

β -amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose

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Summary

The non-protein amino acid β -amino-butyric acid (BABA) protects plants against a wide range of pathogens. We have examined the effectiveness and mode of action of BABA on resistance against two necrotrophic pathogens. Treatment of *Arabidopsis* with BABA induced resistance against *Alternaria brassicicola* and *Plectosphaerella cucumerina* to a similar level by jasmonic acid (JA). Conversely, treatment with benzothiadiazole (BTH), a functional analogue of salicylic acid (SA), had no significant effect on the resistance against both pathogens. BABA-induced resistance against *A. brassicicola* and *P. cucumerina* was unaffected in the JA-insensitive mutant *coi1-1* and the camalexin-deficient mutant *pad3-1*. Moreover, the expression of BABA-induced resistance was not associated with enhanced accumulation of camalexin or enhanced transcription of the JA-inducible *PDF1.2* gene. The expression of BABA-induced resistance against *P. cucumerina* was unaffected in mutants impaired in ethylene (ET) and SA signalling, but was blocked in the abscisic acid (ABA)-deficient mutant *aba1-5*, the ABA-insensitive mutant *abi4-1* and the callose-deficient mutant *pmr4-1*. Upon infection by both pathogens, BABA-treated plants showed an earlier and more pronounced accumulation of callose. Treatment with the callose-inhibitor 2-deoxy-D-glucose (2-DDG) reversed the BABA-induced resistance against *A. brassicicola*. Furthermore, primed callose deposition was absent in BABA-treated *abi4-1* and *pmr4-1* plants upon infection by *P. cucumerina*. Although the expression of BABA-induced resistance was not associated with enhanced transcription of the ABA-inducible *RAB18* gene, application of ABA mimicked the effect of BABA on the level of callose accumulation and resistance. Hence, BABA-induced resistance against necrotrophic pathogens is based on primed callose accumulation, which is controlled by an ABA-dependent defence pathway.

Keywords: BABA-induced resistance, necrotrophic pathogens, abscisic acid, jasmonic acid, salicylic acid, camalexin.

Introduction

The success of a plant to combat microbial pathogens depends on speed. Early recognition of the pathogen is critical if the plant is to mobilise the available biochemical and structural defences to effectively halt the invading pathogen. If the plant fails to do so, the appropriate defences are activated too late, and the pathogen starts colonising the plant tissue. During this stage of infection, the plant responds by activating defences around the sites of pathogen invasion. This so-called basal resistance contributes to slow down the colonisation by the pathogen, but it is too weak and too late to prevent disease.

Biotrophic pathogens, which parasitise living plant cells, are controlled by other basal defence mechanisms as compared to necrotrophic pathogens, which immediately kill host cells to metabolise their contents. In *Arabidopsis*, activation of salicylic acid (SA)-dependent defence mechanisms enhances the level of basal resistance against the biotrophic pathogens *Hyaloperonospora parasitica* and turnip crinkle virus (Delaney *et al.*, 1994; Kachroo *et al.*, 2000; Uknes *et al.*, 1993), whereas jasmonic acid (JA)- and ethylene (ET)-dependent defences yield no resistance against these pathogens (Thomma *et al.*, 1998).

Conversely, JA- and ET-dependent defence mechanisms significantly contribute to the level of basal resistance against necrotrophic pathogens, such as *Botrytis cinerea*, *Alternaria brassicicola* and *Plectosphaerella cucumerina* (Berrocal-Lobo *et al.*, 2002; Thomma *et al.*, 1998, 1999a). Furthermore, the *Arabidopsis* mutant *pad3-1*, which is impaired in the biosynthesis of the phytoalexin camalexin, shows reduced levels of basal resistance against the necrotrophic fungi *A. brassicicola* and *B. cinerea* (Ferrari *et al.*, 2003; Thomma *et al.*, 1999b), but develops wild-type levels of resistance against the biotrophic pathogens *Pseudomonas syringae* and *H. parasitica* (Glazebrook and Ausubel, 1994; Van Wees *et al.*, 2003). Thus, the efficiency of the plant's basal resistance response depends on the challenging pathogen.

Besides basal resistance that is expressed locally upon primary pathogen attack, plants are also capable of enhancing their level of resistance against future pathogen attack. This phenomenon is known as induced resistance and can be triggered by a variety of biotic and abiotic stimuli. The classic example of induced resistance is often referred to as systemic acquired resistance (SAR), and occurs in distal plant parts upon localised infection by a necrosis-inducing pathogen (Ryals *et al.*, 1996). The expression of pathogen-induced SAR depends on endogenous accumulation of SA and is marked by the transcriptional activation of SA-inducible genes encoding pathogenesis-related (PR) proteins (Gaffney *et al.*, 1993; Lawton *et al.*, 1994; Nawrath and Métraux, 1999). Apart from pathogen-induced SAR, selected strains of non-pathogenic rhizobacteria can induce systemic resistance as well. This so-called rhizobacteria-mediated induced systemic resistance (ISR) is not associated with transcriptional activation of genes encoding PR proteins (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997), and functions independently of SA, but requires intact responsiveness to the plant hormones JA and ET (Pieterse *et al.*, 1998). Besides these differences in signalling pathway, ISR and SAR also differ in the spectrum of pathogens against which they yield protection. Whereas SAR is mostly effective against biotrophic pathogens that are resisted by SA-dependent basal resistance, ISR predominantly yields protection against necrotrophic pathogens that are sensitive to JA- and ET-dependent basal resistance (Ton *et al.*, 2002).

Apart from SAR and ISR, treatment with the non-protein amino acid β -amino-butyric acid (BABA) has demonstrated to activate an induced resistance response as well. Although BABA is found rarely as a natural occurring compound in plants, it is a potent inducer of broad-spectrum disease resistance in different plant species (Cohen, 2002; Jakab *et al.*, 2001). Non-protein amino acids, such as BABA and its isomer γ -amino-butyric acid (GABA), have known biological effects in animals and plants. For instance, GABA functions as an inhibitory neurotransmitter

in the central nervous system of animals (Waagepetersen *et al.*, 1999), whereas BABA can act as a partial agonist of the glycine receptor (Schmieden and Betz, 1995). In plants, GABA is produced in response to stress (Shelp *et al.*, 1999), although its ability to induce resistance against plant diseases remains controversial (Cohen, 2002; Shelp *et al.*, 1999). In contrast, the broad-spectrum protective effect of BABA against numerous diseases and abiotic stresses is well documented in plants (Jakab *et al.*, 2001). Research in *Arabidopsis* revealed that the signalling pathway controlling BABA-induced resistance is specific for the challenging pathogen. BABA-induced resistance against the bacterial pathogen *P. syringae* pv. *tomato* DC3000 and the fungal pathogen *B. cinerea* resembles pathogen-induced SAR in that it depends on endogenous accumulation of SA and an intact NPR1 protein (Zimmerli *et al.*, 2000, 2001). Although treatment with BABA alone did not induce the expression of SA- and JA-/ET-inducible PR genes, plants treated with BABA showed a faster and stronger transcription of the SA-inducible PR-1 gene upon infection with *P. syringae*, similar to that observed during the expression of biologically induced SAR (Cameron *et al.*, 1999; Kohler *et al.*, 2002). However, BABA-induced resistance against the oomycete pathogen *H. parasitica* was fully functional in *Arabidopsis* genotypes impaired in SAR and ISR signalling (Zimmerli *et al.*, 2000). This indicates that BABA can activate resistance through a yet unidentified signalling pathway.

A common characteristic of the various induced resistance phenomena is that they are all associated with an enhanced capacity to express cellular defence responses, which are induced specifically upon attack by a pathogen. By analogy with a phenotypically similar phenomenon in mammalian monocytes and macrophages, the capacity to express a faster and stronger basal defence response upon pathogen infection is called priming (Conrath *et al.*, 2002). In *Arabidopsis*, the enhanced resistance by BABA is based on various priming mechanisms. BABA-induced resistance against *P. syringae* and *B. cinerea* was found to correlate with primed transcription of the SA-inducible PR-1 gene in wild-type plants, whereas NahG and *npr1* plants failed to express BABA-induced resistance against these pathogens (Zimmerli *et al.*, 2000, 2001). This clearly demonstrates that priming for SA-inducible defences is the predominant mechanism by which BABA protects against *P. syringae* and *B. cinerea*. In contrast, priming for SA-inducible defences cannot explain the protection against *H. parasitica*, as SA non-accumulating NahG plants and SA-insensitive *npr1* plants were fully capable of expressing BABA-induced resistance against this pathogen (Zimmerli *et al.*, 2000). Interestingly, the expression of the BABA-induced resistance against *H. parasitica* correlated with an earlier and stronger formation of callose-rich papillae around the growing hyphae of the pathogen (Zimmerli *et al.*, 2000), suggesting

that BABA-induced resistance against *H. parasitica* is based on primed callose deposition. However, conclusive evidence that enhanced callose deposition was the actual mechanism behind this BABA-induced resistance was not provided. Recently, we found that BABA is also effective against osmotic stress (G. Jakab and J. Ton, unpublished data). This BABA-induced protection against salt and drought stresses was functional in *Arabidopsis* genotypes impaired in SA, JA or ET signalling, but was blocked in *Arabidopsis* mutants impaired in the production or sensitivity to the abiotic stress hormone abscisic acid (ABA). Additionally, BABA-treated wild-type plants expressed ABA-inducible marker genes at lower salt concentrations than non-induced plants, indicating that BABA-induced protection against osmotic stress is achieved through priming of ABA-dependent defence mechanisms. Hence, BABA triggers a primed state in the plant that enables a more efficient activation of SA-dependent defence mechanisms, callose deposition or ABA-dependent defence mechanisms.

To gain more insight into the SA-independent component of BABA-induced resistance, we investigated the effectiveness and mode of action of BABA against two necrotrophic pathogens that are relatively insensitive to SA-induced resistance. Here, we show that the expression of BABA-induced resistance against *A. brassicicola* and *P. cucumerina* is not based on SA-, JA- or ET-dependent defences, or on enhanced accumulation of camalexin. Instead, BABA-induced resistance against these necrotrophs is primarily based on a primed accumulation of callose. In addition, we provide evidence that this primed callose response is regulated by an ABA-dependent signalling pathway.

Results

Comparison between BABA-induced resistance and benzothiadiazole (BTH)- and JA-induced resistance against A. brassicicola and P. cucumerina

Wild-type *Arabidopsis* exhibits full resistance against the necrotrophic fungus *A. brassicicola* and is therefore unsuitable for studying induced resistance against the fungus. In contrast, the camalexin-deficient mutant *pad3-1* as well as the JA-insensitive mutant *coi1-1* are susceptible to *A. brassicicola* (Thomma *et al.*, 1998, 1999b). To determine whether BABA-induced resistance is effective against *A. brassicicola*, 6-week-old *pad3-1* plants were treated with either water or BABA, and subsequently challenged with *A. brassicicola*. Compared to water-treated control plants, soil drench treatment with 150 μ M BABA resulted in a statistically significant reduction of lesion size at 4 days after challenge inoculation (Figures 1a and 2a). The level of BABA-induced resistance was comparable to the level of

resistance induced by soil drench treatment with 100 μ M JA. In contrast, treatment with 300 μ M of SA analogue BTH yielded no protection against *A. brassicicola*, even though this amount of BTH was enough to activate SA-inducible *PR-1* transcription (data not shown). To determine the effectiveness of BABA-induced resistance against the necrotrophic fungus *P. cucumerina*, 5-week-old Col-0 plants were treated with 150 μ M BABA, and were subsequently challenged with *P. cucumerina* spores. Compared to water-treated control plants, treatment with BABA induced a statistically significant reduction in lesion size at 6 days after challenge inoculation (Figures 1b and 2b). Also in this interaction, the level of BABA-induced resistance was comparable to that of JA-induced resistance. Treatment with 300 μ M BTH resulted in a relatively weak reduction in lesion size compared to control plants, which was not statistically significant (Figure 2b). Apparently, SA-inducible resistance is considerably less effective than BABA- and JA-induced resistance against *P. cucumerina*.

The role of JA- and SA-dependent defences in BABA-induced resistance against A. brassicicola and P. cucumerina

Because treatment with BABA protected *Arabidopsis* to a similar level as treatment with JA (Figure 2), we examined the possibility that BABA-induced resistance against *A. brassicicola* and *P. cucumerina* is based on primed expression of JA-inducible defences. To this end, we tested the effectiveness of BABA in JA-insensitive *coi1-1* plants. Treatment of this mutant with 150 μ M BABA resulted in a statistically significant reduction in lesion size upon infection with *A. brassicicola* and *P. cucumerina* (Figure 3). This indicates that JA-dependent defences are not required for the expression of BABA-induced resistance against both pathogens. To further test the involvement of JA-dependent defence mechanisms, we examined the expression of the JA-inducible *PDF1.2* gene in *pad3-1* and Col plants upon infection with *A. brassicicola* or *P. cucumerina*, respectively. RNA gel blot analysis revealed that treatment with BABA alone did not induce transcription of the *PDF1.2* marker gene (Figure 4). Moreover, pathogen-induced *PDF1.2* transcription was not enhanced in BABA-treated plants compared to water-treated plants, indicating that BABA-induced resistance against *A. brassicicola* and *P. cucumerina* is not based on primed expression of JA-inducible defences. Hybridisation of the blots with a probe for the SA-inducible *PR-1* gene revealed that BABA treatment caused an earlier and stronger induction of *PR-1* upon infection by both pathogens, although treatment with BABA alone had no effect on the gene (Figure 4). It can thus be concluded that BABA primes for SA-dependent defence responses upon infection by both fungi.

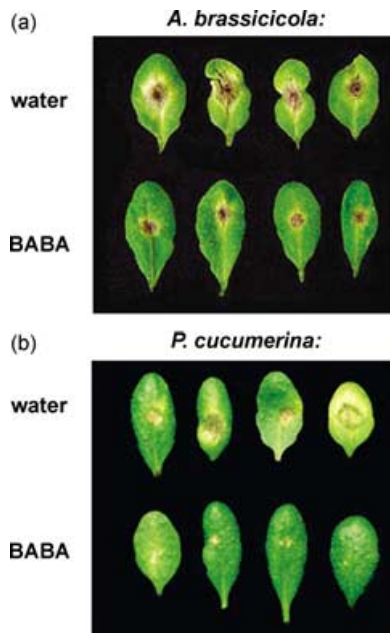


Figure 1. Lesion development on *Arabidopsis* leaves infected with *A. brassicicola* and *P. cucumerina*.

(a) *A. brassicicola*: 6-week-old *pad3-1* plants were soil-drenched with water or 150 μM BABA at 2 days prior to challenge inoculation with 6- μl droplets containing 2×10^6 spores ml^{-1} . Pictures were taken at 4 days after inoculation.

(b) *P. cucumerina*: 5-week-old Col plants were soil-drenched with water or 150 μM BABA at 2 days prior to challenge inoculation with 6- μl droplets containing 5×10^6 spores ml^{-1} . Pictures were taken at 6 days after inoculation.

The role of camalexin in BABA-induced resistance against *A. brassicicola* and *P. cucumerina*

To test whether camalexin plays a role in BABA-induced resistance against *A. brassicicola* and *P. cucumerina*, we quantified BABA-induced resistance in camalexin-deficient *pad3-1* plants. Treatment with BABA induced normal levels of resistance against *A. brassicicola* and *P. cucumerina* (Figures 1a and 3), indicating that camalexin is not required for the induced resistance. Furthermore, quantification of camalexin levels revealed that BABA-treated *coi1-1* and Col plants did not accumulate enhanced levels of camalexin upon infection with *A. brassicicola* or *P. cucumerina*, respectively (Figure 5). In contrast, water-treated control plants accumulated significantly more camalexin than BABA-treated plants. This clearly demonstrates that BABA-induced resistance against *A. brassicicola* and *P. cucumerina* is not based on primed accumulation of camalexin.

BABA-induced resistance against *P. cucumerina* depends on an ABA-dependent signalling pathway

As described above, BABA-induced resistance against *P. cucumerina* is not affected in *pad3-1* and *coi1-1* plants. Similarly, ET-insensitive (*ein2-1*) plants, SA non-accumulat-

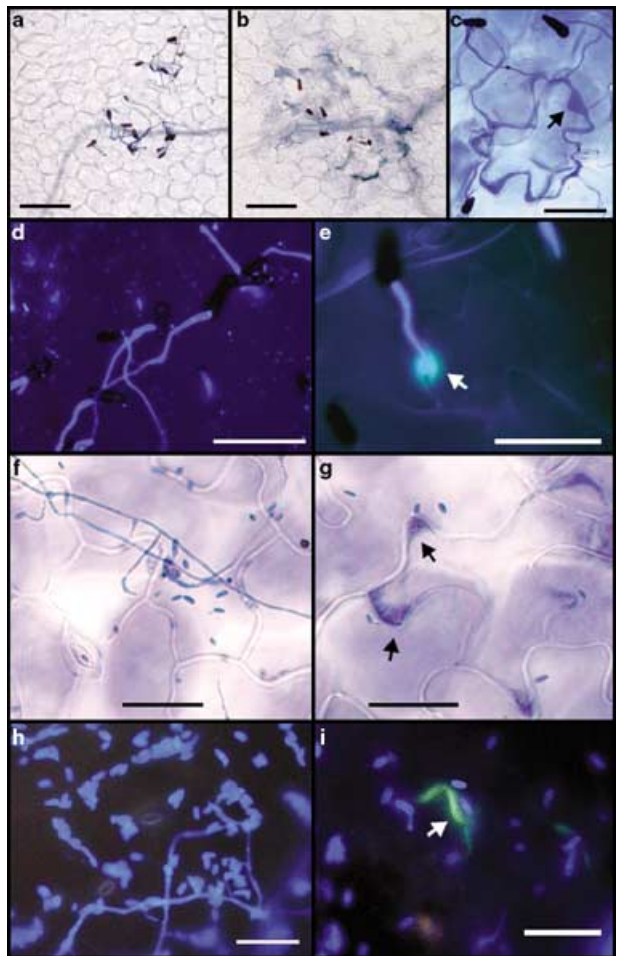


Figure 6. Tissue colonisation by *A. brassicicola* and *P. cucumerina* in water- and BABA-treated *Arabidopsis* at 2 days post-inoculation (dpi).

(a) *A. brassicicola* in water-treated *pad3-1* plants (bar = 100 μm).

(b) *A. brassicicola* in BABA-treated *pad3-1* plants (bar = 100 μm).

(c) Papillae formation (arrow) in an epidermal cell surrounded by germ tubes of *A. brassicicola* in BABA-treated *pad3-1* (bar = 25 μm).

(d) *A. brassicicola* in water-treated *pad3-1* plants (bar = 25 μm).

(e) Callose deposition (arrow) around an appressorium of *A. brassicicola* in BABA-treated *pad3-1* (bar = 25 μm).

(f) *P. cucumerina* in water-treated Col plants (bar = 25 μm).

(g) Papillae formation (arrows) in an epidermal cell surrounded by germinating spores of *P. cucumerina* in BABA-treated Col plants (bar = 25 μm).

(h) *P. cucumerina* in water-treated Col plants (bar = 15 μm).

(i) Callose deposition (arrow) around germinating spores of *P. cucumerina* in BABA-treated Col plants at 2 dpi (bar = 15 μm).

Leaves were stained with lactophenol/trypan blue and analysed by light microscopy (a–c, f, g), or stained with calcofluor/aniline blue and analysed by epifluorescence microscopy (UV; d, e, h, i).

ing NahG plants and SA-insensitive *npr1-1* plants were fully capable of expressing BABA-induced resistance against *P. cucumerina* (Figure 3b). Apparently, camalexin as well as SA-, JA- and ET-dependent defence mechanisms do not contribute to BABA-induced resistance against this pathogen. In contrast, the ABA-deficient mutant *aba1-5* (Koornneef *et al.*, 1982) and the ABA-insensitive mutant *abi4-1* (Finkelstein, 1994), which are both in the background

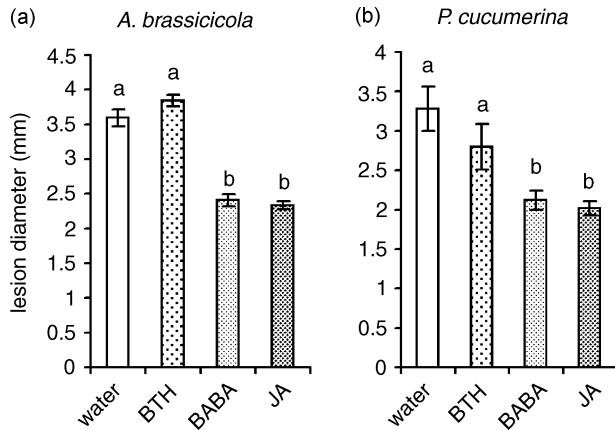


Figure 2. Quantification of BTH-, BABA- and JA-induced resistance against *A. brassicicola* and *P. cucumerina*.

(a) *A. brassicicola*: 6-week-old *pad3-1* plants were soil-drenched with water, 300 μ M BTH, 150 μ M BABA or 100 μ M JA. At 2 days after chemical treatment, 6–8 leaves per plant were challenged by applying 6- μ l droplets containing 2×10^6 spores ml^{-1} . Average lesion diameters for 20–30 plants per treatment were determined at 4 days after inoculation.

(b) *P. cucumerina*: 5-week-old Col plants were chemically treated as described for (a). Challenge inoculation was performed at 2 days after chemical treatment by applying 6- μ l droplets containing 5×10^6 spores ml^{-1} . Average lesion diameters were determined at 6 days after inoculation.

Values presented are means \pm SEM. Different letters indicate statistically significant differences (Fisher's least significant difference (LSD) test; $\alpha = 0.05$).

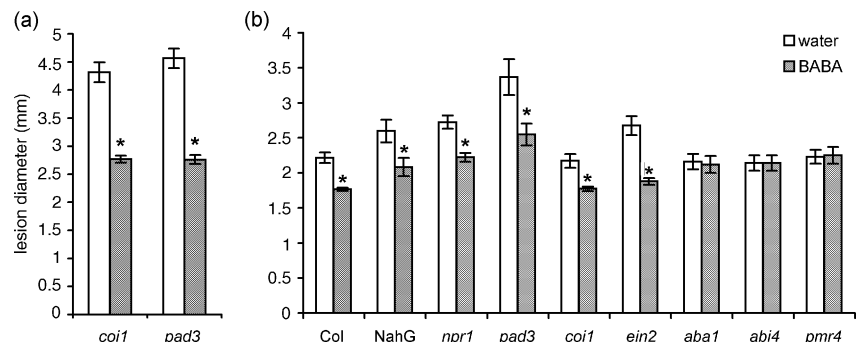
of accession Col-0, were completely blocked in their ability to express BABA-induced resistance against *P. cucumerina* (Figure 3b). This indicates that the expression of BABA-induced resistance against *P. cucumerina* requires intact ABA signalling. Quantification of BABA-induced resistance against *A. brassicicola* in *ein2-1*, *npr1-1*, *NahG*, *aba1-5* and *abi4-1* was not possible, because all these genotypes exhibited full resistance against the fungus (data not shown). To examine whether the expression of BABA-induced resistance against *P. cucumerina* and *A. brassicicola* is accompanied with enhanced production of ABA, we tested the expression of the ABA-inducible *RAB18* gene upon infection by both pathogens. RNA blot analysis showed no enhanced transcription of the *RAB18* gene in water- and

BABA-treated plants upon infection by both pathogens (Figure 4a,b), whereas treatments with 150 mM NaCl or 80 μ M ABA were both effective in inducing the expression of the *RAB18* gene (Figure 4c). These findings suggest that the BABA-induced resistance is not mediated by major rises in ABA levels.

BABA-induced resistance against A. brassicicola is based on a primed accumulation of callose

To gain more insight into the nature of BABA-induced resistance against *A. brassicicola*, cytological observations were performed at the sites of infection. At 2 days after inoculation, approximately half of the spores had germinated on the leaves of *pad3-1* plants. Although treatment with BABA had no obvious effect on the efficiency of spore germination, the length of the germ tubes was significantly reduced on leaves of BABA-treated plants (Figure 6a,b). Furthermore, germinating spores induced significantly more papillae in the epidermal cell layer of BABA-treated plants than in leaves of control plants (Figure 6c). Aniline blue staining revealed that these papillae contained callose (Figure 6d,e). Thus, the expression of BABA-induced resistance against *A. brassicicola* is associated with primed deposition of callose-rich papillae. To determine whether this primed callose response contributes to the BABA-induced resistance against the pathogen, the petioles of detached leaves of 5-week-old *pad3-1* plants were placed in tubes containing 150 μ M BABA with increasing concentrations of the callose inhibitor 2-deoxy-D-glucose (2-DDG; Jaffe and Leopold, 1984). After 24 h of incubation, the leaves were challenged with *A. brassicicola* spores. Callose accumulation was quantified microscopically by determining the percentage of callose-inducing spores at 2 days after inoculation (Figure 7a), and the level of induced resistance was quantified by determining average lesion diameters at 4 days after inoculation (Figure 7b). Leaves incubated in 150 μ M BABA showed a 40% reduction in lesion size compared to water-incubated control leaves (Figure 7a). This enhanced resistance correlated with a threefold increase in the percentage of callose-inducing

Figure 3. Quantification of BABA-induced resistance in different *Arabidopsis* genotypes against *A. brassicicola* (a) and *P. cucumerina* (b). For experimental details, see legend to Figure 2. Asterisks indicate statistically significant differences compared to water-treated control plants (Student's *t*-test; $\alpha = 0.05$).



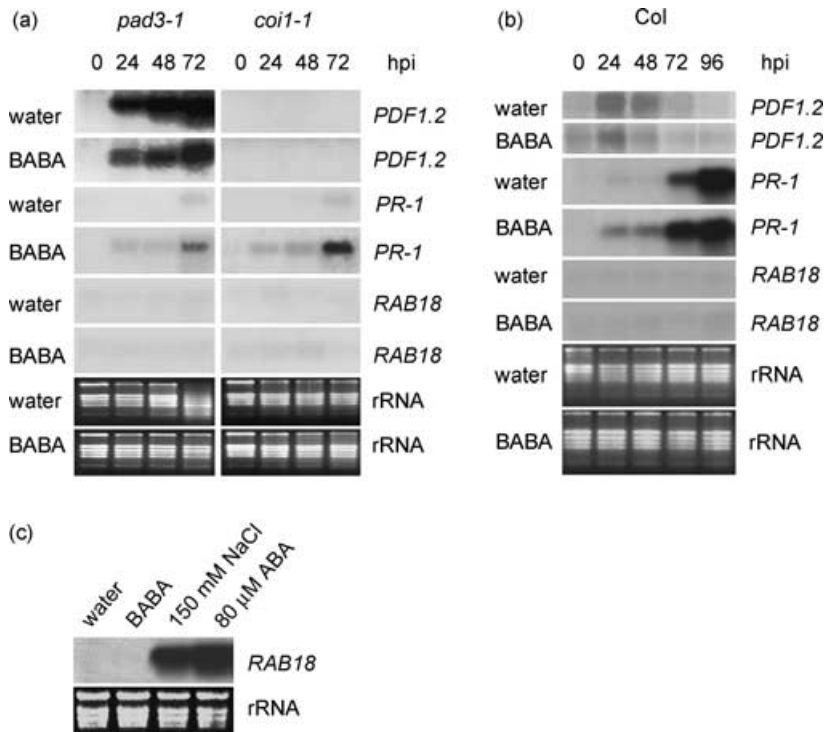


Figure 4. RNA gel blot analysis of *PDF1.2*, *PR-1* and *RAB18* gene expression in water- and BABA-treated plants at different hours after infection with *A. brassicicola* (a) or *P. cucumerina* (b).

Six-week-old plants were soil-drenched with water or 150 μM BABA at 2 days before challenge inoculation with *A. brassicicola* or *P. cucumerina*. Induction of *RAB18* gene expression was verified in 6-week-old *Col* plants at 2 days after treatment with 150 μM BABA, 150 mM NaCl or 80 μM ABA (c). Each time-point represents 20 leaves per treatment collected from four different plants. RNA blots were hybridised with *PR-1*, *PDF1.2* or *RAB18* probes. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading. hpi, hours post-inoculation.

spores (Figure 7b). Co-application of BABA with increasing concentrations of 2-DDG reduced the percentage of callose-inducing spores. At 250 μM 2-DDG, the level of callose-inducing spores was comparable to that in leaves of non-induced plants (Figure 7a). The reduction in callose accumulation correlated with an increase in average lesion size. At a concentration of 250 μM 2-DDG, the resistance-inducing effect of BABA was completely abolished (Figure 7b). Thus, inhibition of callose deposition and

possibly other associated responses by 2-DDG reduces BABA-induced resistance against *A. brassicicola*. Nevertheless, treatment with 2-DDG did not severely affect papillae formation (data not shown). This suggests that papillae can still occur in the absence of callose, but have no further contribution to the BABA-induced resistance.

BABA-induced resistance against P. cucumerina is based on ABA-dependent priming for callose accumulation

To assess the involvement of callose in BABA-induced resistance against *P. cucumerina*, cytological observations were performed at the sites of infection in water- and BABA-treated *Col* plants. As observed during the interaction with *A. brassicicola*, BABA-treated plants developed significantly more callose-containing papillae than non-induced control plants at 2 days after inoculation with *P. cucumerina* (Figure 6f–i). To determine whether this primed callose deposition is responsible for the BABA-induced resistance against *P. cucumerina*, we have determined the level of callose accumulation and BABA-induced resistance in water- and BABA-treated *Col* and *pmr4-1* plants. The *pmr4-1* mutant carries a mutation in the callose synthase gene *AtGSL5* and fails to synthesise callose in response to pathogen infection and wounding (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003). At different time points after inoculation, infected leaves were collected, stained with aniline blue and examined by UV fluorescence microscopy.

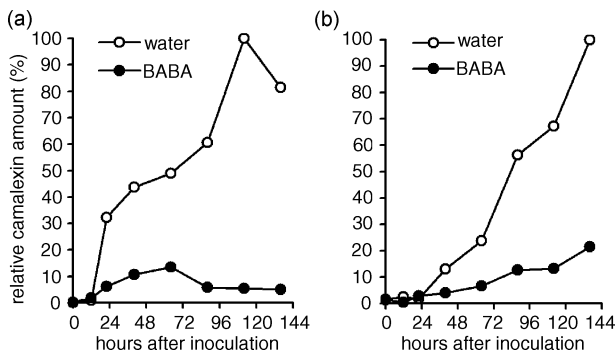


Figure 5. Quantification of camalexin production in water- and BABA-treated *coi-1* (a) and *Col* (b) during infection with *A. brassicicola* and *P. cucumerina*, respectively.

Six-week-old plants were soil-drenched with water or 150 μM BABA at 2 days prior to challenge inoculation. Camalexin was extracted at different time-points after inoculation and quantified by luminescence/fluorescence spectrometry. Each time-point represents six infected leaves collected from three different plants. Camalexin quantities were expressed relative to the sample containing the highest emission value.

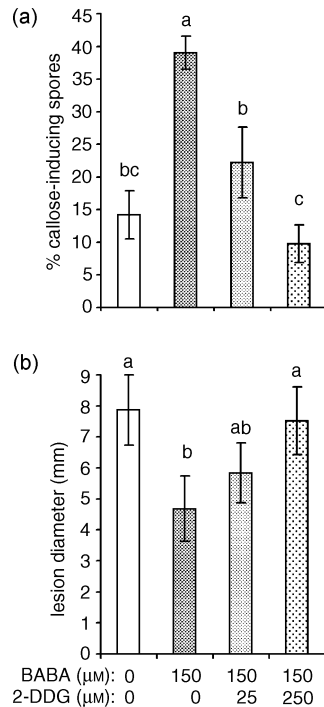


Figure 7. Effect of the callose-inhibitor 2-DDG on the level of callose deposition and BABA-induced resistance against *A. brassicicola*.

Leaves of 5-week-old *pad3-1* plants were detached and incubated for 24 h in Eppendorf tubes containing different concentration of BABA and 2-DDG. Leaves were inoculated with 2×10^6 spores ml^{-1} .

(a) Callose was visualised by aniline blue staining and epifluorescence microscopy (UV) at 2 days post-inoculation (dpi). Data shown are means (\pm SEM; $n = 8$) of the percentage of callose-inducing spores per leaf.

(b) Resistance was quantified by measuring lesion diameters at 4 dpi. Data shown are means (\pm SEM; $n = 15$). Different letters indicate statistically significant differences (Fisher's LSD test; $\alpha = 0.05$).

Callose deposition was quantified by determining the relative number of yellow pixels on digital photographs that correspond to pathogen-induced callose. As shown in Figure 8, leaves of non-induced Col plants transiently increased callose deposition upon *P. cucumerina* infection. Treatment of Col plants with BABA resulted in an earlier and stronger deposition of callose, demonstrating the primed callose response (Figure 8a,b). In contrast, both water- and BABA-treated *pmr4-1* plants failed to accumulate any detectable levels of callose upon infection by *P. cucumerina* (Figure 8a). Moreover, *pmr4-1* plants failed to express BABA-induced resistance against the fungus (Figure 3b), indicating that callose is a critical factor in the establishment of BABA-induced resistance against *P. cucumerina*. As observed in response to treatment with 2-DDG, papillae formation was not severely affected in water- and BABA-treated *pmr4-1* plants (data not shown). This indicates that papillae can still occur in the absence of callose, but have no further contribution to the BABA-induced resistance.

To further investigate the relationship between ABA signalling, callose deposition and BABA-induced resistance

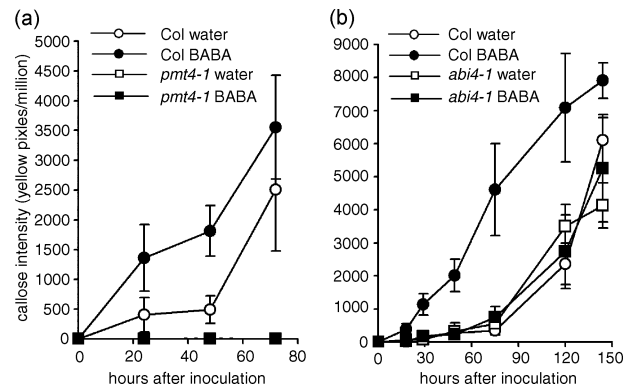


Figure 8. Callose accumulation in water- and BABA-treated Col (a,b), *pmr4-1* (a) and *abi4-1* (b) upon infection by *P. cucumerina*.

Callose was visualised by aniline blue staining and epifluorescence microscopy (UV). Quantification was performed by determining the number of yellow pixels per million pixels corresponding to pathogen-induced callose on digital photographs of infected leaf areas. Data shown are means (\pm SEM; $n = 8$) of the relative number of yellow pixels per photograph.

against *P. cucumerina*, we compared the BABA-induced callose response of Col plants to that of *abi4-1* plants, which, like *aba1-5* and *pmr4-1*, are impaired in BABA-induced resistance against the fungus (Figure 3b). Water-treated *abi4-1* plants showed similar kinetics in callose accumulation as water-treated Col plants, indicating that this mutant, unlike *pmr4-1*, is not affected in pathogen-inducible callose formation (Figure 8b). However, BABA-treated *abi4-1* plants failed to accumulate enhanced levels of callose upon pathogen infection. This indicates that the *abi4-1* mutation affects BABA-induced resistance against *P. cucumerina* by blocking the primed callose deposition.

Application of ABA mimics the effect of BABA on the level of callose deposition and resistance against A. brassicicola and P. cucumerina

To further examine the involvement of ABA in callose-mediated resistance against *A. brassicicola* and *P. cucumerina*, we determined the effect of exogenous application of ABA on the level of callose deposition and pathogen resistance upon infection with *A. brassicicola* and *P. cucumerina*. Soil drench treatment with 80 μM ABA resulted in a fivefold increase in the number of callose-inducing spores at 2 days after challenge inoculation with *A. brassicicola* (Figure 9a). Similarly, the level of callose accumulation in ABA-treated plants following *P. cucumerina* infection was increased 10-fold as compared to water-treated control plants (Figure 9a). This enhanced callose production correlated with increased levels of pathogen resistance, as evidenced by a statistically significant reduction in lesion size at 4 and 6 days after inoculation with *A. brassicicola* and *P. cucumerina*, respectively (Figure 9b). The extent of the ABA-induced effects on callose and pathogen resistance

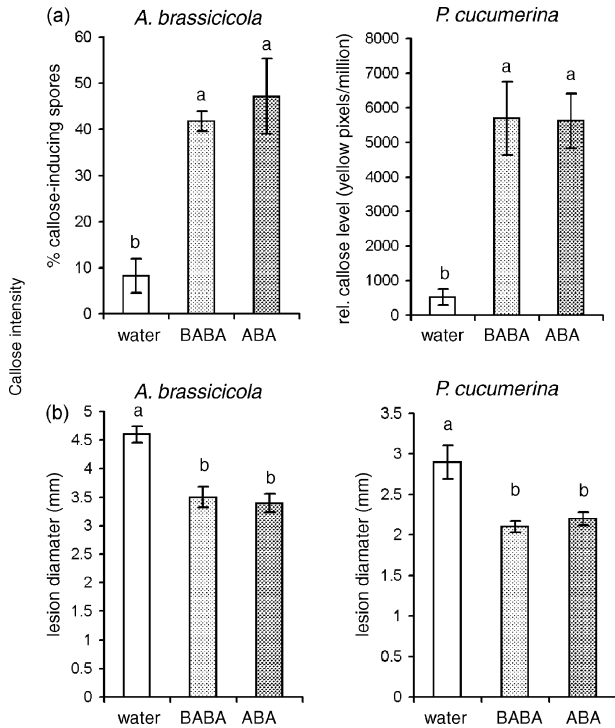


Figure 9. Effect of exogenous application of BABA and ABA on the level of callose deposition (a) and resistance (b) after inoculation with *A. brassicicola* and *P. cucumerina*. Five-week-old *pad3-1* or Col plants were soil-drenched with water, 150 μM BABA or 80 μM ABA at 2 days before challenge inoculation with *A. brassicicola* and *P. cucumerina*, respectively. For further experimental details, see legends to Figures 2 and 8. Different letters indicate statistically significant differences (Fisher's LSD test; $\alpha = 0.05$).

was comparable with that observed after treatment with BABA. Apparently, activation of the ABA response primes for pathogen-inducible callose deposition, causing enhanced levels of resistance against *A. brassicicola* and *P. cucumerina*.

Discussion

In this study, we have investigated the effectiveness and mode of action of BABA-induced resistance against two necrotrophic pathogens. We have shown that treatment with BABA induces resistance against *A. brassicicola* and *P. cucumerina*, which are both unaffected by SA-induced defence mechanisms. This confirms the earlier notion that BABA-induced resistance can be a mechanistically different resistance response than SA-dependent SAR (Zimmerli *et al.*, 2000). To elucidate the mechanisms behind the BABA-induced resistance against *A. brassicicola* and *P. cucumerina*, we examined the involvement of SA-, JA- and ET-dependent defences, camalexin accumulation and callose deposition.

Previously, we reported that BABA-induced resistance against the necrotrophic fungus *B. cinerea* is based on a primed expression of SA-dependent defence mechanisms

(Zimmerli *et al.*, 2001). This conclusion was based on the observation that SA non-accumulating NahG plants failed to express BABA-induced resistance against the fungus, and that BABA-treated wild-type plants showed primed expression of the SA-inducible marker gene *PR-1*. In this study, we found similar priming for *PR-1* expression after infection by *A. brassicicola* and *P. cucumerina* (Figure 4). However, this primed defence reaction cannot explain the BABA-induced resistance against both pathogens. In the first place, treatment with the SA analogue BTH failed to induce significant levels of resistance against *A. brassicicola* and *P. cucumerina* (Figure 2), indicating that activation of SA-inducible defence mechanisms is ineffective against both pathogens. Secondly, BABA-induced resistance against *P. cucumerina* was fully functional in SA non-accumulating NahG plants and SA-insensitive *npr1-1* plants (Figure 3b). Hence, BABA primes for SA-dependent resistance against *A. brassicicola* and *P. cucumerina*, but this primed defence expression is not effective against these pathogens, and can therefore not explain the BABA-induced resistance.

JA- and ET-dependent defences contribute to the level of basal resistance against *A. brassicicola* and *P. cucumerina* (Berrocal-Lobo *et al.*, 2002; Thomma *et al.*, 1998). Accordingly, we found enhanced susceptibility in the JA-insensitive mutant *coi1-1* to *A. brassicicola*, and enhanced susceptibility to *P. cucumerina* in the ET-insensitive mutant *ein2-1* (Figure 3). Despite the apparent function of JA and ET in basal resistance against *A. brassicicola* and *P. cucumerina*, the expression of BABA-induced resistance against both pathogens was not affected by the *coi1-1* and *ein2-1* mutations, respectively (Figure 3). Moreover, transcriptional analysis of the JA- and ET-inducible *PDF1.2* gene did not reveal primed activity of JA- and ET-dependent defence pathway in BABA-treated plants (Figure 4). It can thus be concluded that the protection by BABA against *A. brassicicola* and *P. cucumerina* is not based on potentiation for JA- and ET-inducible defences.

Another factor involved in *Arabidopsis* resistance against necrotrophic fungi is the phytoalexin camalexin. Characterisation of the camalexin-deficient mutant *pad3-1* showed that loss of camalexin confers enhanced susceptibility to *A. brassicicola* (Thomma *et al.* 1999b) and *B. cinerea* (Ferrari *et al.*, 2003). In support of these findings, we found that the level of basal resistance against *A. brassicicola* and *P. cucumerina* is severely affected by the *pad3-1* mutation (Figure 3). However, expression of BABA-induced resistance against both pathogens was unaffected in this mutant (Figure 3), and there was no primed camalexin production in BABA-treated *coi1-1* and wild-type plants upon infection by *A. brassicicola* and *P. cucumerina*, respectively (Figure 5). Hence, camalexin does not contribute to the BABA-induced resistance against both necrotrophic pathogens. Surprisingly, non-induced control plants accumulated significantly more camalexin than BABA-treated plants upon pathogen

infection. This difference can be explained by the higher level of disease incidence in non-induced plants compared to the more resistant BABA-treated plants. Because the level of pathogen colonisation is higher in the more susceptible non-induced plants, there are more cells that are directly attacked by the invading fungus. Consequently, there are more cells producing camalexin as part of the basal resistance response. The reduced camalexin production in BABA-treated plants also indicates that pathogen colonisation in BABA-treated plants is slowed down before the production of camalexin is triggered. This suggests that the mechanism behind BABA-induced resistance acts during an earlier stage of infection than camalexin.

Treatment with BABA resulted in an earlier and more pronounced accumulation of callose upon infection with *A. brassicicola* and *P. cucumerina* (Figures 6–9). Inhibition of this primed callose response by co-application of the callose inhibitor 2-DDG inhibited the BABA-induced resistance against *A. brassicicola* (Figure 7). Similarly, loss of callose accumulation in the *pmr4-1* mutant coincided with its inability to express BABA-induced resistance against *P. cucumerina* (Figures 3b and 8b). Furthermore, the BABA-mediated priming for callose during *P. cucumerina* infection was absent in the *abi4-1* mutant (Figure 8b), which concurrently lacked expression of BABA-induced resistance against this pathogen (Figure 3b). Together, these data strongly suggest that primed callose deposition is critical for the BABA-induced resistance against *A. brassicicola* and *P. cucumerina*.

Callose is an amorphous high-molecular weight β -1,3-glucan that is deposited in the form of papillae upon infection by fungal and oomycetous pathogens. It is thought to act as a physical barrier against fungal colonisation of the intercellular space. Two research groups have independently found that mutations in the callose synthase gene *AtGSL5* of *Arabidopsis*, such as *pmr4-1*, paradoxically confer enhanced resistance against powdery mildew fungi (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003). Blocking the SA-dependent defence pathway in *pmr4-1* by introducing the *NahG* gene, or *pad4* or *npr1* mutations, reversed the enhanced resistance against powdery mildew, but did not restore callose deposition. This indicates that the enhanced resistance of *pmr4-1* is caused by a hyperactive SA response (Nishimura *et al.*, 2003). Such enhanced responsiveness to SA might be caused by a disturbance of cell wall homeostasis that could lead to a conditioning effect on SA-dependent resistance. Alternatively, the hyperactive SA response of *pmr4-1* could suggest that callose or callose synthase negatively regulates the SA pathway. Nishimura *et al.* (2003) hypothesised that rapid callose deposition during the early stages of infection may repress later defence responses that may potentially be harmful for the plant. If a pathogen has evolved mechanisms to overcome the callose defence barrier, conditions become favourable for further

colonisation because of repression of SA-dependent defences by callose. However, *A. brassicicola* and *P. cucumerina* would not benefit from a repressed SA response, as they are not affected by SA-inducible resistance (Figure 2). Moreover, *A. brassicicola* and *P. cucumerina* apparently lack mechanisms to circumvent callose deposition as they are effectively halted by BABA-mediated callose deposition. Interestingly, BABA treatment has been reported to be ineffective against powdery mildew pathogens (Cohen, 2002; L. Zimmerli, personal communication). Therefore, one may envisage that the insensitivity of powdery mildew pathogens to BABA-induced defences is because of their ability to overcome the callose defence barrier.

Treatment with BABA failed to induce resistance against *P. cucumerina* in the ABA-deficient mutant *aba1-5* and the ABA-insensitive mutant *abi4-1* (Figure 3b). Furthermore, we observed that the BABA-mediated priming for callose was absent in *abi4-1* plants (Figure 9), and that exogenous application of ABA mimicked the action of BABA on the level of pathogen resistance and callose accumulation. These results indicate that ABA signalling plays a regulatory role in the primed callose response during BABA-induced resistance. This conclusion is supported by the isolation of a BABA-insensitive mutant carrying a mutation in the 3' untranslated region of a gene that encodes the ABA biosynthetic enzyme zeaxanthin epoxidase (Toquin *et al.*, 2002). Characterisation of this so-called *bai38* mutant revealed that it is impaired in the expression of BABA-induced resistance against *H. parasitica* and salt stress (Toquin *et al.*, 2002; J. Ton, unpublished results). Because expression of BABA-induced resistance against *H. parasitica* also correlates with enhanced callose deposition (Zimmerli *et al.*, 2000), the inability of mutant *bai38* to express BABA-induced resistance against *H. parasitica* supports our conclusion that ABA signalling regulates BABA-mediated priming for callose deposition.

Apart from the well-established role of ABA in the regulation of plant responses to abiotic stress (Xiong *et al.*, 2002), there is increasing evidence that ABA regulates responses to pathogen attack. Audenaert *et al.* (2002) reported that treatment with ABA enhanced the level of susceptibility in tomato to *B. cinerea*. Additionally, they found that the ABA-deficient tomato mutant *sitiens* exhibited enhanced resistance against *B. cinerea*, which correlated with increased activity of phenylalanine ammonia lyase (PAL) during pathogen infection. In addition, these *sitiens* plants exhibited increased sensitivity to BTH treatment in the transcriptional activation of the *PR-1* gene. Apparently, ABA partially represses pathogen-induced activation of SA-dependent defences in tomato. Indications for such negative cross-talk between ABA and SA were also found in *Arabidopsis*. Mohr and Cahill (2003), who reported that treatment of *Arabidopsis* with ABA, conferred partial loss of resistance against an avirulent isolate of *P. syringae* pv. *tomato*, carrying the avirulence gene *avrRpt2*. The

authors speculated that ABA inhibits RPS2-induced resistance by repressing the SA- and NPR1-dependent defence pathway. In this study, we found that ABA has a positive effect on the level of resistance against *P. cucumerina* in *Arabidopsis*. We not only showed that mutations in the ABA signalling pathway impair BABA-induced resistance against *P. cucumerina*, but also found that treatment with ABA induced resistance against *A. brassicicola* and *P. cucumerina*, which coincided with enhanced callose depositions at the sites of pathogen attack (Figure 9). Apparently, ABA represses SA-dependent defence responses, but at the same time, primes for enhanced callose deposition upon pathogen attack. The results obtained in this study show that ABA-dependent priming for callose effectively resists necrotrophic pathogens that are relatively insensitive to SA-inducible resistance. Regarding the recent finding that callose production suppresses SA action (Nishimura *et al.*, 2003), it is tempting to speculate that the negative action by ABA on SA signalling is mediated by enhanced callose accumulation. It remains nevertheless puzzling how BABA can prime for SA-inducible *PR-1* expression and ABA-dependent callose deposition at the same time (Figures 4 and 9). On the other hand, we could not detect enhanced levels of ABA-inducible *RAB18* transcription in BABA-treated plants (Figure 4). This indicates that the expression of BABA-induced resistance against *P. cucumerina* is not mediated by major rises in ABA levels. We therefore hypothesise that small, localised fluctuations in ABA levels regulate the BABA-mediated callose deposition. These ABA levels are too small to induce detectable amounts of *RAB18* gene expression and do not affect the BABA-mediated priming for SA-inducible defences.

It is evident from the present study that BABA triggers a resistance that is mechanistically different from SA-dependent SAR and JA-/ET-dependent ISR. The observed BABA-mediated priming, leading to earlier and stronger callose formation, also explains how various *Arabidopsis* mutants impaired in different basal defence mechanisms against necrotrophic fungi are effectively protected by BABA. This illustrates the importance of callose as an early defensive barrier against fungal pathogens. Our results also point to a function of ABA in the regulation of primed callose deposition. How BABA optimises the plant's basal defence response by interfering in the defence signalling network requires further research into the cross-communication between ABA- and SA-dependent signalling pathways.

Experimental procedures

Biological material

The *Arabidopsis* accession Col-0 was obtained from Lehle Seeds (Round Rock, TX, USA), and the Col-0 mutants *ein2-1*, *aba1-5* and

abi4-1 were obtained from the Nottingham Arabidopsis Stock Centre. Col-0 mutants *npr1-1*, *pad3-1*, *coi1-1* and *pmr4-1* were provided by X. Dong (Duke University, Durham, NC, USA), Jane Glazebrook (University of Maryland, MD, USA), J. Turner (University of East Anglia, Norwich, UK) and M. Nishimura (Stanford University, CA, USA), respectively. The transgenic *Arabidopsis* NahG line was obtained from J. Ryals (Novartis Research Triangle Park, USA). All plant genotypes except *coi1-1* were germinated on a pasteurised mixture of commercial potting soil and perlite (3 : 1). To select homozygous *coi1/coi1* plants, heterozygous *COI1/coi1* segregants were germinated on 0.5× Murashige and Skoog medium (Duchefa B.V. Haarlem, the Netherlands) containing 50 μM JA. At 2 weeks after germination, seedlings of all genotypes were individually transferred to 33-ml pots containing the 3 : 1 soil:perlite mixture. Plants were cultivated at 20°C day/18°C night temperatures with 8.5 h of light per 24 h and 60% relative humidity (RH). To prevent wilting of the *aba1-5* mutant under these conditions, *aba1* plants were maintained at higher humidity by keeping transparent lids on the trays. *A. brassicicola* strain MUCL 20297, kindly provided by B.P.H.J. Thomma (Katholieke Universiteit Leuven, Belgium), and *P. cucumerina*, isolated from naturally infected *Arabidopsis* (accession Landsberg *erecta*), were grown on 19.5 g l⁻¹ potato dextrose agar (Difco, Detroit) at room temperature for 2 weeks before spores were collected and suspended in 10 mM MgSO₄.

Induced resistance bioassays

Treatments with BABA, JA, ABA, salt (NaCl) and BTH were performed with 5–6-week-old plants. Chemicals were dissolved in water and applied as soil drench at the indicated concentrations. Control plants were treated with an equal volume of water. Challenge inoculation was performed 2 days after chemical treatment by applying 6-μl droplets containing 2 × 10⁶ spores ml⁻¹ (*A. brassicicola*) or 5 × 10⁶ spores ml⁻¹ (*P. cucumerina*) to six to eight fully expanded leaves. After challenge inoculation, plants were maintained at 100% RH. Disease symptoms were evaluated 4–6 days after inoculation by determining the average lesion diameters in 20–30 plants per treatment. Pathogen colonisation was examined microscopically at 2 days after inoculation in infected leaves that were stained by lactophenol-trypan blue (Koch and Slusarenko, 1990). To determine the effect of the callose inhibitor 2-DDG on BABA-induced resistance against *A. brassicicola*, leaves of 5-week-old *pad3-1* plants were detached and placed in Eppendorf tubes containing 1 ml of the different BABA and 2-DDG solutions. After 24 h incubation, 20 leaves per treatment were challenge-inoculated. At 2 days after challenge inoculation, five leaves were collected for callose staining. Disease symptoms were evaluated in the remaining 15 leaves at 4 days after inoculation.

Quantification of callose deposition

Leaves were collected at the time points indicated and incubated overnight in 95% ethanol. De-stained leaves were washed in 0.07 M phosphate buffer (pH 7), incubated for 15 min in 0.07 M phosphate buffer containing 0.005% calcofluor (fluorescent brightener; Sigma, USA) and 0.01% aniline blue, and subsequently washed in 0.07 M phosphate buffer containing only 0.01% aniline blue (pH 7) to discard excess amounts of the calcofluor. Observations were performed with an epifluorescence microscope with UV filter (BP, 340–380 nm; LP, 425 nm). Callose depositions in response to *A. brassicicola* infection were quantified by determining the average percentage of callose-inducing spores per infected leaf ($n = 8$). Callose

deposition in response to *P. cucumerina* infection was quantified from digital photographs of aniline blue-stained leaves (600× final magnification; 150 pixels inch⁻¹). Yellow spots corresponding to stained callose were analysed for number of pixels using ADOBE PHOTOSHOP 5.5 software. Callose intensity was expressed as the average number of yellow pixels/million pixels on digital photographs of infected leaf areas ($n = 8$).

Camalexin quantification

The method for camalexin detection was based on the procedure described by Glazebrook and Ausubel (1994). For each sample, six infected leaves from three different plants were pulverised in liquid N₂ and incubated for 25 min in 80% methanol at 55°C. After centrifugation (10 000 *g* for 5 min), the supernatant was evaporated under vacuum. The residue was extracted two times with 100 µl of chloroform, after which both fractions were combined and evaporated under vacuum. The residue was dissolved in 15 µl of chloroform and spotted on silica gel thin layer chromatography (TLC) plates (Silica gel 60 F254; Merck, Darmstadt, Germany), which were developed with ethylacetate:hexane 9 : 1 (v/v). After drying, camalexin spots were marked under UV light (approximately 345 nm), scraped off and taken up in 2 ml of 100% methanol. Fractions were scanned in a luminescence/fluorescence spectrometer (Perkin Elmer, Buckinghamshire, UK) at an excitation wavelength of 315 nm and an emission wavelength of 385 nm. Camalexin quantities were expressed relative to the sample containing the highest emission value.

RNA gel blot analysis

Total RNA was extracted by homogenising frozen leaf tissue in extraction buffer (0.35 M glycine, 0.048N NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% (w/v) SDS; 1 ml g⁻¹ leaf tissue). The homogenates were extracted with phenol and chloroform and the RNA was precipitated using LiCl, as described by Sambrook *et al.* (1989). For RNA gel blot analysis, 12.5 µg of RNA was denatured using glyoxal and DMSO (Sambrook *et al.*, 1989). Subsequently, samples were electrophoretically separated on 1.5% agarose gels and blotted onto Hybond N+ membranes (Amersham, 's-Hertogenbosch, the Netherlands) by capillary transfer. The electrophoresis and blotting buffers consisted of 10 and 25 mM sodium phosphate (pH 7.0), respectively. RNA gel blots were hybridised and washed as described previously by Pieterse *et al.* (1994). DNA probes were labelled with α-³²P-dCTP by random primer labelling (Feinberg and Vogelstein, 1983). Probes to detect *PDF1.2* and *RAB18* transcripts were prepared by PCR with primers based on GenBank sequences At5g44420 and At5g66400, respectively. Probes to detect *PR-1* transcripts were derived from a *PR-1* cDNA clone (Uknes *et al.*, 1992). Equal loading was visualised by ethidium bromide staining of rRNA.

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