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Interplay between JA, SA and ABA signalling during basal and induced resistance against *Pseudomonas syringae* and *Alternaria brassicicola*

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Summary

We have examined the role of the callose synthase PMR4 in basal resistance and β-aminobutyric acid-induced resistance (BABA-IR) of *Arabidopsis thaliana* against the hemi-biotrophic pathogen *Pseudomonas syringae* and the necrotrophic pathogen *Alternaria brassicicola*. Compared to wild-type plants, the *pmr4-1* mutant displayed enhanced basal resistance against *P. syringae*, which correlated with constitutive expression of the *PR-1* gene. Treating the *pmr4-1* mutant with BABA boosted the already elevated levels of *PR-1* gene expression, and further increased the level of resistance. Hence, BABA-IR against *P. syringae* does not require PMR4-derived callose. Conversely, *pmr4-1* plants showed enhanced susceptibility to *A. brassicicola*, and failed to show BABA-IR. Wild-type plants showing BABA-IR against *A. brassicicola* produced increased levels of JA. The *pmr4-1* mutant produced less JA upon *A. brassicicola* infection than the wild-type. Blocking SA accumulation in *pmr4-1* restored basal resistance, but not BABA-IR against *A. brassicicola*. This suggests that the mutant's enhanced susceptibility to *A. brassicicola* is caused by SA-mediated suppression of JA, whereas the lack of BABA-IR is caused by its inability to produce callose. *A. brassicicola* infection suppressed ABA accumulation. Pre-treatment with BABA antagonized this ABA accumulation, and concurrently potentiated expression of the ABA-responsive *ABI1* gene. Hence, BABA prevents pathogen-induced suppression of ABA accumulation, and sensitizes the tissue to ABA, causing augmented deposition of PMR4-derived callose.

Keywords: BABA, PMR4, ABA, induced resistance, callose, JA.

Introduction

For effective expression of plant resistance against microbial pathogens, early recognition of the intruder is critical. Recognition, followed by mobilization of biochemical and structural defences, leads to efficient halting of the invading pathogen. Comparisons of global gene expression between incompatible and compatible plant–pathogen interactions have revealed that the difference between resistance and susceptibility is quantitative and temporal, rather than qualitative (Maleck *et al.*, 2000; Tao *et al.*, 2003). Hence, activation of inducible defence mechanisms upon infection by virulent pathogens is usually too weak and too late to prevent disease. However, upon perception of specific stimuli, plants are capable of enhancing their level of basal resistance against future attacks by virulent pathogens. This induced resistance may be triggered by various biotic and abiotic agents, such as predisposal infection with an avirulent pathogen (systemic acquired resistance, SAR) (Durrant and Dong, 2004), non-pathogenic rhizobacteria (induced systemic resistance, ISR) (Pieterse *et al.*, 1998), volatiles that are emitted from insect-infested plants (Engelberth *et al.*, 2004; Ton *et al.*, 2006) or the chemical agent β-aminobutyric acid (BABA-induced resistance, BABA-IR) (Ton *et al.*, 2005;

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Zimmerli *et al.*, 2000). Interestingly, ISR and BABA-IR are not based on direct defence activation by the resistance-inducing agent, but on faster and stronger activation of inducible defence mechanisms once the plant is exposed to stress. This sensitization for defence is called 'priming', and brings the plant to an alarmed or primed state of defence (Prime-A-Plant Group, 2006). Recently, it was demonstrated that priming for defence yields enhanced resistance with minimal reduction of plant growth and seed set (van Hulten *et al.*, 2006). Hence, priming is an important regulatory system that increases the plant's ability to survive in hostile environments.

Resistance triggered by BABA has served as a model to study the molecular and physiological mechanisms behind priming for defence (Jakab *et al.*, 2005; Ton and Mauch-Mani, 2004; Ton *et al.*, 2005; Zimmerli *et al.*, 2000, 2001). BABA is a potent inducer of resistance against an exceptionally wide range of organisms such as viruses, bacteria, oomycetes, fungi, nematodes and aphids, and even some types of abiotic stress (Cohen, 2002; Hodge *et al.*, 2006; Jakab *et al.*, 2001; Zimmerli *et al.*, 2000, 2001, 2007). In Arabidopsis, BABA-IR is based on various priming mechanisms. Protection against *Pseudomonas syringae* and *Botrytis cinerea* by BABA functions via priming for salicylic acid (SA)-inducible defence mechanisms, whereas BABA-IR against *Hyaloperonospora parasitica*, *Plectosphaerella cucumerina* and *Alternaria brassicicola* is based on priming for resistance at the cell wall through the formation of callose-rich papillae (Ton and Mauch-Mani, 2004; Zimmerli *et al.*, 2000, 2001). Priming for SA-dependent resistance is regulated by the cyclin-dependent kinase-like protein IBS1, whereas BABA-induced priming of callose deposition involves proteins that play a role in regulation of phosphoinositides and biosynthesis or perception of abscisic acid (ABA) (Ton and Mauch-Mani, 2004; Ton *et al.*, 2005).

The plant hormone ABA has mostly been considered to act as a negative regulator of disease resistance, as it can interfere with signal transduction pathways that are controlled by other defence-related plant hormones such as SA, jasmonic acid (JA) or ethylene (ET) (Audenaert *et al.*, 2002; Flors *et al.*, 2005; Mauch-Mani and Mauch, 2005; Mohr and Cahill, 2003). Interestingly, in the case of BABA-IR against oomycetes and fungi, disruption of the ABA signalling pathway results in loss of BABA-induced priming for formation of callose-rich papillae, which illustrates that ABA can also act as a positive regulator of disease resistance through potentiation of callose deposition (Ton and Mauch-Mani, 2004; Ton *et al.*, 2005).

Callose is an amorphous, high-molecular-weight β-1,3-glucan. During infection by oomycetes or fungi, callose is deposited in papillae formed beneath the infection sites. It acts as a physical barrier or as a matrix that concentrates antimicrobial compounds at the attempted sites of fungal penetration (Aist, 1976; An *et al.*, 2006). Recently, the *PMR4/GSL5* gene (At4g03550) was identified and characterized as a glucan callose synthase that is responsible for the production of pathogen-inducible callose (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003 Østengaard *et al.*, 2002). Paradoxically, mutations in this gene confer enhanced resistance rather than enhanced susceptibility to various powdery mildew species (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003). This resistance is based on an enhanced SA response, indicating a negative cross-talk between the callose response and SA signalling (Nishimura *et al.*, 2003). It was hypothesized that callose deposition during the early stages of fungal infection can inhibit relatively late SA-dependent defence mechanisms that are potentially harmful to the plant.

In this study, we have examined the role of the callose synthase gene *PMR4* in basal resistance and BABA-IR against *P. syringae* and *A. brassicicola*. We show that mutations in PMR4 not only influence disease resistance as a result of impaired callose production, but also as a result of cross-talk effects between SA and JA signalling. In addition, we provide novel evidence regarding the role of ABA as possible a target for virulence mechanisms of *A. brassicicola*, and describe how BABA might interfere with this postulated fungal manipulation of the plant.

Results

PMR4 suppresses SA-dependent basal resistance against P. syringae *pv. tomato DC3000*

The callose-deficient *pmr4-1* mutant displays elevated levels of SA-dependent resistance (Nishimura *et al.*, 2003). In agreement with this, the number of diseased leaves in *pmr4-1* plants was reduced by 50% compared to wild-type Col-0 plants (Figure 1a). Furthermore, in water-treated *pmr4-1* plants, the levels of bacterial growth were reduced by 10-fold compared to that of wild-type Col-0 plants 3 days after dip inoculation with *P. syringae* DC3000 (Figure 1b). Hence, *pmr4-1* plants exhibited enhanced levels of basal resistance to *P. syringae*. Non-induced *pmr4-1* plants showed enhanced expression of the SA-inducible marker gene *PR-1*, which remained unaltered at various time points after infection with *P. syringae* (Figure 2). The slight differences in expression levels are due to unequal loading of the gel. These results corroborate those of Nishimura *et al.* (2003), who demonstrated that various pathogen- and SA-inducible genes are upregulated in *pmr4-1*. Hence, PMR4 suppresses SA-dependent basal defence mechanisms against *P. syringae*. Accordingly, blocking the SA pathway, as seen in *NahG pmr4-1* (data not shown) or *sid2-1 pmr4-1* double mutants (Figure 1) restores the basal susceptibility. However, the plants remain insensitive to BABA-IR.

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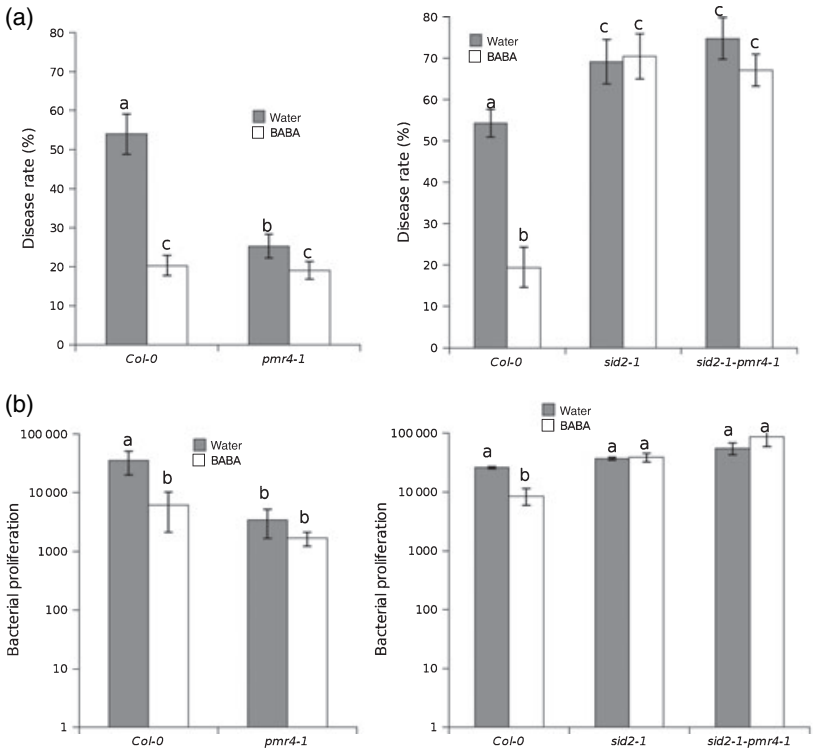

Figure 1. BABA-IR against *Pseudomonas syringae* pv. tomato DC3000 in wild-type plants (Col-0) and *pmr4-1, sid2-1 and sid2-1 pmr4-1* mutants.
Five-week-old plants were soil-drenched with BABA to a final concentration of 250 μM, and 2 days later were challenge-inoculated with a bacterial suspension of *P. syringae* pv. tomato DC3000 at 1.5×10^7 colony-forming units ml^{-1}. Data are from a representative experiment that was repeated with similar results.
(a) Disease symptoms were determined 3 days after inoculation and quantified as the proportion of leaves with symptoms. Data presented are the means of the percentage of diseased leaves per plant (±SD). Different letters indicate statistically significant differences compared with non-induced control plants (LSD test; $P < 0.05$, $n = 20–25$).
(b) Bacterial growth in the leaves was determined over a 3-day time interval. Values presented are means (±SD) of the log of the proliferation values. Different letters indicate statistically significant differences compared with non-induced control plants (LSD test; $P < 0.05$, $n = 5$).

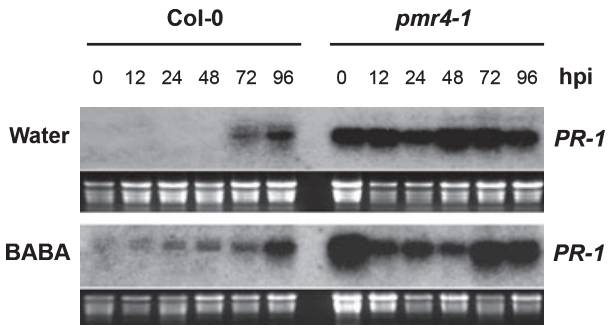

Figure 2. RNA gel blot analysis of *PR-1* expression in wild-type (Col-0) and *pmr4-1* plants.
Five-week-old plants were treated with either water or BABA (250 μM). Two days after induction treatment, the plants were inoculated with *Pseudomonas syringae* pv. tomato DC3000 (for experimental details, see Figure 1). Total RNA was extracted from the leaves of five plants at 0, 12, 24, 48, 72 and 96 h after inoculation. RNA gel blots were hybridized with P^{32}-labelled *PR-1* probes. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading. This experiment was repeated with similar results.

PMR4-derived callose is not essential for BABA-IR against P. syringae

Treatment with BABA reduced disease symptoms and bacterial proliferation of *P. syringae* in wild-type plants (Figure 1a,b). This correlated with accelerated *PR-1* expression upon infection by *P. syringae*. The observed enhancement of the level of *PR-1* gene expression following treatment of *pmr4-1* plants with BABA cannot be attributed definitively to the treatment itself due to the unequal loading of the gel. However, the concurrent slightly improved level of disease suppression indicates that the mutant's constitutive expression of SA-dependent defences might be further boosted by BABA. This also demonstrates that PMR4-derived callose does not play a role in BABA-IR against *P. syringae*.

PMR4 controls both basal resistance and BABA-IR against A. brassicola

Classically, Arabidopsis is considered to be resistant towards infection by *A. brassicicola* (Thomma *et al.*, 1998, 2000). By isolating the fungus from chlorotic tissue of infected *pad3-1* mutant plants, growing it on PDA containing 300 mM of $CaCO_3$ to induce sporulation, and incubating the spores in Gamborg B5 medium for 2 h prior to inoculation, we obtained an aggressive strain that was able to successfully infect Col-0. Four days after inoculation, water-treated wild-type plants displayed necrosis at the inoculation site, which was surrounded by a wide chlorotic halo. Trypan blue staining revealed that the diseased areas were extensively colonized by fungal hyphae (Figure 3a). Interestingly, the *pmr4-1* mutant was more susceptible to *A. brassicicola*, as evidenced by a 40% increase in lesion size and a higher density of fungal hyphae compared to wild-type plants (Figure 3b). Pre-treating wild-type plants with BABA reduced the lesion size by 50% (Figure 3b), and in most cases prevented colonization by *A. brassicicola* (Figure 3a).

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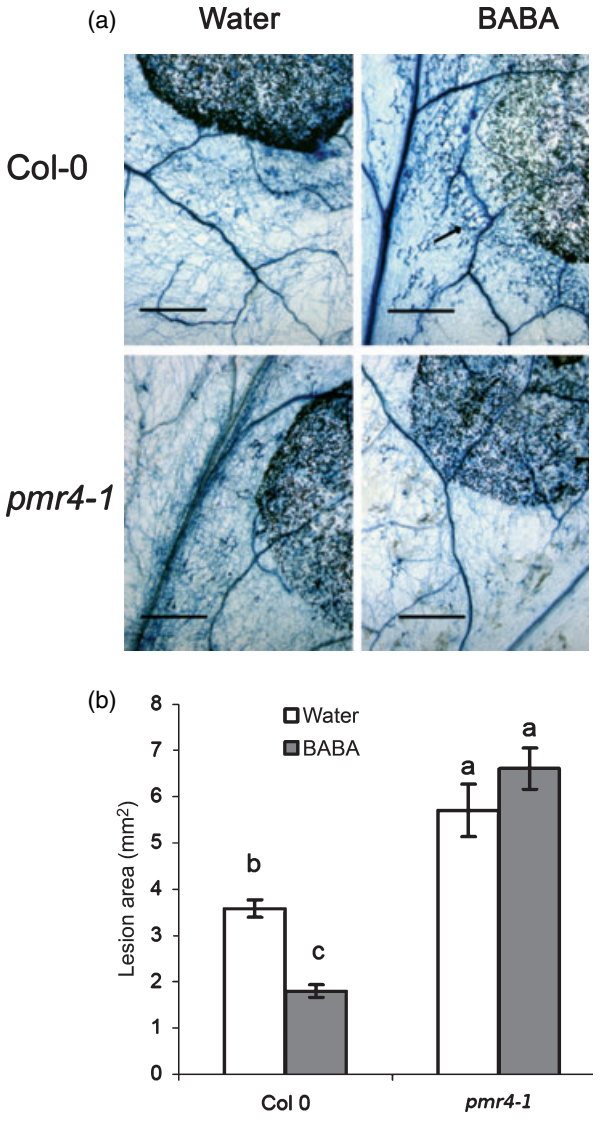

Figure 3. Quantification of BABA-induced resistance against *Alternaria brassicicola* in wild-type Col-0 and *pmr4-1* plants.
(a) Five-week-old plants were soil-drenched with water or 150 μM BABA. At 2 days after chemical treatment, 6–8 leaves per plant were challenged by applying 6 μl droplets containing 2×106 spores ml^{-1} of *A. brassicicola*. Images show tissue colonization by *A. brassicicola* in water- and BABA-treated Col-0 and *pmr4-1* plants at 4 days post-inoculation (scale bar = 200 μm). The arrow indicates necrotic cells around the inoculation site. Leaves were stained with lactophenol/Trypan blue and analysed by light microscopy.
(b) Lesion diameters for 20 plants per treatment were determined 4 days after infection. Values are means ± SD (n = 20).

Apparently, BABA effectively induces resistance against *A. brassicicola* in Col-0 plants. Conversely, pre-treatment of *pmr4-1* plants with BABA failed to reduce lesion size and fungal colonization (Figure 3a,b). Thus, PMR4 not only contributes to basal resistance against *A. brassicicola*, it is also essential for BABA-IR against this fungus.

Role of pathway cross-talk and callose in PMR4-mediated basal resistance and BABA-IR against A. brassicicola

JA plays important roles in basal resistance against *A. brassicicola* (Thomma *et al.*, 1998). To investigate whether the reduced basal resistance in *pmr4-1* is related to perturbations in JA production, its accumulation was quantified in water- and BABA-treated wild-type and *pmr4-1* plants. In control and inoculated wild-type plants, there was no difference in the timing and intensity of JA accumulation between water and BABA treatments (Figure 4). This supports our previous finding that JA plays no role in BABA-IR against *A. brassicicola* (Ton and Mauch-Mani, 2004).

Intriguingly, despite the enhanced degree of colonization by the pathogen (Figure 3), both water- and BABA-treated *pmr4-1* plants produced less JA than wild-type plants upon *A. brassicicola* infection (Figure 4), suggesting a suppression of JA-dependent basal defence mechanisms by the *pmr4-1* mutation. *sid2-1 pmr4-1* plants, which do not accumulate SA, were tested to investigate whether the enhanced SA response in *pmr4-1* (Figure 1) is causing the mutant's enhanced susceptibility via inhibition of JA accumulation. Introduction of the *NahG* transgene or the *sid2-1* mutation into the *pmr4-1* background restored basal resistance but not callose deposition (Figure 6a,b), suggesting that the enhanced susceptibility of *pmr4-1* to *A. brassicicola* is caused by SA-mediated suppression of JA, and not by the mutant's inability to produce callose.

The role of callose in BABA-IR against *A. brassicicola* was assessed by monitoring callose accumulation at various time points after challenge inoculations. BABA-treated Col-0 plants showed faster and stronger callose deposition at 48 h after infection, illustrating the primed callose response (Figure 5a). Interestingly, Col-0 control plants reacted locally by accumulating callose at the sites of infection, while BABA-treated Col-0 plants also showed callose accumulation several cell layers distal from the sites of spore application. In contrast to wild-type plants, both *pmr4-1* and *NahG pmr4-1 or sid2-1 pmr4-1* plants failed to accumulate callose after treatment with water or BABA (Figure 6a,b). Despite their restored level of basal resistance, *NahG pmr4-1* and *sid2-1 pmr4-1* mutants, like *pmr4-1*, remained incapable of BABA-IR against *A. brassicicola*. Hence, the inability to show BABA-IR in *pmr4-1* is not caused by SA-mediated suppression of JA signalling, but rather by the mutant's inability to produce callose.

The ABA-deficient mutant npq2-1 *is impaired in BABA-IR and BABA-induced priming for callose against* A. brassicicola

Previously, we found that the ABA signalling mutants *aba1* and *abi4* were impaired in BABA-IR and BABA-induced priming for callose upon infection by the necrotrophic

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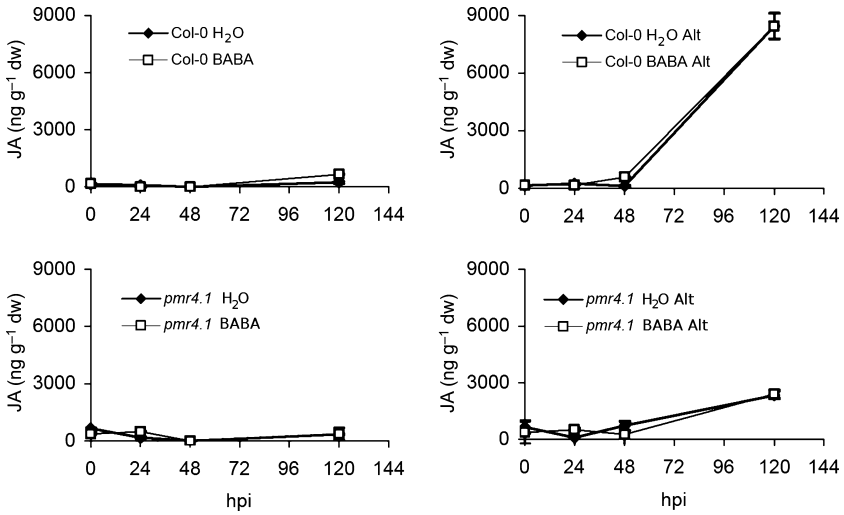

Figure 4. Effect of *Alternaria brassicicola* inoculation on JA accumulation in water- and BABA-treated Col-0 and *pmr4-1* plants.
Five-week-old plants were soil-drenched with water or BABA, and 48 h later 6–8 leaves per plant were challenged by applying 6 μl droplets containing 2×10^6 spores ml^{-1} of *A. brassicicola*. Infected leaves were collected at various time points, and JA levels were determined in freeze-dried material by HPLC-MS. The results shown are mean JA levels ± SD ($n = 5$). Data are from a representative experiment that was repeated with similar results.

fungus *P. cucumerina* (Ton and Mauch-Mani, 2004). To further investigate the relationship between ABA and BABA-induced priming for callose, we tested the ABA-deficient mutant *npq2-1* for callose deposition and BABA-IR against *A. brassicicola*. Interestingly, *npq2-1* not only failed to show BABA-IR against *A. brassicicola*, but water-treated *npq2-1* plants also showed reduced levels of resistance to *A. brassicicola* in comparison to water-treated Col-0 plants (Figure 6a).

Furthermore, *npq2-1* plants were completely blocked with regard to the ability to accumulate augmented levels of callose (Figure 6b). Hence, NPQ2/ABA1-mediated ABA production contributes to basal resistance against *A. brassicicola*, and is essential for BABA-IR and BABA-induced priming for callose against infection by *A. brassicicola*.

BABA prevents the suppression of ABA accumulation induced by A. brassicicola *infection, and sensitizes the plant's responsiveness to ABA*

To further examine the role of ABA in BABA-induced resistance, we quantified ABA production and ABA-inducible *ABI1* gene expression in control- and BABA-treated plants at various time points after infection by *A. brassicicola*. Remarkably, control-treated plants exhibited a dramatic reduction in ABA biosynthesis 24 h after inoculation with *A. brassicicola*; this reduction was suppressed in BABA-treated plants (Figure 7a). A similar pattern for ABA kinetics was present in *pmr4-1* (Figure 7a), but *npq2-1* did not produce detectable amounts of ABA (data not shown). Basal ABA levels did not differ between wild-type and *pmr4-1* plants, and ranged between 500 and 800 ng g^{-1} DW (data not shown).

To assess the impact of these ABA fluctuations, expression of the ABA-responsive gene *ABI1* was quantified (Leung *et al.*, 1994). Surprisingly, a transient induction of *ABI1* expression was observed 24 h after pathogen challenge in control-treated wild-type plants (Figure 7b). This transient induction was strongly potentiated by pre-treatment with BABA in both wild-type and *pmr4-1* plants (Figure 7b). Collectively, these data suggest that BABA prevents a pathogen-mediated reduction in ABA biosynthesis, and concurrently sensitizes the tissue for ABA-induced *ABI1* expression. The observation that *pmr4-1* behaved similarly upon BABA treatment to wild-type plants suggests that PMR4 acts downstream of ABA signalling in the pathway leading to BABA-induced priming for callose.

Exogenous application of ABA primes for callose and induces resistance against A. brassicicola *in both wild-type and* pmr4-1 *plants*

To further investigate the relationship between ABA signalling and PMR4, we treated wild-type and *pmr4-1* plants with either water (control) or 80 μM ABA, and quantified the level of induced resistance and callose deposition upon infection with *A. brassicicola*. In agreement with previous observations (Ton and Mauch-Mani, 2004), 80 μM ABA induced resistance in Col-0 against *A. brassicicola* (Figure 8a), which correlated with enhanced callose deposition (Figure 8b). These results reinforce the link between ABA and callose deposition, and demonstrate that exogenous application of ABA can prime the plant for enhanced callose deposition upon pathogen infection. Interestingly, *pmr4-1* plants were also protected by treatment with ABA, which correlated with an increase in callose deposition (Figure 8a,b). Hence, ABA primes for enhanced callose production that, at least partially, originates from other callose synthases than PMR4. To confirm that *pmr4-1* is specifically blocked in pathogen-inducible callose synthesis, we performed a BABA-induced sterility test by applying increasing concentrations of BABA and quantifying the amount of callose deposition in the

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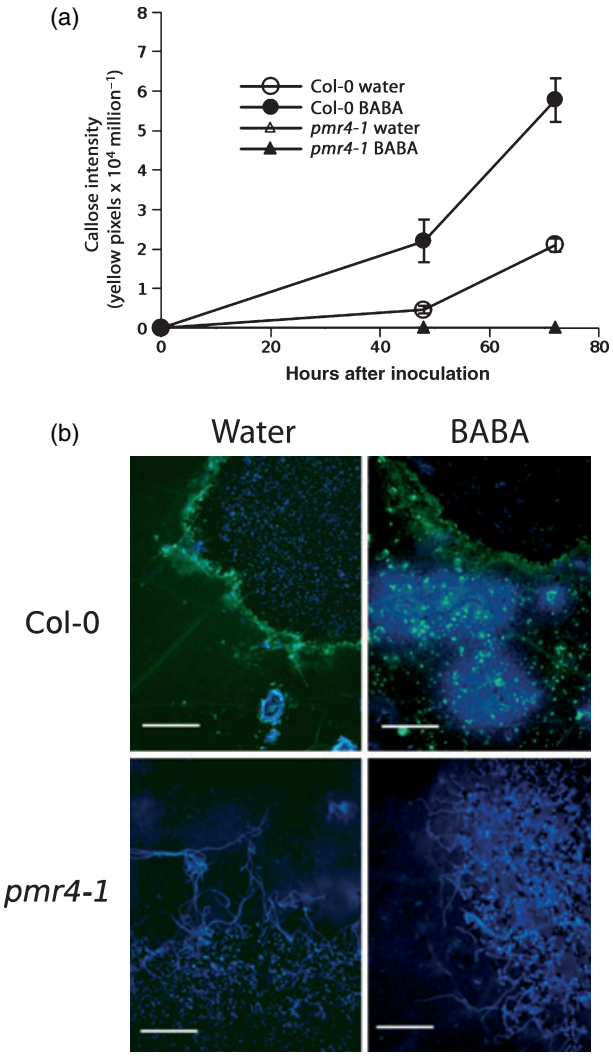

Figure 5. Callose accumulation in water- and BABA-treated Col-0 and *pmr4-1* upon inoculation by *Alternaria brassicicola*.
(a) Callose was visualized by aniline blue staining and epifluorescence microscopy. Quantification was performed by determining the number of yellow pixels (corresponding to pathogen-induced callose) per million pixels on digital photographs of infected leaf areas. Data shown are means ± SD (n = 8) of the relative number of yellow pixels per photograph.
(b) Micrographs showing *A. brassicicola* infection in Arabidopsis Col-0 and *pmr4-1* 4 days after inoculation with or without BABA treatment. Fungal structures are stained bright blue and callose is stained yellow (scale bar = 200 μm).

siliques. As expected, BABA induced prominent callose depositions in the siliques of both Col-0 and *pmr4-1* plants (data not shown). This clearly shows that additional callose synthases can be induced by BABA or ABA.

Discussion

This study was undertaken to analyse the role of PMR4, a pathogen-inducible callose synthase of Arabidopsis, in the interplay between the SA, JA and ABA signalling and callose deposition in basal resistance and BABA-IR against hemibiotrophic bacteria (*P. syringae*) and a necrotrophic fungus (*A. brassicicola*).

PMR4 suppresses SA-dependent basal resistance against Pseudomonas

Confirming previous findings (Nishimura *et al.*, 2003; Ton and Mauch-Mani, 2004), the *pmr4-1* mutant displayed constitutive expression of *PR-1*, a marker gene for the SA signalling pathway. This correlated with elevated levels of resistance to *P. syringae.* Hence, PMR4 suppresses basal resistance against *P. syringae* through negative regulation of SA-dependent responses. BABA-IR in Arabidopsis against *P. syringae* pv. *tomato* DC3000 depends on the SA- and *NPR1*/*NIM1*/*SAI1*-dependent pathway (Zimmerli *et al.*, 2000), and is characterized by augmented expression of the SA-inducible marker gene *PR-1*. SA also plays a role in basal defence against *P. syringae* (Katagiri *et al.*, 2002) and in the regulation of stomatal closure against bacterial invasion (Melotto *et al.*, 2006). Thus, SA is important for both BABA-induced and basal resistance against *Pseudomonas.*

Here, we show that a mutation in the *PMR4-1* gene confers resistance to *P. syringae*, correlating with enhanced constitutive expression of *PR-1*. This enhanced expression could be the consequence of negative regulation of the SA signalling pathway by callose itself or by the callose synthase PMR4 (Nishimura *et al.*, 2003). The *pmr4-1* mutant is blocked in this negative cross-talk, and therefore shows a stronger SA response. Alternatively, the negative effect of PMR4 on SA signalling might also be due to the influence of the hormone ABA. ABA accumulation suppresses SA-dependent defence in plants (Audenaert *et al.*, 2002; Mohr and Cahill, 2007), and callose deposition has been shown to be tightly correlated with ABA accumulation or sensitivity (Figure 8) (Kaliff *et al.*, 2007 Ton and Mauch-Mani, 2004; de Torres-Zabala *et al.*, 2007). It can therefore be speculated that up-regulation of SA synthesis in the *pmr4-1* mutant could also be due to alterations in the ABA levels. The *pmr4-1* mutant is still sensitive to BABA treatment, leading to a marginally higher increase of the already enhanced levels of *PR-1* (Figure 2). In conclusion, PMR4-1 acts as a negative regulator of basal defence against *P. syringae* by repressing the SA pathway, and is not essential for BABA-IR against this pathogen. However, it should be kept in mind that basal resistance probably consists of combinations of defence responses whose exact contributions cannot be easily determined. Interestingly, *pmr4-1* shows enhanced basal resistance against the oomycete *H. parasitica* (Nishimura *et al.*, 2003). As BABA-IR against this biotroph is mediated by priming for callose (Ton *et al.*, 2005; Zimmerli *et al.*, 2000), the mutant *pmr4-1* consequently lacks BABA-IR against *H. parasitica* (data not shown).

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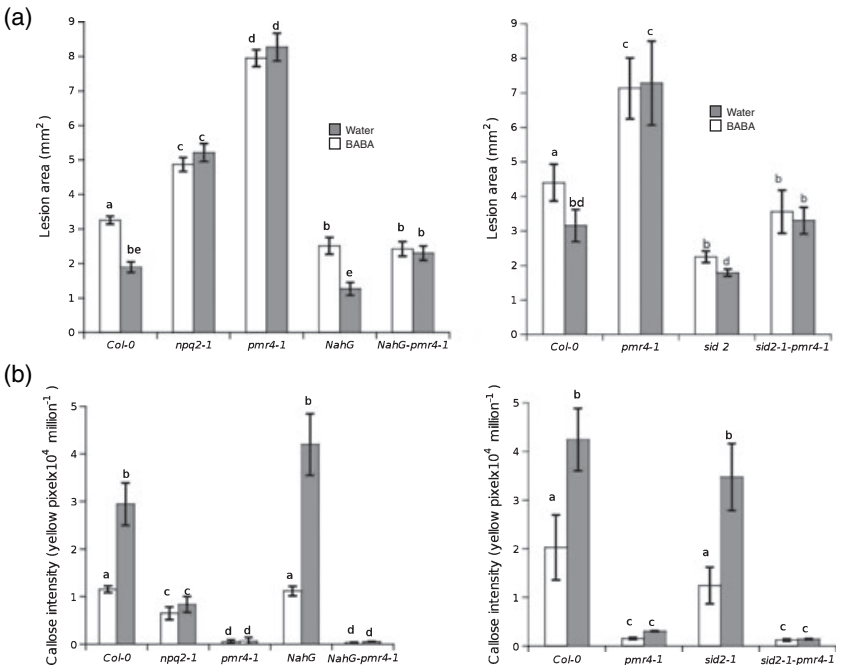

Figure 6. BABA-IR and callose accumulation in Col-0, *npq2-1*, *pmr4-1*, *NahG*, *NahG pmr4-1*, *sid2-1 and sid2-1 pmr4-1* Arabidopsis genotypes inoculated with *Alternaria brassicicola*.
(a) Lesion diameter in water- and BABA-treated plants 5 days after infection with *A. brassicicola*. For experimental details, see legend to Figure 3. Values are means ± SD. Letters indicate statistically significant differences compared with non-induced control plants (LSD test; $P < 0.05$, $n = 20$).
(b) Effect of application of BABA on the level of callose deposition 48 h after inoculation with *A. brassicicola*. For further experimental details, see legend to Figure 5. Data shown are means ± SD ($n = 8$).

Basal resistance and BABA-IR against A. brassicicola *are both dependent on PMR4*

Because the interaction between *A. brassicicola* and wild-type (Col-0) Arabidopsis is generally considered to be incompatible, most studies have been performed using the susceptible, camalexin-deficient mutant *pad3-1* (Thomma *et al.*, 2000; Ton and Mauch-Mani, 2004). Here, we describe an aggressive strain of *A. brassicicola* that can successfully infect the Arabidopsis accession Col-0 (Figure 3b). In this interaction, Col-0 plants accumulate callose around the infection site, but, as in other compatible interactions, this defence reaction is not fast enough to stop the pathogen. Callose accumulation is an important defence barrier against necrotrophs (Glazebrook, 2005). Accordingly, the lack of callose accumulation mediated by pathogen infection in *pmr4-1* makes it hyper-susceptible to the necrotroph *A. brassicicola*. However, *pmr4-1* also shows repressed JA production (Figure 4). The simultaneous occurrence of both, lack of callose deposition and reduced JA accumulation in the *pmr4-1* mutant makes assessment of the contribution of each of these factors difficult. Basal resistance of *pmr4-1* towards *A. brassicicola* was restored by introducing the *NahG* transgene or the *sid2-1* mutation (Figure 5), presumably by rescuing the mutant from JA repression. Therefore, JA repression seems to have a greater influence on basal resistance of *pmr4-1* against *A. brassicicola* than the lack of callose deposition.

BABA-IR against *A. brassicicola*, however, is JA-independent, as neither Arabidopsis Col-0 nor *pmr4-1* plants showed an increase in JA accumulation attributable to BABA treatment after mock or *A. brassicicola* inoculation (Figure 4). Although the JA mutant *coi1-1* shows enhanced susceptibility to this pathogen, it is not affected with regard to BABA-IR against either *A. brassicicola* or *Pl. cucumerina* (Ton and Mauch-Mani, 2004). Moreover, transcriptional analysis of the JA- and ET-inducible gene *PDF1.2* did not reveal any primed activity of the JA/ET pathway in BABA-treated Arabidopsis plants (Ton and Mauch-Mani, 2004). Therefore, it has been suggested that BABA-IR against *A. brassicicola* was not based on potentiation of JA- and ET-inducible defences (Ton and Mauch-Mani, 2004).

Suppression of ABA accumulation is a potential virulence mechanism of A. brassicicola *that can be antagonized by BABA*

Historically, the plant hormone ABA is best known to be involved in the control of abiotic stress processes, but, in recent years, it has also been shown to mediate plant responses to pathogens (reviewed by Flors *et al.*, 2005; Mauch-Mani and Mauch, 2005). Previous results (Ton and Mauch-Mani, 2004) also suggest possible regulation of BABA-induced priming of callose by ABA. Here, we describe the downregulation of ABA accumulation in Arabidopsis within the first 24 h after *A. brassicicola* inoculation of both wild-type and *pmr4-1* plants (Figure 7a). This strongly suggests that suppression of ABA accumulation could be a virulence mechanism in *A. brassicicola*, especially as this effect has not been described during interactions with other pathogens. Suppression of plant defences plays a crucial role in causing plant diseases: the inhibition of callose

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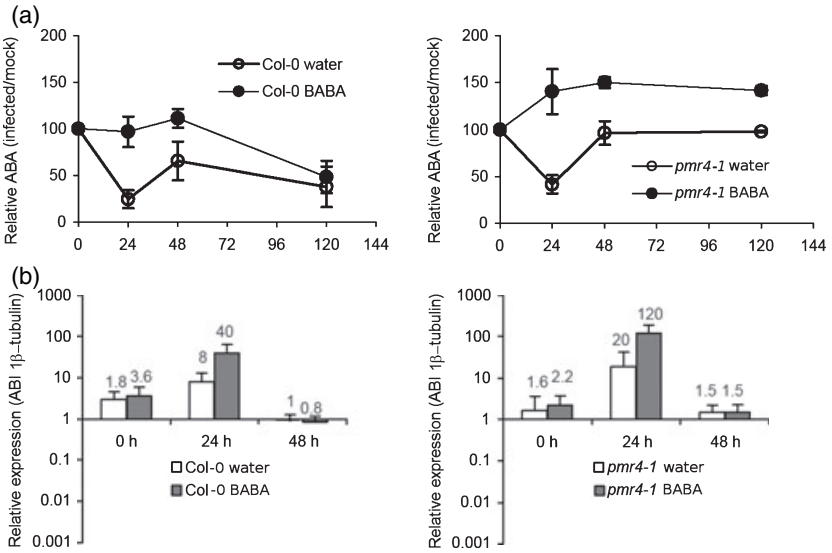

Figure 7. Effect of *Alternaria brassicicola* infection on ABA accumulation and *ABI1* gene expression levels in water- and BABA-treated Col-0 and *pmr4-1* plants.
(a) Relative ABA accumulation in water- and BABA-treated plants upon infection by *A. brassicicola*. Plants were treated and inoculated as described in Figure 3. Plant tissue was collected at various time points, and ABA levels were determined in freeze-dried material by HPLC-MS. Results are means ± SD (n = 5). Data are from a representative experiment that was repeated with similar results.
(b) Total RNA was isolated from infected leaves at 0, 24 and 48 h after inoculation, converted to cDNA, and subjected to quantitative RT-PCR analysis. The *ABI1* transcript levels in untreated and BABA-treated wild-type and *pmr4-1* plants were normalized to the expression of β-tubulin measured in the same sample and expressed logarithmically. Results are the means of two independent experiments with similar results.

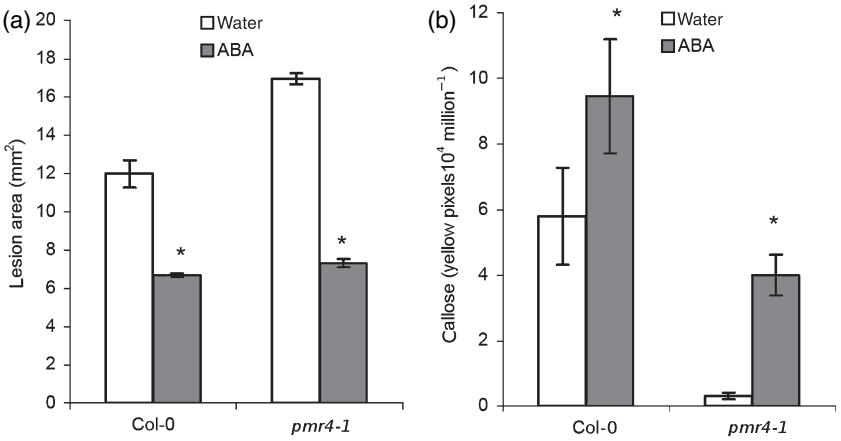

Figure 8. Quantification of ABA-induced resistance and ABA-induced callose deposition against *Alternaria brassicicola* in Col-0 and *pmr4-1 Arabidopsis* genotypes.
(a) Four-week-old plants were soil-drenched with water or 80 μM ABA. At 2 days after chemical treatment, 6–8 leaves per plant were challenged by applying 6 μl droplets containing 2 × 106 spores ml^{-1} of *A. brassicicola*. Average lesion diameters for 15–20 plants per treatment were determined 5 days after inoculation. Values are means ± SD. Asterisks indicate statistically significant differences compared with non-induced control plants (LSD test; $P < 0.05$, n = 20).
(b) Callose accumulation in water- and ABA-treated Col-0 and *pmr4-1* plants upon infection by *A. brassicicola*. Callose was visualized by aniline blue staining and epifluorescence microscopy. Quantification was performed by determining the number of yellow pixels (corresponding to pathogen-induced callose) per million pixels on digital photographs of infected leaf areas. Data shown are means ± SD of the relative number of yellow pixels per photograph. Asterisks indicate statistically significant differences compared with non-induced control plants (LSD test; $P < 0.05$, n = 8).

production by xanthans secreted by *Xanthomonas* spp. (Yun *et al.*, 2006) and the inhibition of ABA-induced stomatal closure against *P. syringae* by coronatine (Melotto *et al.*, 2006) are recent examples. Here, we show that *A. brassicicola* might employ a similar virulence strategy by suppressing ABA accumulation. In order to substantiate this claim, dependence on a specific pathogen-delivered effector(s) that is also crucial for pathogen virulence will have to be demonstrated. Interestingly, there is no downregulation of ABA accumulation following colonization by *A. brassici-*

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cola in plants pre-treated with BABA, and, concomitantly, priming for callose deposition is only observed in plants with a non-mutated *PMR4* (Figures 6b and 7a). A lack of BABA-IR similar to that seen in the *pmr4-1* mutant has been observed in *aba1-5* and *abi4-1* mutants after infection with *Pl. cucumerina* (Ton and Mauch-Mani, 2004). Both mutants are also impaired in primed callose deposition. Interestingly, BABA treatment also leads to a transient induction of *ABI1* expression in Col-0 and *pmr4-1*, but, as mentioned, callose production is only induced when PMR4 is functional (Figures 6b and 7b). Hence, BABA sensitizes the tissue for ABA perception and primes for callose deposition by inducing *ABI1* upstream of PMR4. The *aba1*-allelic mutant *npq2* is also impaired in BABA-IR and strongly affected with regard to its basal and induced callose accumulation. Accordingly, it is also more susceptible to *A. brassicicola* compared to Col-0 (Figure 6).

Although a role for ABA in BABA-IR is clearly shown by the results presented here, the specific role of ABA in defence in general still remains unclear and seems to strongly depend on the mode of pathogenicity of the attacking pathogen. In contrast to the described downregulation of ABA accumulation by *A. brassicicola* (Figure 7), infection of Arabidopsis with *P. syringae* (de Torres-Zabala *et al.*, 2007) or with *Leptosphaeria maculans* (Kaliff *et al.*, 2007) has been shown to lead to an increase in ABA levels. However, it is not clear whether ABA can induce additional defence mechanisms as it is able to induce resistance in *pmr4-1* while BABA cannot. In addition to the *PMR4* gene, 12 additional callose synthase-like genes are present in the TAIR database. The induction of callose by ABA shows that other functionally redundant β-1,3-glucan synthases can be induced by ABA treatment, causing enhanced levels of resistance to *A. brassicicola* (Figure 8). ABA can negatively regulate SA and JA signalling (Anderson *et al.*, 2004; Mauch-Mani and Mauch, 2005; Mohr and Cahill, 2007), but, on the other hand, there is no evidence for downregulation of ABA by SA. The mutant *pmr4-1* over-produces SA, but no reduction in ABA accumulation was observed (Figure 7).

Interplay between defence signalling, ABA and BABA in wild-type and pmr4-1 *mutants following infection with* A. brassicicola

We have shown that PMR4 strongly influences both basal and BABA-induced resistance against *A. brassicicola*. Infection with *A. brassicicola* leads to repression of ABA accumulation in both wild-type and mutant (Figures 7a and 9a,c). BABA treatment antagonizes this repression (Figures 7a and 9b,d), and potentiates expression of the ABA-responsive gene *ABI1* (Figure 7b). Therefore, ABA is proposed to be the signalling molecule mediating the BABA-induced callose priming that seems to be the main defence mechanism underlying BABA-IR against *A. brassicicola*. The mutation in PMR4 leads to derepression of SA expression. As a consequence, SA levels increase and JA production is repressed, causing the hyper-susceptible phenotype observed in *pmr4-1* towards the necrotrophic fungus *A. brassicicola* (Figure 9c). The basal resistance of *pmr4-1* towards *A. brassicicola* can be restored by either suppressing SA accumulation through *NahG* or preventing SA biosynthesis by the *sid2-1* mutation (Figure 6). In both cases, *pmr4-1* is rescued from the JA repression. However, because of the lack of priming for callose deposition, the *pmr4-1* mutant does not show BABA-IR (Figure 9d).

Our observations provide more insight into the interplay between various defence signalling pathways during BABA-IR against biotic stress. BABA not only acts by potentiating an under-expressed defence pathway(s), but is also able to specifically antagonize virulence mechanisms of the pathogen as seen in the case of downregulation of ABA accumulation following infection with *A. brassicicola*. Use of BABA is emerging as an interesting research tool that, in combination with mutants, could help to further elucidate the role of ABA in resistance mechanisms against necrotrophic pathogens.

Experimental procedures

Biological material

The Arabidopsis accession Col-0 was obtained from X. Dong (Duke University, Durham, NC, USA), and the Col-0 mutants *pmr4-1*, *NahG pmr4-1* and *sid2-1 pmr4-1* were provided by M. Nishimura (Stanford University, CA, USA); *npq2-1* was obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK). All plant genotypes were germinated in soil, and, 2 weeks after germination, seedlings were individually transferred to 33 ml pots containing commercial potting soil (TKS1, Floragard GmbH; http://www.floragard.de). Plants were cultivated at 20°C day/18°C night temperatures with 8.5 h of light per 24 h and 60% relative humidity. The virulent strain of *P. syringae* pv. tomato DC3000 (Whalen *et al.*, 1991) was grown overnight at 28°C in liquid King's medium B (King *et al.*, 1954). Bioassays with necrotrophs were performed using *A. brassicicola* maintained as described by Ton and Mauch-Mani (2004) with a few changes. The fungus was grown on medium containing 19 g l^{-1} potato dextrose agar (Duchefa; http://www.duchefa.com), 20 g l^{-1} of sucrose and 30 g l^{-1} of $CaCO_3$. In the absence of light, this medium strongly induces sporulation of the fungus. Spores from 10–15-day-old plants were maintained in Gamborg B5 liquid medium (Duchefa) supplemented with sucrose (10 mM) and KH_2PO_4 (10 mM) for 2 h prior to inoculation. Bioassays on BABA-treated *pmr4-1* mutants inoculated with *H. parasitica* were performed using isolate NOCO as described previously (Ton *et al.*, 2005).

Pseudomonas syringae *bioassays*

Two-week-old seedlings were individually transferred to 33 ml pots. Five-week-old plants were soil-drenched with water (control) or a solution of BABA at a final concentration of 250 μM. Two days after chemical treatment, plants were inoculated by dipping the leaves in

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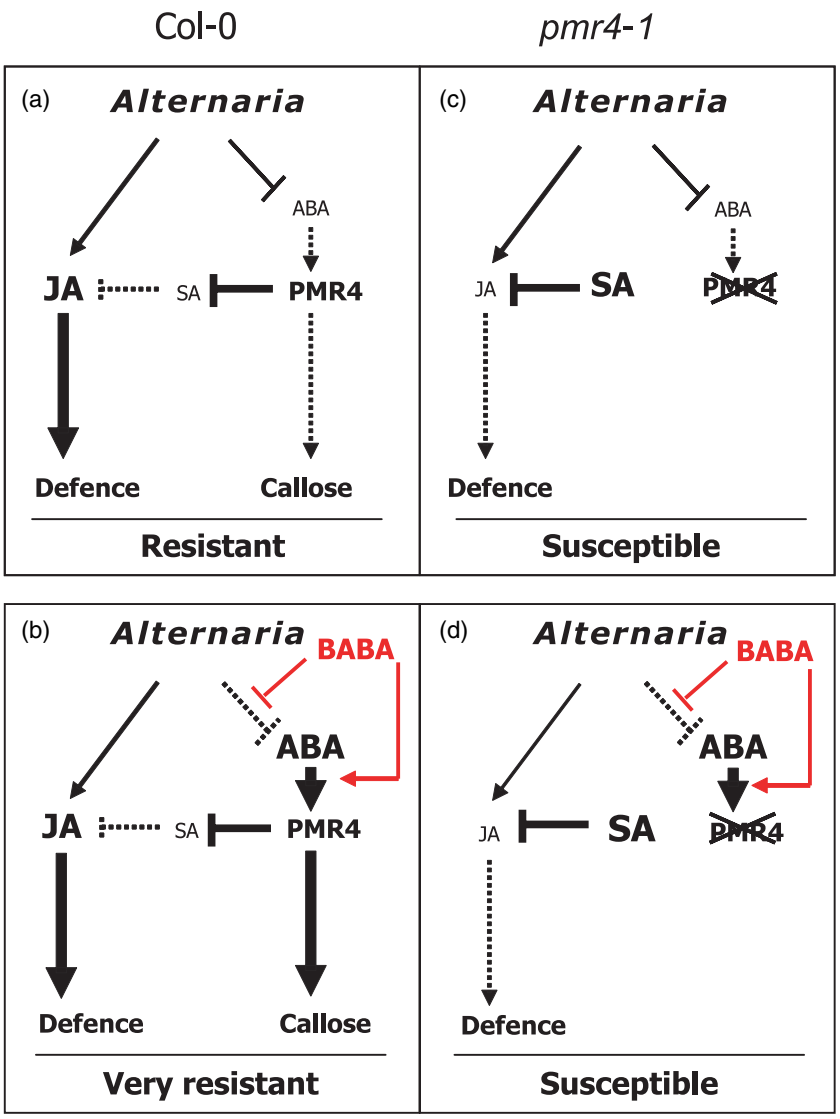

Figure 9. Model for the interplay of JA, SA and ABA in wild-type Col-0 plants and *pmr4-1* mutants during infection with *Alternaria brassicicola* with or without BABA treatment.
Infection of the plants with *A. brassicicola* leads to a repression of ABA accumulation in both genotypes.
(a) The low ABA content in the wild-type leads to a weak induction of callose deposition via PMR4. PMR4 also represses SA accumulation, alleviating its repression of JA accumulation.
(b) BABA treatment antagonizes the repression of ABA. The resulting higher levels of ABA lead to a stronger accumulation of callose, making the plants highly resistant.
(c) In the *pmr4-1* mutant, both callose accumulation and the repression of SA accumulation are abolished. The resulting higher levels of SA lead to repression of JA accumulation and consequently to a lowered JA-dependent defence.
(d) Antagonizing ABA suppression using BABA has no effect in *pmr4-1* as PMR4 is mutated. Callose cannot accumulate and the high SA levels repress JA accumulation and its effects on defence.

a suspension of *P. syringae* containing 1.5×10^7 colony-forming units ml^{-1} in 10 mM $MgSO_4$, 0.01% v/v Silwet L-77 (Lehle Seeds; www.arabidopsis.com). Three days after challenge inoculation, the percentage of leaves with symptoms was determined per plant (n = 20–25). Leaves showing necrotic or water-soaked lesions surrounded by chlorosis were scored as diseased. Bacterial growth in the leaves was determined by collecting replicate samples from five plants per genotype. Approximately 30 min after challenge inoculation and 3 days later, leaf samples were collected, weighed, rinsed in water, and homogenized in 10 mM $MgSO_4$. Serial dilutions were plated on selective King's medium B agar, supplemented with 100 mg l^{-1} cycloheximide and 50 mg l^{-1} rifampicin. After incubation at 28°C for 2 days, the number of rifampicin-resistant colony-forming units per gram of infected leaf tissue was determined, and bacterial proliferation over the 3-day time interval was calculated.

Alternaria brassicicola *bioassays*

Treatments with BABA (150 μM) were performed with 5–6-week-old plants. For ABA experiments, an 80 μM solution of abscisic acid was applied by soil drenching to 4-week-old plants in order to avoid ABA-induced senescence that might influence the resistance to *A. brassicicola*.. Two days later, plants were challenged by applying 6 μl drops of 2×10^6 spores ml^{-1} to 6–8 fully expanded leaves. The challenged plants were maintained at 100% relative humidity. Disease symptoms were evaluated by determining the average lesion diameter in 20–30 plants per treatment. Pathogen colonization was examined microscopically in infected leaves stained with lactophenol/Trypan blue. Callose deposition was determined in infected leaves at various time points after inoculation, using calcofluor/aniline blue staining and subsequent analysis by epifluorescence microscopy. Staining and quantifications were performed as described previously by Ton and Mauch-Mani (2004).

RNA gel blot analysis

RNA gel blot analysis was performed as described previously (Zimmerli *et al.*, 2000). The membrane was probed with ^{32}P-labelled DNA (Prime-a-Gene labelling system, Promega, http://www.promega.com/) encoding pathogenesis-related protein *PR-1* (Uknes *et al.*, 1992).

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Quantitative real-time RT-PCR analysis of transcripts

Gene expression by quantitative real-time RT-PCR was performed using RNA samples extracted from leaf tissue using the Total Quick RNA cells and tissues kit (Talent; http://www.spin.it/talent). Arabidopsis leaf tissue samples for RNA isolations were collected at 0, 24 and 48 h after inoculation. Leaf tissue from five plants each of the mutant and wild-type plants was collected. For quantitative real-time RT-PCR experiments, 1.5 μg of total RNA was digested using 1 unit of RQ1 RNase-Free DNase (Promega; http://www.promega.com) in 1 μl of DNase buffer and up to 10 μl of Milli-Q water, and incubated for 30 min at 37°C. After incubation, 1 μl of RQ1 DNase stop buffer was added, and the solution was incubated again at 65°C for 10 min to inactivate the DNase. Highly pure RNA was used for the RT reaction. The RT reaction was performed by adding 2 μl of RT buffer, 2 μl of 5 mM dNTP, 2 μl of 10 μMoligo$(dT)_{15}$ primer (Promega), 1 μl of 10 U μl^{-1} Rnasin RNase inhibitor (Promega) and 1 μl of Omniscript reverse transcriptase (Qiagen, http://www.qiagen.com/). The reaction mixture was incubated at 37°C for 60 min. Less than 10% of the volume of the RT reaction was used for the quantitative PCR. Forward and reverse primers (0.3 μM)) were added to 25 μl of QuantiTect™ SYBR Green PCR reaction buffer (Qiagen), 2 μl of cDNA and Milli-Q sterile water up to 50 μl total reaction volume. Quantitative PCR was carried out using the Smart Cycler II sequence detector (Cepheid; http://www.cepheid.com). PCR cycling conditions comprised an initial HotStarTaq (Qiagen; http://www.qiagen.com) polymerase activation step at 95°C for 15 min, followed by 45 cycles of 95°C for 15 sec, 59°C for 30 sec and 72°C for 30 sec. A melting curve analysis was performed at the end of the PCR reaction to confirm the product purity. Differences in cycle numbers during the linear amplification phase between samples containing cDNA from treated and untreated plants were used to determine differential gene expression. Expression of the β-tubulin gene of Arabidopsis using primers 5′-AATGAGTGACACACTTGGAATCCTT-3′ and 3′-TGTTGTAGCTCTTGCCTTAGC-5′ was used as an internal standard. Primers for *ABI1* (At4g26080) were 5′-CGGCAAAACTGCACTTCCAT-3′ and 3′-AAGTCACCTTACTCGAGCAC-5′.

Determination of ABA and JA levels

Fresh material was frozen in liquid nitrogen and lyophilized. Before extraction, a mixture of internal standards containing 100 ng [^{2}H6]-ABA and 100 ng prostaglandin B1 (Pinfield-Wells *et al.*, 2005) was added. Dry tissue (0.05 g) was immediately homogenized in 2.5 ml of ultrapure water. After centrifugation (5000 ***g***, 40 min), the supernatant was recovered and adjusted to pH 2.8 with 6% acetic acid, and subsequently partitioned twice against an equal volume of diethyl ether. The aqueous phase was discarded, and the organic fraction was evaporated in a Speed Vaccuum Concentrator (Eppendorf; www.eppendorf.com) at room temperature and the solid residue re-suspended in 1 ml of a water/methanol (90:10) solution and filtered through a 0.22 μm cellulose acetate filter. A 20 μL aliquot of this solution was then directly injected into the HPLC system. Analyses were carried out using a Waters Alliance 2690 HPLC system (Milford, MA, USA) with nucleosil ODS reversed-phase column (100 x 2 mm i.d.; 5 μm; Scharlab, Barcelona, Spain; http://www.scharlab.es). The chromatographic system was interfaced to a Quatro LC (quadrupole-hexapole-quadrupole) mass spectrometer (Micromass; http://www.micromass.co.uk). The Masslynx NT version 3.4 (Micromass) software was used to process the quantitative data from calibration standards and the plant samples. The MASSLYNX NT software version 3.4 (Micromass) was used to process the quantitative data from calibration standards and the plant samples.

ABA-induced protection against necrotrophs is dependent on the plant age: in older *Arabidopsis* plants, ABA treatment might lead to senescence in older leaves, which then could more efficiently be infected by necrotrophs. We therefore performed all ABA experiments in young 4-week-old plants.

BABA-induced sterility assays

Sterility was induced as described previously (Jakab *et al.*, 2001). Two weeks after the onset of flowering, siliques were collected and stained with aniline blue for callose detection. To quantify BABA-induced sterility, the average number of seeds per silique was determined in 6-week-old plants.

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