1 (efr), fls2c (fls2) and fls2 efr cerk1 mutants were previously described (Zipfel et al., 2004; Zipfel et al., 2006; He et al., 2007; Gimenez-Ibanez et al., 2009; Schwessinger et al., 2011). The pepr1-1, pepr1-2, and pepr2-1 mutants (Yamaguchi et al., 2010) were obtained from the Nottingham Arabidopsis Stock Centre (NASC, Nottingham, UK). The pepr1/pepr2 double mutant (Krol et al., 2010) was obtained from Dirk Becker (Department of Molecular Plant Physiology and Biophysics, University of Wuerzburg, Wuerzburg, Germany). The pen2-1 (Lipka et al., 2005) and AtrbohD (Torres et al., 2002) mutants were obtained from Jonathan Jones (The Sainsbury Laboratory, Norwich, UK). The pad3 and cyp79b2/cyb79b3 double mutants (Glazebrook and Ausubel, 1994; Zhao et al., 2002) were used in a previous study (Kettles et al., 2013).

Preparation of aphid-derived extract and fractions for elicitation experiments

Apterous late instar and adult aphids were collected using a moist paintbrush, placed in a 2 ml Eppendorf tube and snap-frozen in liquid nitrogen. The aphids were ground to a fine powder using a pre-chilled mortar and pestle. The powder was then transferred to a 50 mL Corning tube (Corning, New York, USA) on ice using a pre-chilled spoon. Sterile, distilled water was added to the ground powder and thoroughly mixed with a pipette to generate 20 mg (wet weight) per mL of whole aphid-derived extract.

GPA-derived extracts were further processed as described (De Vos and Jander, 2009; Schafer et al., 2011). The ground aphid powder was resuspended in sterile 0.025 M potassium phosphate buffer (KH_2PO_4, pH 6.8). The extract was centrifuged at 13200 rpm for 15 min at 4°C and the supernatant was collected. For fractionation of GPA-derived extract, the supernatant was filtered by centrifuging at 13200 rpm for 15 min at 4°C using a 10-kD cutoff-column (Ultracel 10K membrane, Millipore, Carrigtwohill, Co. Cork, Ireland). The fraction remaining in the upper part of the column was the >10 kD fraction. The fraction that passed through the column was retrieved by placing the column upside-down in a fresh
centrifuge tube and centrifuging it at 1000 x g for 2 min. It was then filtered by centrifuging at 13200 rpm for 15 min at 4°C using a 3 kD cutoff-column (Ultracel 3K membrane, Millipore). The fraction that passed through the column was the <3 kD fraction, whilst the fraction that remained in upper part of the column was the 3-10 kD fraction. The 3-10 kD fraction was retrieved by placing the column upside-down in a fresh centrifuge tube at centrifuging at 1000 x g for 2 min. After filtering, all fractions were adjusted to their original volume using potassium phosphate buffer.

GPA-derived extract was denatured by boiling for 10 min or degraded in a final concentration of 0.2 μg/μL of proteinase K (Sigma-Aldrich, St Louis, MO, USA) at 37°C for 30 min.

Saliva collection

GPA saliva was collected using a Parafilm sachet. Two 500 mL plastic tumblers (Sainsbury’s Supermarkets Ltd, London, UK) had several small holes pierced in them with a hot syringe (Terumo, Egham, Surrey, UK). Approximately 1000 adult GPA from the Chinese cabbage stock cage, amounting to a weight of 0.2 g (50 adult GPA weighed 0.01 g), were added to one of the tumblers. The other tumbler served as a no aphid control. A thin layer of Parafilm (Brand GMBH, Wertheim, Germany) was stretched over each tumbler, and 1 mL of sterile, distilled water pipetted onto the Parafilm. A second layer of Parafilm was then stretched over each tumbler. The tumblers were placed underneath a sheet of yellow plastic (Lincoln Polythene Ltd, Lincoln, Lincolnshire, UK) to enhance feeding activity, in a CER with 14 h day (90 μmol m⁻² sec⁻¹ at 18°C) and a 10 h night (15°C) photoperiod. After 24 hours the saliva/water was collected from both tumblers under sterile conditions. The 3 to 10 kD fraction of the saliva and control was obtained using centrifugal filters as described above. After filtering, the saliva and control were adjusted to their original volume using sterile, distilled water.

Induced-resistance assays

Induced-resistance fecundity assays were carried out using a modified protocol as described (De Vos and Jander, 2009). Experiments were conducted in a CER with an 8-h day (90 μmol m⁻² sec⁻¹ at 18°C) and 16-h night (16°C) photoperiod. To obtain aphids of the approximately the same age, five-week old Col-0 Arabidopsis plants were potted into one litre round black pots (13 cm diameter, 10 cm tall) that were caged inside clear plastic tubing (10 cm diameter, 15 cm tall) (Jetran tubing, Bell Packaging), which was pushed
inside the soil of the pot and capped at the top with white gauze-covered plastic lid. Each plant was seeded with 20 adult GPA. After 24 hours, all adults were removed from the Col- plants whilst the nymphs remained on the plants for 10 days.

For treatment of plants with aphid elicitors, five-week old Arabidopsis plants in black plastic pots (base measurement 3.5 cm x 3.5 cm, top measurement 5.5 cm x 5.5 cm, height 5.5 cm) were infiltrated with the GPA-derived extracts on the first fully expanded leaf using a needless 1-mL syringe (Terumo). The extracts being tested were diluted 1:10 with distilled water or potassium phosphate buffer as appropriate. The 3-10 kD fraction of GPA saliva was diluted 1:2 with distilled water. Control plants were infiltrated with distilled water or potassium phosphate buffer without GPA-derived extract. The infiltrated leaves were marked. The plants were used for aphid reproduction assays after 24 hours.

To assay aphid reproduction on the infiltrated leaves, one aged adult of 10 days old was placed in a clip cage using a moist paintbrush and the cage was placed on the infiltrated leaf at one aphid per plant. Plants were returned to the experimental CER and left for 10 days. After 10 days, the number of aphids in each clip cage was counted. Each experiment included 10 plants per condition and/or genotype unless otherwise stated. Each plant was randomly placed in a tray of 42 x 52 x 9 cm. Each experiment was repeated at least three times on different days to generate data from at least three independent biological replicates. Leaves that had shrivelled up and died, thus killing all the aphids, were removed from the analysis.

GPA whole plant fecundity assays

GPA whole plant fecundity assays were carried out as previously described (Kettles et al., 2013). Experiments were conducted in a CER with an 8-h day (90 μmol m⁻² sec⁻¹ at 18°C) and 16 h night (16°C) photoperiod. Four-week old Arabidopsis plants were potted into one litre round black pots and caged in clear plastic tubing as described above. Each plant was seeded with five adult GPA. After 48 hours, all adults were removed from test plants whilst the nymphs remained at five nymphs per plant. These nymphs developed into adults and started producing their own nymphs at about day 8. The number of nymphs and surviving adults were counted on days 11 and 14 in which the nymphs were removed at each count. The total number of nymphs produced per live adult was calculated for each time point and combined. Each experiment included 5 plants per genotype and each plant was randomly placed in a tray of 42 x 52 x 9 cm. Each experiment was repeated three times on different days to generate data from three independent biological replicates.
Pea aphid survival assays

To obtain pea aphid adults of the same age, 50 adult pea aphids were transferred to three mature *V. faba* plants between three to four weeks old and placed in 24 cm x 54 cm x 47 cm cages. The cage was placed in a CER with a 14-h day (90 μmol m⁻² sec⁻¹ at 18°C) and a 10 h night (15°C) photoperiod. After 24 hours all adults were removed from the plants whilst the nymphs remained. Pea aphid adults of 10-14 days old were used for survival experiments on Arabidopsis. The survival experiments on Arabidopsis were conducted in a CER with an 8-h day (90 μmol m⁻² sec⁻¹ at 18°C) and 16-h night (16°C) photoperiod. Five 10-14 day adult pea aphids were placed in one clip cage using a moist paintbrush. The clip cages were clipped on one leaf per plant of seven-week old Arabidopsis plants potted in black plastic pots (base measurement 3.5 cm x 3.5 cm, top measurement 5.5 cm x 5.5 cm, height 5.5 cm). To ascertain pea aphid survival on Col-0 Arabidopsis the number of aphids remaining alive on days 3 to 7 was counted. To compare survival on Col-0 and bak1-5 Arabidopsis the number of adult aphids remaining alive on days 3 and 4 were recorded, and the average of these two readings taken. Each experiment consisted of 5 plants per genotype. Each plant was randomly placed in a tray of 42 x 52 x 9 cm. The experiments were repeated at least four times on different days to generate data from at least four independent biological replicates.

Measurements of ROS bursts

Measurements of ROS bursts to the peptide flg22 (QRLSTGSRINSKDDAAGLQIA) (Felix et al., 1999) (Peptron, Daejeon, South Korea) and GPA-derived extracts were carried out as previously described (Bos et al., 2010). One leaf disc was taken from each of the two youngest fully expanded leaves of five-week old Arabidopsis plants using a circular cork borer (diameter 4 mm). The leaf discs were floated on water overnight in 96-well plates (Grenier Bio-One, Stonehouse, Gloucestershire, UK). Flg22 (final concentration 100 nM unless stated otherwise) or GPA-derived extract (final concentration 5 mg/mL unless otherwise stated) were added to a solution containing 20 μg/mL horseradish peroxidase (HRP) (Sigma-Aldrich) and 21 nM of the luminol derivative 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H)dione (L-012) (Nishinaka et al., 1993) (Wako, Osaka, Japan). Before the experiment began the water was removed from the wells and replaced with 100 μL of HRP and L-012 solution containing flg22, GPA-derived extract or water/buffer controls. ROS burst assays to proteinase K were conducted with 100 μg of
proteinase K (Sigma-Aldrich) or 100 μg of proteinase K boiled for 10 min. Luminescence
was captured using a Photek camera system (Photek, St Leonards on Sea, East Sussex,
UK) and analysed using company software and Microsoft® Office Excel (Microsoft, London,
UK). Experiments were repeated at least three times on different days to generate
independent biological replicates.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) assays
Two Arabidopsis leaf discs were taken from each of the two youngest fully expanded leaves
of five-week old Col-0 plant using a circular cork borer with a diameter of 6 mm. The leaf
discs were floated on water overnight in 96-well plates (Grenier Bio-One). Before the
experiment began the water was removed and leaf discs were exposed to 100 μL of water
(control), 100 nM flg22 (in water) and 20 mg/mL GPA-derived extract (in water) for 1 hour.
Eight leaf discs under the same treatment were pooled generating one sample. Samples
were ground in chilled 1.5 mL Eppendorf tubes using disposable pellet pestles (Sigma-
Aldrich). Total RNA was extracted using Tri-Reagent (Sigma-Aldrich) and included a
DNaseI treatment (RQ1 DNase set; Promega, Madison, WI, USA). cDNA was synthesised
from 1 μg RNA using the M-MLV-RT Kit (Invitrogen, Carlsbad, CA, USA) and oligo-dT
primer, following the manufacturer’s instructions. cDNA from these reactions was diluted
1:10 with distilled H2O before qRT-PCR.

Each reaction consisted of 20 μL containing 25 ng of cDNA and 0.5 μM of each primer
(Table 1) added to SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) in a single well
of a 96-well plate white ABgene PCR plate (Thermo Scientific, Loughborough,
Leicestershire, UK). Reactions for the target and reference genes and corresponding
controls were combined in one 96-well plate, which was placed in a CFX96 Real-Time
System with a C1000 Thermal Cycler (Bio-Rad, Hemel Hempstead, Hertfordshire, UK).
PCRs were carried out using the following thermocycle: 3 min at 95°C, followed by 40
cycles of (30 s at 95°C, 30 s at 60°C, 30 s at 72°C), followed by melt curve analysis: 30 s at
50°C (65–95°C at 0.5°C increments, 5 s for each).

Using a selection of candidates previously identified as superior reference genes
(Czechowski et al., 2005), we selected Arabidopsis genes GAPDH (At1g13440) and TIP41
(At4g34270) as the most stable across a range of mock, flg22 and GPA-derived extract-
exposed Arabidopsis leaf disc RNA samples by geNORM analysis (Vandesompele et al.,
2002). All primers are listed in Table S1.
To calculate the relative expression levels of target genes, mean \( C_t \) values for each sample–primer pair combination were calculated from three replicate reaction wells. Mean \( C_t \) values were then converted to relative expression values using efficiency of primer pair -\( \Delta C_t \). The geometric mean of the relative expression values of the reference genes was calculated to produce a normalization factor unique to each sample that was used to calculate the relative expression values for each gene of interest in each sample. These values from independent biological replicates were compared using a described method (Willems et al., 2008).

Callose staining

The first two fully expanded leaves of 5-week-old Arabidopsis plants were infiltrated using a 1 ml syringe with buffer (control), 100 nM flg22 (in buffer) and 20 mg/mL GPA-derived extract (in buffer). After 24 hours, one leaf disc was taken from each infiltrated leaf using a circular cork borer with a diameter of 5 mm. To remove chlorophyll from the leaf discs, the discs were placed in 70% ethanol for 1 hour, 95% ethanol with chloroform overnight (18 hours) and 100% ethanol for 2 hours. The discs were then rehydrated for 30 minutes in 70% ethanol, 30 min in 50% ethanol and 30 min in 67 mM K2HPO4 at pH 9.5. Staining with 0.1% aniline blue in 67 mM K2HPO4 at pH 9.5 was carried out for one hour. Leaf discs were mounted in glycerol and viewed under a Nikon Eclipse 800 microscope (Nikon, Tokyo, Japan) using a UV filter (BP 340-380 nm, LP 425 nm). An image was taken of the entire field of view of the centre of each leaf disc under 10x magnification (1.34 mm – 1.34 mm by 1 mm). The images were analysed using ImageJ (National Institutes of Health, USA) to count the number of callose deposits.

Statistical analyses

Statistical analyses were conducted using Genstat v.12 (VSN International, Hemel Hempstead, UK). Aphid survival or fecundity assays and callose deposition were analysed by classical linear regression analysis using a Poisson distribution within a generalized linear model (GLM). ROS burst assays comparing two conditions were analysed with Student’s \( t \)-tests and those comparing more than two conditions with ANOVA. The qRT-PCR data were analysed using classical linear regression analysis within a GLM in which the means were compared by calculating \( t \) probabilities within the GLM.
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**Figure legends**

**Figure 1.** Plant defense elicitations to GPA-derived extract are characteristic of PAMP-triggered immunity. (A) GPA-derived extract elicits the expression of PTI marker genes. Bars show the means ± SE of target gene expression levels of four independent experiments (n = 3 per experiment). Asterisks indicate significant differences in GPA fraction compared to water (t probabilities calculated within GLM) with *P < 0.05 compared to water control for each gene and **P < 0.05 between flg22 and GPA-derived extract treatment. (B) GPA-derived extract elicits callose deposition. Data shown are mean callose deposits produced per 1.34 mm² of leaf upon each treatment with means ± SE of three independent experiments (n = 12 leaf discs per experiment). Different letters indicate significant differences between the treatments (t probabilities calculated within GLM) at P < 0.05 (n = 36, F<sub>2,103</sub> = 2039.93). (C and D) Col-0 leaf discs were elicited with water, 12.5 nM flg22 (in water) and GPA-derived extract (in water) and ROS bursts in these leaf discs were measured using luminol-based assays at (C) 0 to 60 min and (D) 60 to 600 min after elicitation. RLU = Relative light units. Graphs show means ± SE of n = 32 leaf discs per replicate. Data of one representative experiment is shown. The experiment was repeated three times with similar results.

**Figure 2.** Plant defense elicitations to GPA-derived extract require components of PAMP-triggered immunity. (A) GPA-derived extract elicits a ROS burst in wild type Col-0 that is reduced in bak1-5 and absent in the AtrbohD mutant. ROS bursts were measured over a 600 min period. Graph shows means ± SE of n = 16 leaf discs per replicate. Open symbols represent water treated leaf discs, closed symbols represent GPA-derived extract treated leaf discs. Data of one representative experiment is shown. The experiment was repeated three times with similar results. (B) GPA-derived extract-elicited callose deposition is significantly reduced in bak1-5 and pen2-1. Data shown are mean callose deposits produced per 1.34 mm² of leaf upon each
treatment with means ± SE of three independent experiments (n = 12 leaf discs per replicate). Different letters indicate significant differences between the treatments (t probabilities calculated within GLM) at $P < 0.05$ ($n = 36, F_{10,323} = 1388.15$).

Figure 3. Plant defense responses elicited by GPA-derived extract are dependent on BAK1 and PAD3. (A and B) Induced-resistance to (A) GPA-derived extract and (B) GPA 3-10 kD fraction is dependent on BAK1. Bars show the means ± SE of total nymphs produced per plant of (A) six and (B) three independent experiments. The nymph counts were normalized with the water or buffer controls set at 100%. Asterisks indicate significant differences to GPA fraction compared to water/buffer (t probabilities calculated within GLM) with (A) *$P < 0.001$ (Col-0 wild type, $n = 60, F_{1,19} = 17.88$) and $P = 0.063$ ($bak1-5$ mutant $n \geq 57, F_{1,115} = 3.45$) and (B) *$P = 0.005$ (Col-0 wild type, $n \geq 28, F_{1,56} = 8.065$) and $P = 0.835$ ($bak1-5$ mutant, $n \geq 25, F_{1,53} = 0.043$). (C) Induced-resistance to GPA-derived extract is dependent on PAD3. Bars show the means ± SE of total nymphs produced per plant of three independent experiments. Nymph counts were normalized with the water control set at 100%. Asterisks indicate significant differences compared to water control (t probabilities calculated within GLM) with *$P < 0.001$ (Col-0, $n \geq 23, F_{1,46} = 15.5$), $P = 0.384$ ($cyp79b2/cyp79b3$ mutants, $n \geq 16, F_{1,36} = 0.76$) and $P = 0.188$ ($pad3$ mutant, $n \geq 19, F_{1,41} = 1.73$). (D) GPA-derived extract-triggered PAD3 expression is not dependent on BAK1 or AtRbohD. Bars show the means ± SE of target gene expression levels of three independent experiments ($n = 3$ per experiment). Expression levels were normalized with the water control of Col-0 set at 1. Asterisks indicate significant differences compared to water control (t probabilities calculated within GLM) with *$P < 0.05$.

Figure 4. GPA-derived extract eliciting activities disappear upon boiling and proteinase K treatments. (A) Boiled GPA-derived extract does not elicit a ROS burst. ROS bursts were measured over a 600 min period. Bars show means ± SE of $n = 16$ leaf discs per replicate. Data of one representative experiment is shown. The experiment was repeated three times with similar results. Bars marked with different letters indicate significant differences at $P < 0.05$ using analysis of variance (ANOVA). (B) Boiled and proteinase K-treated GPA-derived extract do not elicit induced-resistance. Bars show the means ± SE of total nymphs produced per plant of three independent experiments. Bars marked with different letters indicate significant differences at $P < 0.05$ (t probabilities calculated within GLM) ($n = 30, F_{3,119} = 7.688$). (C) The 3 to 10 kD fraction of GPA-derived extract elicits ROS bursts. ROS bursts were measured over an 800 min period. Bars show...
means ± SE of n = 16 leaf discs per replicate. Data of one representative experiment is shown. The experiment was repeated three times with similar results. Letters indicates significant differences at $P < 0.05$ using ANOVA. (D) 3 to 10 kD and >10 kD GPA-derived extracts elicit induced-resistance. Bars show the means ± SE of total nymphs produced per plant of six independent experiments. Letters indicate significant differences at $P < 0.05$ (t probabilities calculated within GLM) ($n = 60, F_{3,237} = 6.051$).

**Figure 5.** Plant immune responses to individual GPA-derived elicitor fractions are BAK1 dependent. (A) BAK1 is involved in Arabidopsis ROS burst to GPA-derived elicitors. ROS bursts were measured in response to buffer and 2.5 mg/mL 3-10 kD GPA-derived extract over an 800 min period. Bars show means ± SE of n = 8 leaf discs per replicate. Data of one representative experiment is shown. The experiment was repeated three times with similar results. Asterisk indicates significant differences at $P < 0.05$ between GPA-derived extract ROS burst in Col-0 and bak1-5 using Student’s t-test. (B and C) The ROS burst of Arabidopsis to GPA-derived elicitors is not reduced in mutants of known PRR genes. ROS bursts were measured in response to 2.5 mg/mL 3-10 kD GPA-derived extract over an 800 min period. Bars show means ± SE of n = 16 leaf discs per replicate. Data of one representative experiment is shown. The experiment was repeated three times with similar results. Letters indicates significant differences at $P < 0.05$ using ANOVA. (D) Induced-resistance to GPA 3-10 kD and >10 kD fractions is dependent on BAK1. Bars show the means ± SE of total nymphs produced per plant of four independent experiments ($n = 8$ per experiment). Nymph counts were normalized with the buffer control set at 100%. Asterisks indicate significant differences at $P < 0.05$ (t probabilities calculated within GLM) (Col-0 n ≥ 28, $F_{2,86} = 8.14$. bak1-5 n ≥ 25, $F_{2,80} = 1.53$).

**Figure 6.** BAK1 is involved in pea aphid resistance.
(A) Elicitors derived from several aphid species trigger upregulation of PTI marker genes. Bars show the means ± SE of target gene expression levels of four biological replicates (n = 3 per replicate). Asterisks indicate significant differences in aphid-derived extracts compared to water (t probabilities calculated within GLM) with $*P < 0.05$. (B) Pea aphids do not survive beyond 6 days on Col-0 Arabidopsis. Data show the percentage of aphids alive at a given time point with means ± SE of four biological replicates with n = 5 per replicate. The time point at which 50% of pea aphids are still alive is indicated. (C) Pea aphids survive better on Arabidopsis bak1-5 plants. Bars show the percentage of aphids alive between
days 3 and 4 with means ± SE of six biological replicates with n = 5 per replicate. Asterisk indicates significant difference in aphid survival (t probabilities calculated within GLM) (n = 30, \( F_{1,59} = 5.028 (\*P = 0.025) \).
Figure 1. Plant defense elicitations to GPA-derived extract are characteristic of PAMP-triggered immunity. (A) GPA-derived extract elicits the expression of PTI marker genes. Bars show the means ± SE of target gene expression levels of four independent experiments (n = 3 per experiment). Asterisks indicate significant differences in GPA fraction compared to water (t probabilities calculated within GLM) with *P < 0.05 compared to water control for each gene and **P < 0.05 between flg22 and GPA-derived extract treatment. (B) GPA-derived extract elicits callose deposition. Data shown are mean callose deposits produced per 1.34 mm² of leaf upon each treatment with means ± SE of three independent experiments (n = 12 leaf discs per experiment). Different letters indicate significant differences between the treatments (t probabilities calculated within GLM) at P < 0.05 (n = 36, F 2,103 = 2039.93). (C and D) Col-0 leaf discs were elicited with water, 12.5 nM flg22 (in water) and GPA-derived extract (in water) and ROS bursts in these leaf discs were measured using luminol-based assays at (C) 0 to 60 min and (D) 60 to 600 min after elicitation. RLU = Relative light units. Graphs show means ± SE of n = 32 leaf discs per replicate. Data of one representative experiment is shown. The experiment was repeated three times with similar results.
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(B) GPA-derived extract-elicited callose deposition is significantly reduced in bak1-5 and pen2-1. Data shown are mean callose deposits produced per 1.34 mm² of leaf upon each treatment with means ± SE of three independent experiments (n = 12 leaf discs per replicate). Different letters indicate significant differences between the treatments (t probabilities calculated within GLM) at \( P < 0.05 \) (n = 36, \( F_{10,323} = 1388.15 \)).
Figure 3. Plant defense responses elicited by GPA-derived extract are dependent on BAK1 and PAD3. (A and B) Induced-resistance to (A) GPA-derived extract and (B) GPA 3-10 kD fraction is dependent on BAK1. Bars show the means ± SE of total nymphs produced per plant of (A) six and (B) three independent experiments. The nymph counts were normalized with the water or buffer controls set at 100%. Asterisks indicate significant differences to GPA fraction compared to water/buffer (t probabilities calculated within GLM) with (A) *P < 0.001 (Col-0 wild type, n = 60, F_1,19 = 17.88) and P = 0.063 (bak1-5 mutant n ≥ 57, F_1,115 = 3.45) and (B) *P = 0.005 (Col-0 wild type, n ≥ 28, F_1,56 = 8.065) and P = 0.835 (bak1-5 mutant, n ≥ 25, F_1,53 = 0.043). (C) Induced-resistance to GPA-derived extract is dependent on PAD3. Bars show the means ± SE of total nymphs produced per plant of three independent experiments. Nymph counts were normalized with the water control set at 100%. *P < 0.001 (Col-0, n ≥ 23, F_1,46 = 15.5), P = 0.384 (cyp79b2/cyp79b3 mutants, n ≥ 16, F_1,36 = 0.76) and P = 0.188 (pad3 mutant, n ≥ 19, F_1,41 = 1.73). (D) GPA-derived extract-triggered PAD3 expression is not dependent on BAK1 or AtRbohD. Bars show the means ± SE of target gene expression levels of three independent experiments (n = 3 per experiment). Expression levels were normalized with the water control of Col-0 set at 1. Asterisks indicate significant differences compared to water control (t probabilities calculated within GLM) with *P < 0.05.
Figure 4. GPA-derived extract eliciting activities disappear upon boiling and proteinase K treatments. (A) Boiled GPA-derived extract does not elicit a ROS burst. ROS bursts were measured over a 600 min period. Bars show means ± SE of n = 16 leaf discs per replicate. Data of one representative experiment is shown. The experiment was repeated three times with similar results. Bars marked with different letters indicate significant differences at \( P < 0.05 \) using analysis of variance (ANOVA). (B) Boiled and proteinase K-treated GPA-derived extract do not elicit induced-resistance. Bars show the means ± SE of total nymphs produced per plant of three independent experiments. Bars marked with different letters indicate significant differences at \( P < 0.05 \) (t probabilities calculated within GLM) \( (n = 30, F_{3,119} = 7.688) \). (C) The 3 to 10 kD fraction of GPA-derived extract elicits ROS bursts. ROS bursts were measured over an 800 min period. Bars show means ± SE of n = 16 leaf discs per replicate. Data of one representative experiment is shown. The experiment was repeated three times with similar results. Letters indicates significant differences at \( P < 0.05 \) using ANOVA. (D) 3 to 10 kD and >10 kD GPA-derived extracts elicit induced-resistance. Bars show the means ± SE of total nymphs produced per plant of six independent experiments. Letters indicate significant differences at \( P < 0.05 \) (t probabilities calculated within GLM) \( (n = 60, F_{3,237} = 6.051) \).
Figure 5. Plant immune responses to individual GPA-derived elicitor fractions are BAK1 dependent. (A) BAK1 is involved in Arabidopsis ROS burst to GPA-derived elicitors. ROS bursts were measured in response to buffer and 2.5 mg/mL 3-10 kD GPA-derived extract over an 800 min period. Bars show means ± SE of n = 8 leaf discs per replicate. Data of one representative experiment is shown. The experiment was repeated three times with similar results. Asterisk indicates significant differences at \( P < 0.05 \) between GPA-derived extract ROS burst in Col-0 and bak1-5 using Student’s t-test. (B and C) The ROS burst of Arabidopsis to GPA-derived elicitors is not reduced in mutants of known PRR genes. ROS bursts were measured in response to 2.5 mg/mL 3-10 kD GPA-derived extract over an 800 min period. Bars show means ± SE of n = 16 leaf discs per replicate. Data of one representative experiment is shown. The experiment was repeated three times with similar results. Letters indicates significant differences at \( P < 0.05 \) using ANOVA. (D) Induced-resistance to GPA 3-10 kD and >10 kD fractions is dependent on BAK1. Bars show the means ± SE of total nymphs produced per plant of four independent experiments (n = 8 per experiment). Nymph counts were normalized with the buffer control set at 100%. Asterisks indicate significant differences at \( P < 0.05 \) (t probabilities calculated within GLM) (Col-0 n ≥ 28, \( F_{2,86} = 8.14 \). bak1-5 n ≥ 25, \( F_{2,80} = 1.53 \)).
Figure 6. BAK1 is involved in pea aphid resistance.

(A) Elicitors derived from several aphid species trigger upregulation of PTI marker genes. Bars show the means ± SE of target gene expression levels of four biological replicates (n = 3 per replicate). Asterisks indicate significant differences in aphid-derived extracts compared to water (t probabilities calculated within GLM) with *$P < 0.05$.

(B) Pea aphids do not survive beyond 6 days on Col-0 Arabidopsis. Data show the percentage of aphids alive at a given time point with means ± SE of four biological replicates with n = 5 per replicate. The time point at which 50% of pea aphids are still alive is indicated.

(C) Pea aphids survive better on Arabidopsis bak1-5 plants. Bars show the percentage of aphids alive between days 3 and 4 with means ± SE of six biological replicates with n = 5 per replicate. Asterisk indicates significant difference in aphid survival (t probabilities calculated within GLM) (n = 30, $F_{1,59} = 5.028$ (*$P = 0.025$).