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Priming for enhanced defence responses by specific inhibition of the Arabidopsis response to coronatine

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SUMMARY

The priming agent β-aminobutyric acid (BABA) is known to enhance Arabidopsis resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 by potentiating salicylic acid (SA) defence signalling, notably *PR1* expression. The molecular mechanisms underlying this phenomenon remain unknown. A genome-wide microarray analysis of BABA priming during *Pst* DC3000 infection revealed direct and primed up-regulation of genes that are responsive to SA, the SA analogue benzothiadiazole and pathogens. In addition, BABA was found to inhibit the Arabidopsis response to the bacterial effector coronatine (COR). COR is known to promote bacterial virulence by inducing the jasmonic acid (JA) response to antagonize SA signalling activation. BABA specifically repressed the JA response induced by COR without affecting other plant JA responses. This repression was largely SA-independent, suggesting that it is not caused by negative cross-talk between SA and JA signalling cascades. Treatment with relatively high concentrations of purified COR counteracted BABA inhibition. Under these conditions, BABA failed to protect Arabidopsis against *Pst* DC3000. BABA did not induce priming and resistance in plants inoculated with a COR-deficient strain of *Pst* DC3000 or in the COR-insensitive mutant *coi1-16*. In addition, BABA blocked the COR-dependent re-opening of stomata during *Pst* DC3000 infection. Our data suggest that BABA primes for enhanced resistance to *Pst* DC3000 by interfering with the bacterial suppression of Arabidopsis SA-dependent defences. This study also suggests the existence of a signalling node that distinguishes COR from other JA responses.

Keywords: *Arabidopsis thaliana,* beta-aminobutyric acid, priming, coronatine, jasmonic acid, defence response.

INTRODUCTION

Plants are able to protect themselves against attacking pathogens through constitutive and inducible defences. Following specific stimulation, the plant's resistance level can increase, leading to protection against future pathogen attack, a phenomenon referred to as induced resistance. Several types of induced resistance, such as systemic acquired resistance (SAR) and induced systemic resistance (ISR), can be distinguished based on differences in their signalling pathways and their spectra of effectiveness (Durrant and Dong, 2004; Van Wees *et al.*, 2008). Application of the chemical β-amino-butyric acid (BABA) is known to induce resistance. BABA-induced resistance (BABA-IR) confers protection against a broad spectrum of biotic and abiotic stresses (Zimmerli *et al.*, 2001; Prime-A-Plant Group *et al.*, 2006; Zimmerli *et al.*, 2008). The sensitization of stress responsiveness during induced resistance, which is not only observed in plants but also in animals, is called priming (Prime-A-Plant Group et al., 2006; Pham *et al.*, 2007; Beckers *et al.*, 2009; Jung *et al.*, 2009). Priming boosts the plant's defensive capacity and brings it into an alarmed state of defence. Priming offers low-cost protection under conditions of relatively high disease pressure (van Hulten *et al.*, 2006).

Plants are able to respond to pathogenic *Pseudomonas* bacteria through perception of pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs activates a

downstream signalling cascade that results in expression of PAMP-triggered immunity (Jones and Dangl, 2006; Boller and He, 2009). However, virulent bacteria can weaken the effectiveness of PAMP-triggered immunity by injecting effector proteins into plant cells using type III secretion systems, allowing enhanced proliferation of the bacteria in the intercellular space (Jones and Dangl, 2006; Boller and He, 2009). In addition to the generation of type III secretion system-dependent protein effectors, *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) produces the molecule effector coronatine (COR). COR is a jasmonyl-isoleucine (JA-Ile) mimic that suppresses salicylic acid (SA)-mediated host responses and therefore increases the bacterial virulence (Brooks *et al.*, 2005; Katsir *et al.*, 2008a). COR also acts as a virulence factor to suppress stomatal defence (Melotto *et al.*, 2006), and is involved in systemic induced susceptibility (Cui *et al.*, 2005).

We have previously shown that BABA-IR against *Pst* DC3000 is characterized by potentiated expression of the *PR1* gene, which requires a functional SA signalling and an intact NPR1 protein (Zimmerli *et al.*, 2000; Ton *et al.*, 2005). This BABA-induced priming of SA-dependent defences was shown to be regulated by the cyclin-dependent kinase-like protein IBS1 (Ton *et al.*, 2005). To obtain more general insight into the priming phenomenon, we further analysed the mechanism of action of the priming agent BABA upon infection of Arabidopsis by virulent bacteria.

Here we present evidence that the resistance induced by BABA against *Pst* DC3000 is based on interference of this chemical with COR-mediated bacterial suppression of the plant's SA-dependent defence response.

RESULTS

BABA directly up-regulates and potentiates gene expression during bacterial infection

To analyse the effect of BABA on Arabidopsis gene expression levels at the genome-wide scale, we compared the transcriptomes of BABA- and water-treated Arabidopsis without infection or during infection with virulent *Pst* DC3000. BABA treatment significantly altered the expression levels of 55 genes, of which 46 were up-regulated (Table S1). Gene ontology vocabulary analysis of the up-regulated genes according to molecular function (Berardini *et al.*, 2004) revealed an over-representation of stress-responsive genes (20.2% compared to 4.6% in the whole genome), and genes responsive to abiotic or biotic stimulus (18.7% compared to 4.3% in the whole genome). This confirms previous observations (Zimmerli *et al.*, 2008). Comparison with published microarray datasets of genes activated by treatment with benzothiadiazole (BTH) or SA, and during infection by non-host (*Blumeria graminis*) or host (*Golovinomyces cichoracearum*) fungi, avirulent (*Pst* DC3000 *avrRpm1*) bacteria and the peptide representing the PAMP flagellin (flg22) revealed numerous co-regulated genes (Figure 1a and Table S2). Together, these observations indicate that, similarly to BTH (Lawton *et al.*, 1996), BABA directly induces pathogen-responsive genes.

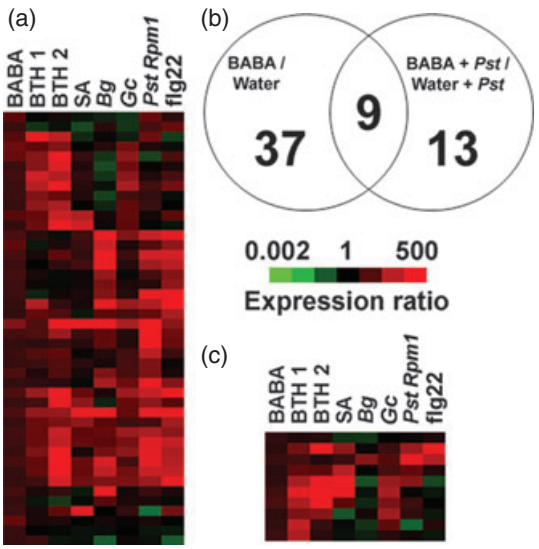

Figure 1. BABA up-regulates and primes BTH-, SA-, pathogen- and PAMP-responsive genes.
(a) Hierarchical clustering analysis of genes co-expressed with 44 BABA up-regulated genes in response to BTH, SA, *Blumeria graminis* (*Bg*), *Golovinomyces cichoracearum* (*Gc*), *Pseudomonas syringae* pv. *tomato* DC3000 *avrRpm1* (*Pst Rpm1*) or flg22. Values are colour-coded as indicated by the scale. Expression values are given in Table S2.
(b) BABA primes the expression of 13 genes. Venn diagram representation of genes either directly up-regulated (BABA/water) or primed by BABA (BABA + *Pst*/water + *Pst*).
(c) The majority of BABA-primed genes are BTH-, SA- and pathogen-responsive. Co-expression analysis of ten BABA-primed genes as in (a). Values are colour-coded as indicated by the scale. Expression values are given in Table S4.

BABA priming of induced defences against *Pst* DC3000 is characterized by potentiated expression of the SA-inducible marker gene *PR1* (Figure S1a) (Zimmerli *et al.*, 2000; Ton *et al.*, 2005). Microarray analysis revealed that 22 genes were significantly up-regulated in BABA-treated Arabidopsis at 22 h post-inoculation with *Pst* DC3000 (Table S3). Of these, 13 transcripts specifically accumulated after bacterial inoculation (Figure 1b). Typically, BABA treatment alone did not significantly alter their expression levels, but did potentiate their up-regulation in response to bacterial inoculation. These genes are thus primed by BABA. As expected, *PR1* belongs to this group of genes (Table S4). Comparison with the above mentioned microarray datasets revealed that the majority of these primed genes are also responsive to BTH and SA treatments, and, with the exception of *B. graminis*, are up-regulated by pathogens (Figure 1c and Table S4). However, most of the primed genes were not responsive to flg22 treatment (Figure 1c and Table S4). This confirms, at a genome-wide level, that BABA primes SA-responsive genes.

BABA inhibits the Arabidopsis response to COR

Forty-four transcripts were found to be down-regulated by BABA during *Pst* DC3000 infection (Table S3). Twenty-nine are known to be COR-responsive (Figure 2a and Table S5) (Thilmony *et al.*, 2006). The bacterial effector COR is a JA-Ile mimic that suppresses SA-mediated defences (Brooks *et al.*, 2005; Katsir *et al.*, 2008a). Comparisons with published microarray datasets revealed that a great majority of these genes are also up-regulated by methyl-jasmonate (MeJA) (Figure 2a and Table S5). To validate the microarray data, we analysed the effect of BABA after bacterial infection on the expression levels of: At4g02360, *COR-INDUCED* (*CORI1*, At1g19670; *CORI3*, At4g23600), *JASMONATE-ZIM-DOMAIN (JAZ10*, At5g13220), *JASMONIC ACID CARBOXYL METHYLTRANSFERASE* (*JMT*, At1g19640) and *PLANT DEFENSIN 1.2* (*PDF1.2a*, At5g44420). In addition, we also tested the response of two key players in the JA response (Gfeller *et al.*, 2010): *JAZ1* (At1g19180) and *LIPOXYGENASE 2* (*LOX2*, At3g45140). Analysis by real-time quantitative RT-PCR showed that *Pst* DC3000-induced up-regulation of these COR/JA-responsive genes was counteracted by BABA (Figure 2b). In addition, we evaluated expression profiles of the JA-dependent defence marker gene *PDF1.2* at various time points after pathogen inoculation. The transient pathogen-induced expression of *PDF1.2* during the first 24 h of bacterial infection was repressed by BABA (Figure S1b). Thus, BABA probably inhibits the JA response induced by COR. To exclude the possibility that the suppression of COR-inducible genes was due to reduced colonization or direct inhibition of COR production by *Pst* DC3000 in BABA-treated plants, we tested whether BABA could also inhibit COR responsiveness upon exogenous application of purified COR. To this end, we sprayed Arabidopsis plants with 0.5 μM COR and evaluated the levels of transcript accumulation of COR-responsive genes. As with bacterial infection, BABA repressed induction of the eight selected COR/JA-responsive genes (Figure 2c). Expression of the COR/JA-responsive *VEGETATIVE STORAGE PROTEIN 1* (*VSP1*, At5g24780) was also visualized in transgenic plants carrying a P_{VSP1}:luciferase or a P_{VSP1}:β-glucuronidase construct (Ellis and Turner, 2001). As expected, the COR-induced activity of luciferase or

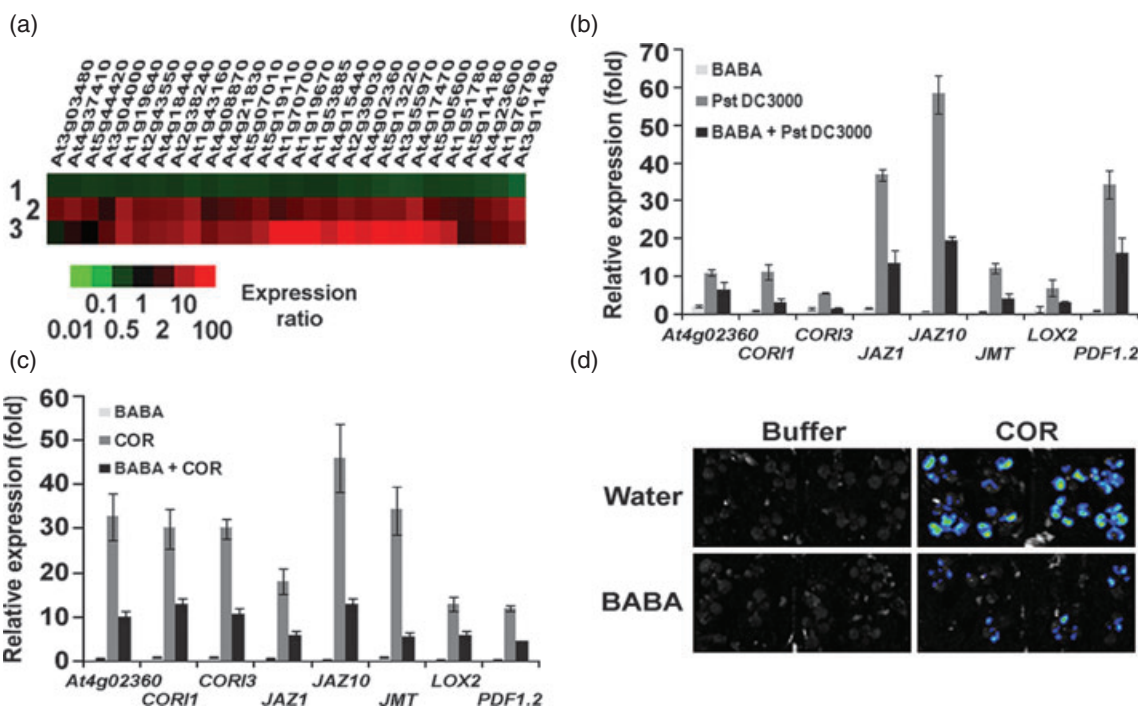

Figure 2. BABA inhibits the COR-induced JA response.
(a) Bacteria-mediated accumulation of COR-responsive transcripts is repressed by BABA. For row 1, RNA samples for microarray analyses were collected 22 h after dip-inoculation with *Pst* DC3000. Row 1: BABA + *Pst* DC3000/water + *Pst* DC3000. Row 2: *Pst* DC3000/*Pst* DC3118 COR^-. Data from Thilmony *et al.* (2006). Row 3: MeJA/mock. Data from NASCArrays, experiment reference number 174. Expression values are given in Table S5. Values are colour-coded as indicated by the scale.
(b) Real-time quantitative RT-PCR analysis of the bacterial-mediated accumulation of eight COR/JA-responsive transcripts. RNA samples were collected 24 h after dip inoculation with *Pst* DC3000.
(c) BABA inhibition of COR-responsive transcript accumulation after treatment with 0.5 μM purified COR. Samples were collected 4 h after treatment.
(d) BABA effect on *VSP1* promoter activity after treatment with purified COR. Representative examples of 2-week-old P_{VSP1}:luciferase transgenic plants 24 h after treatment with 0.5 μM COR.
For all real-time quantitative RT-PCR results, fold expression was calculated relative to water control (defined value of 1). Error bars are SD (n = 3 technical replicates). All experiments were repeated at least twice with similar results.

β-glucuronidase was suppressed by pre-treatment with BABA (Figure 2d and Figure S2). Hence, BABA inhibits both biologically and chemically induced COR responses in Arabidopsis.

BABA suppresses the COR response in SA-deficient mutants

BABA primes the SA response during bacterial infection (Zimmerli *et al.*, 2000; Ton *et al.*, 2005). As SA signalling is antagonistic to the JA pathway (Kunkel and Brooks, 2002), we tested whether the observed inhibition of the COR response by BABA is SA-dependent. To this end, we quantified COR responsiveness in the SA biosynthesis mutant *sid2-1* (Nawrath and Metraux, 1999) and the SA- signalling mutants *pad4-1* (Glazebrook *et al.*, 1997) and *npr1-1* (Cao *et al.*, 1994) upon treatment with purified COR. Although the level of suppression was slightly lower in SA mutants than the Col-0 wild-type control (compare Figure 3 with Figure 2c), accumulation of COR-responsive transcripts was inhibited by BABA in the three mutants (Figure 3a–c). These observations suggest that BABA-mediated inhibition of the COR response is largely SA-independent. Thus BABA-induced suppression of the COR response is probably not the result of negative cross-talk between SA and JA.

High concentrations of purified COR block BABA-IR against *Pst* DC3000

To investigate the interaction between BABA and COR signalling, we tested the effect of BABA on the response to relatively high concentrations of 5 μM of purified COR. COR was infiltrated into leaves to ensure a better controlled treatment. Infiltration of COR only did not induce the formation of necroses (data not shown). BABA no longer repressed the Arabidopsis response to these relatively high amounts of COR (Figure S3). Under these conditions, leaves of BABA-treated plants were not protected against *Pst* DC3000. This effect was observed at both bacterial titre and symptom levels (Figure 4a,b). Hence, high amounts of COR suppress BABA-IR against *Pst* DC3000. This finding suggests that inhibition of the COR response is necessary for BABA-IR against this pathogen.

BABA priming and BABA-IR both depend on a functional COR response

To further assess the biological significance of the observed inhibition by BABA of the plant response to COR, we quantified the priming level of the SA-dependent gene *PR1* in wild-type plants upon infection with a COR-deficient strain of *Pst* DC3000 (*Pst* DC3000 COR$^-$) (DB29) (Brooks *et al.*, 2004), as well as in COR-insensitive *coi1-16* plants upon infection with *Pst* DC3000 wild-type bacteria (Ellis and Turner, 2002). As expected, primed induction of *PR1* was observed in Col-0 Arabidopsis upon infection with the *Pst* DC3000 wild-type strain, but strongly reduced in plants

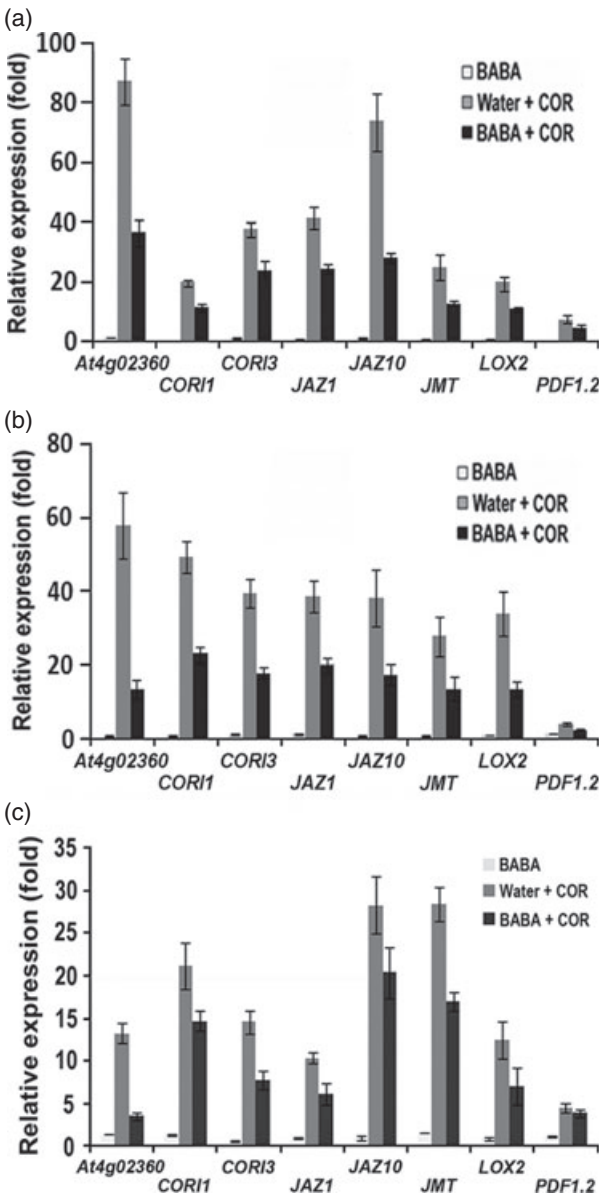

Figure 3. BABA suppression of the COR response occurs in SA biosynthesis and signalling mutants.
(a–c) Real-time quantitative RT-PCR for eight COR-responsive genes in *sid2-1* (a), *pad4-1* (b) and *npr1-1* (c) mutants. RNAs were extracted 4 h after treatment with 0.5 μM purified COR. Values are expression levels relative to the water control (defined value of 1). Error bars are SD (n = 3 technical replicates). Experiments were repeated twice with similar results.

altered in their COR response (Figure 5a). The mutant *coi1-16* also carries a mutant allele of *PEN2* (Westphal *et al.*, 2008). We thus tested BABA priming of *PR1* in e *pen2* mutant. Priming of *pen2* was not altered (Figure S4), ruling out the possibility that the defective priming of *PR1* observed in *coi1-16* is caused by a mutation in *PEN2*. As primed expression of the SA response is critical for BABA-IR against *Pst* DC3000 (Zimmerli *et al.*, 2000; Ton *et al.*, 2005), we

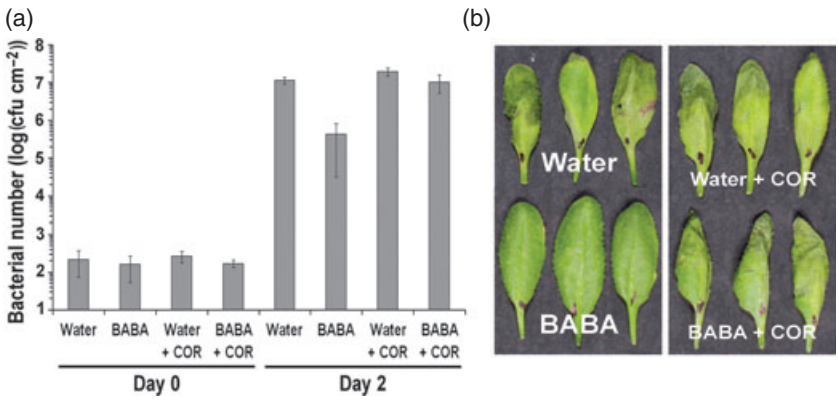

Figure 4. High concentrations of purified COR alter BABA protection.
(a, b) Bacterial growth (a) or symptoms (b) were evaluated 2 days after infiltration with *Pst* DC3000 (10^6 cfu/ml). Bacterial growth data are means ± SD for three independent biological replicates. Pictures of representative leaves were taken 2 days after inoculation. For all experiments, treatments were performed by syringe infiltration of a solution of 5 μM of purified COR or a mixed solution of 5 μM COR and bacteria. All experiments were repeated at least three times with similar results.

further tested whether BABA-IR is functional in Arabidopsis wild-type plants against *Pst* DC3000 COR⁻ bacteria, and in the *coi1-16* mutant against *Pst* DC3000 wild-type bacteria. As shown in Figure 5(b), BABA did not provide a clear protection in both combinations. Although *coi1-16* or Col-0 plants infected with *Pst* DC3000 COR⁻ without BABA were more resistant than water-treated Col-0 infected with *Pst* DC3000, they still harboured about 10 times more bacteria than BABA-treated Col-0. This observation suggests that *coi1-16* or Col-0 plants infected with *Pst* DC3000 COR⁻ could still reach a higher level of protection if normally responsive to BABA. Although reduced priming may be caused by lower bacterial titres in COR⁻ or *coi1-16* infected plants, these data suggest that both BABA-induced priming of the SA response and BABA-IR against *Pst* DC3000 are dependent on a functional COR response.

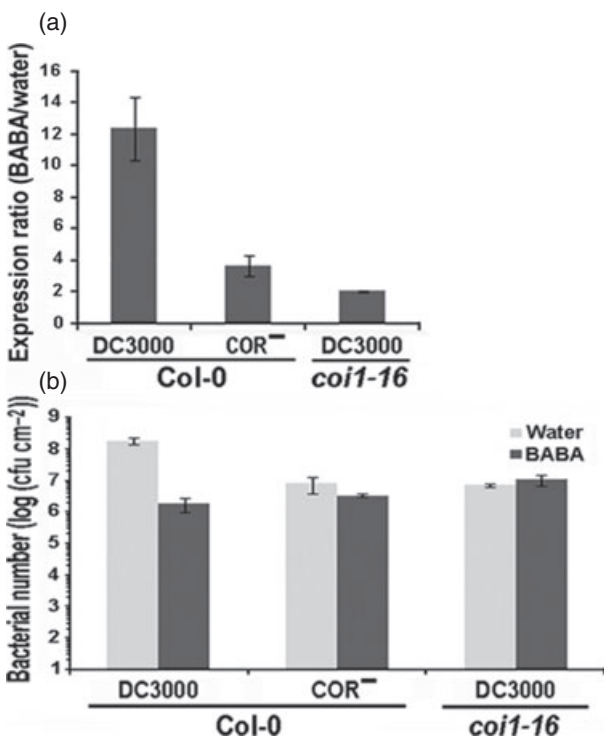

Figure 5. BABA priming and BABA-IR are dependent on a functional COR response.
(a) BABA priming of *PR1* expression is defective in Arabidopsis plants that are altered in their COR response. Samples were collected 22 h after dip inoculation with *Pst* DC3000 (DC3000) or *Pst* DC3000 COR⁻ (COR⁻). For each condition, values are the relative expression ratio of BABA- to water-treated bacteria-infected plants (defined value of 1). Error bars are SD (n = 3 technical replicates). All experiments were repeated three times with similar results.
(b) BABA does not protect COR response-defective Arabidopsis plants. Bacterial growth was evaluated 2 days after bacterial dip inoculation. Bacterial growth data are means ± SD of three technical replicates. Experiments were repeated three times with similar results. Representative data are shown.

BABA does not repress JA signalling

To determine whether BABA acts specifically on the JA response induced by COR or non-specifically on JA signalling, we tested whether BABA inhibits the JA response after exogenous application of either MeJA or JA. To ensure that the JA response was not saturated by these exogenously applied chemicals, we applied concentrations of MeJA or JA that induce gene up-regulation at similar levels to those observed after bacterial inoculation (Figure 2b), and analysed JA-inducible gene expression at early time points. Time course analyses with two MeJA concentrations (40 and 160 nM) revealed that BABA-treated plants did not show suppression of MeJA-induced expression of eight JA/COR-responsive genes up to 16 h after treatment (Figure 6a–c and Figure S5a–c). Furthermore, BABA did not suppress luciferase activity of P_{VSP1}:luciferase transgenic plants after treatment with four concentrations of MeJA (Figure 6d). Five-week-old plants were also treated with 50 or 100 μM JA, and relative gene expression levels were analysed 4 h later by real-time quantitative RT-PCR. As for MeJA treatment, up-regulation of gene expression was not inhibited by BABA (Figure 6e,f). In addition, BABA did not repress the expression of JA-responsive genes in 2-week-old plantlets treated with 25 or 50 μM JA (Figure S6). The effects of BABA on the plant response to mechanical wounding, another JA-dependent response, were also evaluated. BABA did not

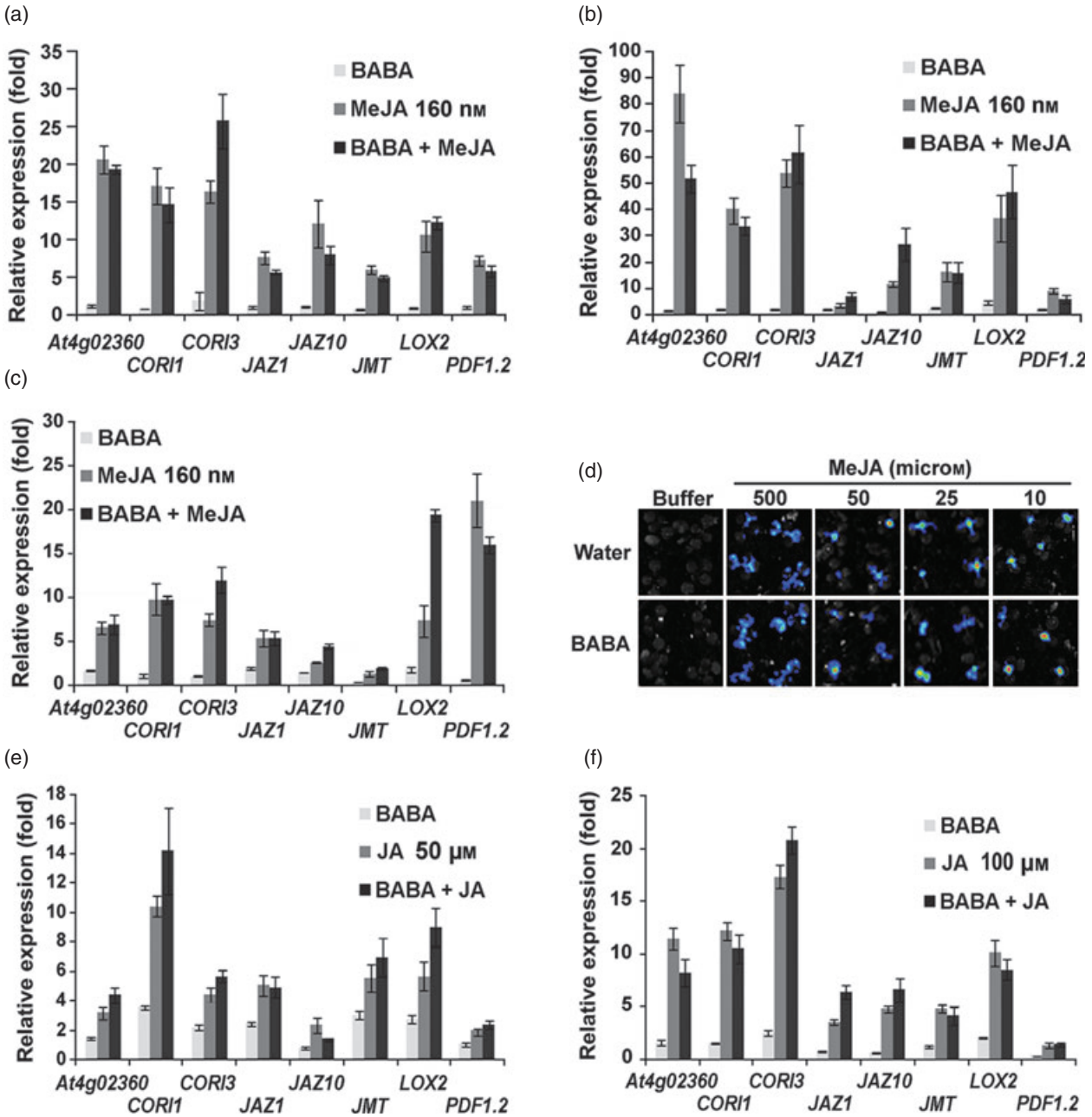

Figure 6. BABA does not inhibit the JA response upon JA or MeJA treatment.
(a–c) The response to MeJA is not altered by BABA. Relative expression levels of eight JA/COR-responsive genes were determined by real-time quantitative RT-PCR after 4 h (a), 8 h (b) or 16 h (c) treatment with MeJA.
(d) BABA does not alter the *VSP1* promoter activity upon treatment with various concentrations of MeJA. Luciferase activity in P_{VSP1}:luciferase transgenic plants was evaluated 16 h after MeJA treatment at the indicated concentrations.
(e, f) BABA does not alter the Arabidopsis response to two concentrations of JA. The relative expression levels of eight JA-responsive genes were evaluated after 4 h of JA treatment by real-time quantitative RT-PCR. For all real-time quantitative RT-PCR analyses, relative expression levels were compared to water-treated controls (no MeJA or JA) (defined value of 1). Error bars are SD (n = 3 technical replicates). All experiments were repeated at least twice with similar results.

repress the JA-dependent wound response at 0.5, 1, 2 or 4 h after wounding (Figure 7a–d). Together, these observations are consistent with a model where BABA does not suppress the entire JA response, but specifically inhibits the COR-induced JA response.

BABA inhibits the COR-dependent re-opening of stomata upon bacterial infection

Plants have developed mechanisms to close stomata during bacterial invasion (Melotto *et al.*, 2006; Zeng *et al.*, 2010).

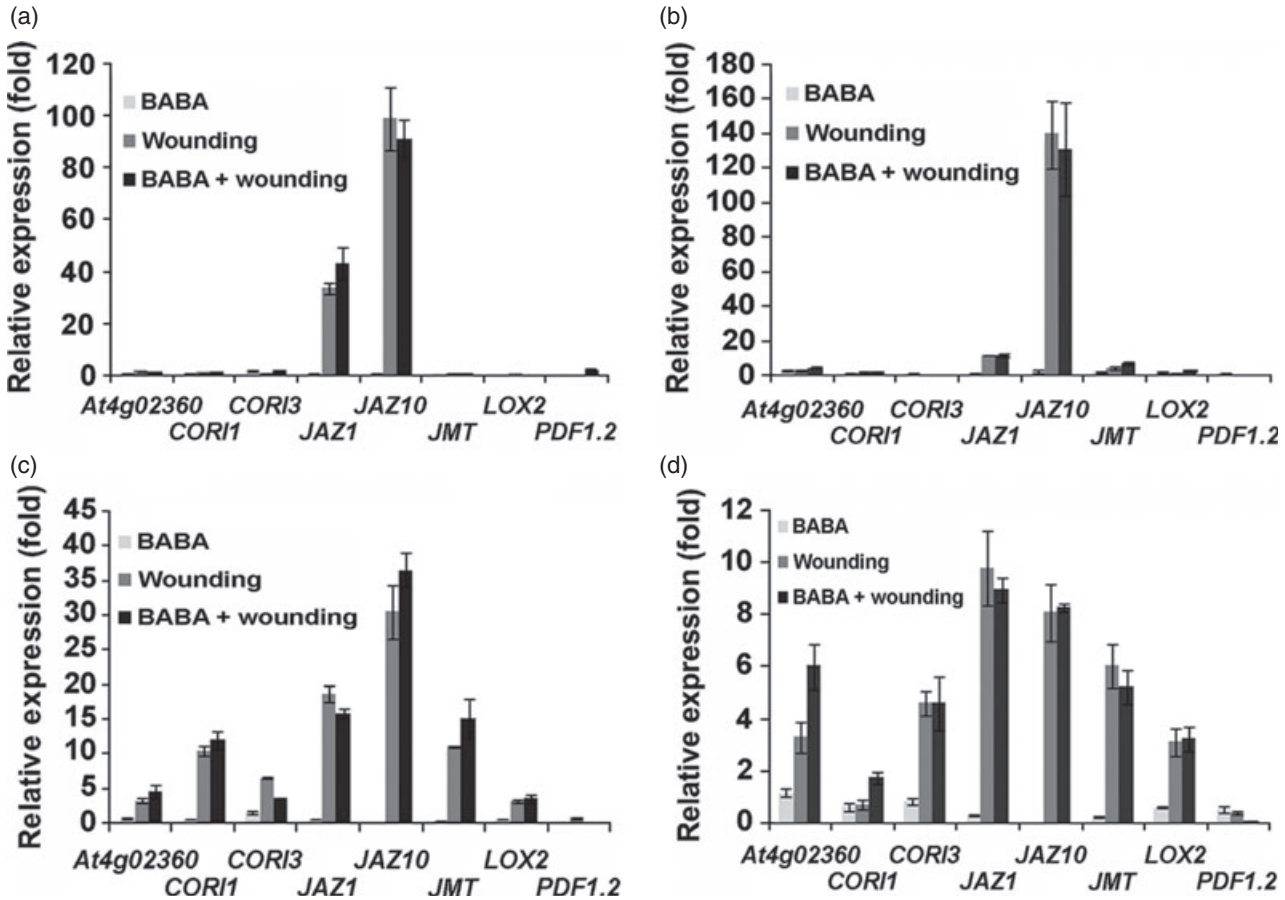

Figure 7. BABA does not alter the expression of JA/COR-responsive genes upon wounding.
(a–d) Relative expression levels of eight JA/COR-responsive genes were monitored at 0.5 h (a), 1 h (b), 2 h (c) and 4 h (d) after wounding by real-time quantitative RT-PCR. Expression levels were compared to water-treated, non-wounded controls (defined value of 1). Error bars are SD (n = 3 technical replicates). Experiments were repeated at least twice with similar results.

Stomatal closure is abscisic acid- and SA-dependent, and COR is necessary for stomatal re-opening upon infection by virulent bacteria such as *Pst* DC3000 (Melotto *et al.*, 2006; Zeng *et al.*, 2010). To further document the possible inhibitory effect of BABA on COR action, stomatal closure after inoculation with *Pst* DC3000 or *Pst* DC3000 COR$^-$ was analysed in water- and BABA-treated Arabidopsis. As expected (Melotto *et al.*, 2006), bacteria induced closure of stomata at 1 h post-inoculation, and stomata re-opened in a COR-dependent manner at 3 h post-inoculation (Figure 8a,b). BABA did not have a direct effect on stomatal movement (Figure 8a,b), confirming observations by Jakab *et al.* (2005). However, BABA did inhibit the COR-dependent re-opening of stomata by *Pst* DC3000 (Figure 8a). Unlike COR, MeJA treatment causes stomatal closure (Suhita *et al.*, 2004). These results further suggest that BABA specifically inhibits COR action in Arabidopsis.

DISCUSSION

The non-protein amino acid BABA increases plant resistance against biotic stress through priming of stress defence responses (Prime-A-Plant Group et al., 2006). Typically, BABA enhances Arabidopsis resistance to the virulent bacterial pathogen *Pst* DC3000 by potentiating mRNA accumulation of the SA-dependent marker *PR1* (Zimmerli *et al.*, 2000; Ton *et al.*, 2005). Priming of *PR1* expression is critical for BABA-induced resistance to virulent bacteria, as cyclin-dependent kinase-like *ibs1* mutants show defective *PR1* priming and concomitantly lose BABA-induced resistance to *Pst* DC3000 (Ton *et al.*, 2005). To further decipher the BABA-mediated priming mechanisms, we analysed the genome-wide BABA-priming transcriptome upon infection with *Pst* DC3000. Treatment with BABA induced the up-regulation of numerous genes that are BTH-, SA-, pathogen- or flg22-responsive. These results contrast with those of a previously published study, in which BABA did not up-regulate such genes (Zimmerli *et al.*, 2008). A lower BABA concentration and analysis at a later time point may explain this discrepancy. In addition, whole-genome microarrays were used in this study, but only slightly more than one-third of the genome was represented on the microarrays used by Zimmerli *et al.* (2008). The difference observed may be due

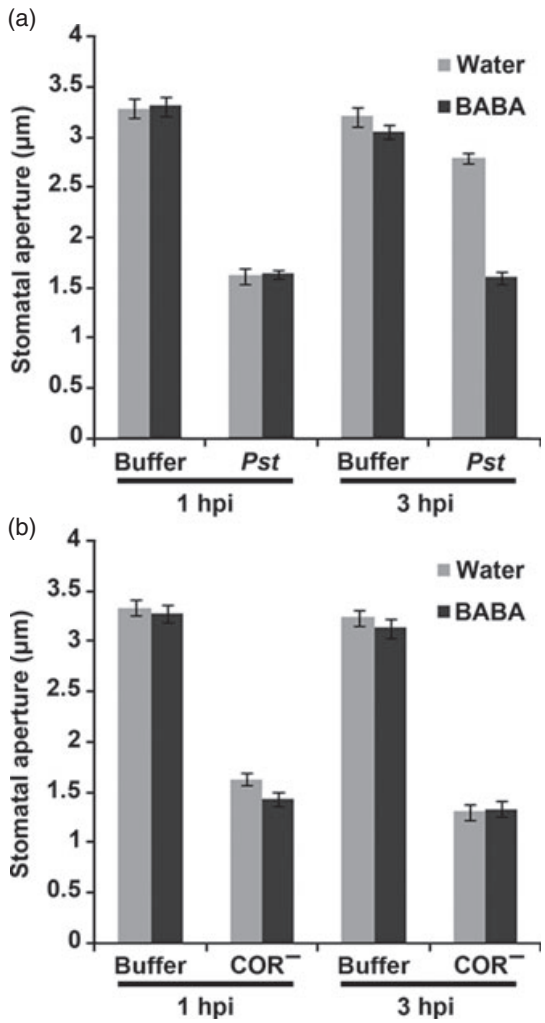

Figure 8. The COR-dependent re-opening of stomata upon *Pst* DC3000 infection is blocked by BABA.
Stomatal apertures in epidermal peels of 5-week-old water- or BABA-treated Arabidopsis plants exposed to $MgSO_4$ buffer or *Pst* DC3000 (*Pst*) (a) or *Pst* DC3000 COR$^-$ (COR$^-$) (b) were analysed at 1 and 3 h post-inoculation. Results are means ± SE (n > 60 stomata). Experiments were repeated three times with similar results.

to a biased distribution of gene functions on the microarray used during the first study (Zimmerli *et al.*, 2008). Nevertheless, both microarray studies showed up-regulation of numerous stress-responsive genes.

Twenty-two genes showed BABA-mediated potentiated expression upon bacterial infection. BABA-IR against *Pst* DC3000 is SA-dependent (Zimmerli *et al.*, 2000; Ton *et al.*, 2005). Confirming these observations, about half of the primed genes were found to be early SA-responsive ges (Figure 1c). Other BABA primed genes such as *PR1* and *PR5* are known late SA-responsive genes (Uknes *et al.*, 1992). BABA thus primes the accumulation of SA-responsive mRNAs upon *Pst* DC3000 infection. By contrast, BABA primes for ABA-dependent callose deposition after attack by necrotrophs (Ton and Mauch-Mani, 2004). None of the BABA-primed genes uncovered in this study are ABA-responsive (data not shown). Corroborating this observation, callose synthase PMR4-derived callose deposition appears to play no role in BABA-IR against *Pst* DC3000 (Flors *et al.*, 2008). Together, these data suggest that the pool of BABA-primed genes differs depending on the challenging pathogen. BABA priming is thus likely to be pathogen-specific. Analysing the BABA-primed transcriptome after infection with necrotrophs should further clarify this assumption.

How does BABA prime the appropriate defence response to *Pst* DC3000 without potentiating other non-specific Arabidopsis defence responses? Here we show that BABA inhibits the Arabidopsis response to the *Pst* DC3000 molecule effector COR. COR has been proposed to suppress SA-mediated defence responses by activation of the JA signalling pathway, which leads to increased bacterial virulence (Brooks *et al.*, 2005). BABA inhibition of the COR-induced JA response may thus restore an earlier activation of SA signalling, leading to a potentiated SA-dependent defence response. Importantly, BABA-induced priming and BABA-IR were greatly reduced in plants altered in their COR response, which strongly suggests a biological significance of the observed BABA inhibition of the Arabidopsis response to COR. As COR is only produced by certain pathovars of *Pseudomonas syringae* (Bender *et al.*, 1999), the specific inhibition of the Arabidopsis response to COR by BABA may explain the targeted BABA priming of SA defence signalling upon *Pst* DC3000 infection. A small level of priming was still observed upon *Pst* DC3000 COR$^-$ infection (Figure 5a). This indicates that BABA may alter the effect of other effectors than COR. Some bacterial protein effectors are known to modify host defence by targeting JA signalling (Zhao *et al.*, 2003). BABA primes *PR1* mRNA accumulation after treatment with the SA analogue BTH (Van der Ent *et al.*, 2009), implying that BABA can prime SA signalling independently of COR. Similarly, our microarray data demonstrated direct up-regulation of SA-responsive genes by BABA. In addition, BABA induced direct up-regulation of flg22-responsive genes, suggesting an effect on PAMP-triggered immunity. BABA probably acts at multiple levels to prime for enhanced defence responses. Indeed, priming of plants infected with pathogens that do not produce COR was also observed (Ton and Mauch-Mani, 2004). It is tempting to speculate that priming in these circumstances reflects inhibition of effectors other than COR.

Here we show that BABA specifically inhibits the COR response. It is very unlikely that BABA directly affects the JA signalling in Arabidopsis as only the COR-induced JA response is repressed, without an effect on other JA responses. BABA inhibition of the COR-dependent

re-opening of stomata by virulent *Pst* DC3000 further suggests that BABA specifically represses COR action in Arabidopsis. Corroborating these data, BABA does not repress JA accumulation upon infection with the necrotroph *Alternaria brassicicola* (Flors *et al.*, 2008). This observation is surprising as COR is known to mimic JA-Ile and bind to the same COI1–JAZ complex (Katsir *et al.*, 2008b; Yan *et al.*, 2009). This result implies the existence of a regulatory node that can distinguish COR-mediated JA responses from other JA responses downstream of COR/JA perception. As recently proposed (Katsir *et al.*, 2008b), it is likely that the type of JA response is determined by the specificity of COI1-bioactive JA–JAZ and JAZ–transcription factor interactions. BABA may act at this level to repress a COR-specific response without affecting other JA responses. Indirect inhibition through interaction of BABA with other hormones cannot be ruled out (Navarro *et al.*, 2008). However, SA signalling does not appear to be implicated in such a mechanism (Figure 3).

In summary, this study suggests that BABA primes SA signalling through inhibition of COR-mediated bacterial manipulation of the SA-dependent Arabidopsis defence response. Our results provide a novel conceptual advance on priming. Plants can indeed be prepared to modulate the outcome of the race between activation and effector inhibition of the defence responses to the plant's advantage. In addition, although both COR and JA bind to the same COI1–JAZ complex (Katsir *et al.*, 2008a), the JA responses induced by COR or other bioactive JAs are differently regulated by BABA. Determining the COR regulatory node targeted by BABA will pinpoint key elements involved in the distinctive responses to COR and JA.

EXPERIMENTAL PROCEDURES

Biological materials

Arabidopsis thaliana (L. Heynh.) Columbia (Col-0) were grown in commercial potting soil/perlite (3:2) at 22°C day and 18°C night temperature with 9 h light per 24 h for 5 weeks. The P_{VSP1}:β-glucuronidase transgenics were obtained from J.G. Turner (Department of Biological Sciences, University of East Anglia, Norwich, UK). The Col-0 background mutants *sid2-1* and *npr1-1* were provided by C. Nawrath (Department of Plant Molecular Biology, University of Lausanne, Lausanne, Switzerland); *pad4-1* and *coi1-16* were obtained from the Arabidopsis Biological Resource Center and J.G. Turner, respectively. Bacterial strains *Pst* DC 3000, *Pst* DC 3000 COR$^-$ (DB29) and the *hrcC* mutant (CB200) were donated by B.N. Kunkel (Department of Biology, Washington University, St Louis, MO). Bacteria were cultivated at 28°C/340 rpm in King's B medium containing rifampicin (*Pst* DC3000), rifampicin, spectinomycin and kanamycin (DB29), or rifampicin and kanamycin (CB200).

Bacterial inoculations

Leaves were dipped in a bacterial suspension of 5×10^7 cfu/ml, or as indicated, in 10 mM $MgSO_4$ containing 0.01% Silwet L-77 (Lehle Seeds, http://www.arabidopsis.com/) for 15 min. For inoculation by infiltration, leaves were syringe-infiltrated with the indicated bacterial concentration as described previously (Zimmerli *et al.*, 2000).

Chemical and wounding treatments

Two days before bacterial inoculation, plants were soil-drenched with BABA (Fluka, http://www.sigmaaldrich.com) at a final concentration of 200 μM, or with water (control). Unless further specified, plants were sprayed with 0.5 μM COR (Sigma, http://www.sigmaaldrich.com/) or 50 or 100 μM JA (Sigma) in 0.1% w/v Tween-20. MeJA treatments were performed as described previously (Zimmerli *et al.*, 2004). Briefly, plants were placed in sealed 5 L boxes containing 40 μl of either ethanol (control) or 5 or 20 mM MeJA (Sigma) to produce the final specified concentration for the indicated period of time. Wounding of leaves was performed by multiple pinching with a forceps.

GUS assay

β-glucuronidase activity was determined as described previously (Zimmerli *et al.*, 2004).

Luciferase assay

Two-week-old MeJA- or COR-exposed seedlings were sprayed with 0.4 mM D-luciferin (Synchem, http://www.synchem.de/) supplemented with 0.01% Triton X-100. After 10 min incubation in the dark, the luminescence of COR- and MeJA-exposed seedlings was recorded using an LAS-3000 luminescent image analyser (Fujifilm, http://www.fujifilm.com/) for 1 and 2 h, respectively.

Stomatal assay

Plants were kept under light (approximately 100 μmol m^{-2} sec^{-1}) for at least 3 h to allow opening of stomata before the start of the experiments. The epidermis of three fully expanded leaves from three plants (9 leaves in total) was peeled off and placed on glass slides with the cuticle side in contact with 10 mM $MgSO_4$ buffer or bacterial suspensions (10^8 cfu/ml *Pst* DC3000 or *Pst* DC3000 COR$^-$ in 10 mM $MgSO_4$ buffer). At various time points, images of random regions were taken using an Olympus DP72 microscope digital camera and application software DP2-BSW (http://www.olympus-global.com/). The width of the stomatal aperture was measured using the 'measure' function of ImageJ (http://rsb.info.nih.gov/ij/).

Real-time quantitative RT-PCR

Leaf samples from 6–8 plants per treatment were harvested at the indicated time points, flash frozen in liquid N_2, and kept at –80°C. Total RNA was isolated using a RNeasy plant mini kit (Qiagen, http://www.qiagen.com/). Complementary DNA was synthesized from 2 μg of total RNA using oligo(dT) primers and the reverse transcriptase from the M-MLV kit (Invitrogen, http://www.invitrogen.com/). An iCycler sequence detection system (Bio-Rad, http://www.bio-rad.com/) and SYBR Green PCR Master Mix (Bio-Rad) were used for real-time PCR analysis. The thermal cycling program was 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec, 54°C for 35 sec and 72°C for 35 sec. UBQ10 (At4g05320) and EF-1α (At5g60390) were used as internal references. Primer efficiency values (close to 100%) were taken into account for calculation of relative expression. Primer sequences are shown in Table S6.

Microarray analysis

BABA was applied 2 days 22 h before RNA sample collection. Samples were collected 22 h after dip inoculation with *Pst* DC3000. RNA samples from three independent biological replicates of (i) BABA- and water-treated plants and (ii) BABA- and water-treated *Pst*

DC3000-inoculated plants were prepared. Total RNA was isolated from eight liquid N_2-frozen Arabidopsis rosettes per treatment group as described previously (Zimmerli *et al.*, 2000). mRNA from leaves was amplified using the MessageAmp^{TM} aRNA II kit (Ambion, http://www.ambion.com/). Five micrograms of amplified RNA were reverse-transcribed into cyanin 3- or cyanin 5-labelled cDNA, purified using Qiaquick^{TM} columns (Qiagen, http://www.qiagen.com) and hybridized on custom microarrays produced by the Lausanne Genomic Technologies Facility containing 25 000 gene-specific tags for the *Arabidopsis thaliana* genome (Hilson *et al.*, 2004).

Microarray data analysis

After scanning of microarrays, the resulting TIFF (tagged image file format) images corresponding to the Cy5 and Cy3 fluorescence emission channels were extracted using GenePix Pro 6.0 software (Molecular Devices, http://www.moleculardevices.com/). Statistical analysis of the data was performed using the LimmaGui software package (Wettenhall and Smyth, 2004). Raw data without background subtraction were print-tip lowess normalized (Yang *et al.*, 2002) to calculate M values. Statistical analysis was performed by pairwise comparison of plants treated with water versus plants treated with BABA, and plants treated with water and infected with *Pseudomonas* versus plants treated with BABA and infected with *Pseudomonas*. The genes shown in Figure 1(a,c) were clustered using Cluster (using absolute correlation uncentred and average linkage) and Treeview software (Eisen *et al.*, 1998). Microarray data have been deposited in the Gene Expression Omnibus (GEO accession number GSE16434, http://www.ncbi.nlm.nih.gov/geo/).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Time course analysis of the SA- and JA-dependent defence response.

Figure S2. Visualization of BABA effect on *VSP1* promoter activity after purified COR treatment.

Figure S3. High concentrations of COR block BABA inhibition of the COR response.

Figure S4. The *pen2* mutant shows normal BABA-induced *PR1* priming.

Figure S5. The Arabidopsis response to MeJA is not altered by BABA.

Figure S6. BABA does not inhibit the JA response of 2-week-old Arabidopsis plantlets.

Table S1. Differentially expressed transcripts in leaves from BABA-treated plants versus leaves from water-treated control plants.

Table S2. Comparison of up-regulated genes from Table S1 with published microarray datasets of genes activated by benzothiadiazole or salicylic acid, various pathogens or the PAMP flagellin flg22.

Table S3 Differentially expressed transcripts in leaves from BABA-treated plants after infection with *Pst* DC3000 versus leaves from water-treated control plants after infection with *Pst* DC3000.

Table S4. Comparison of up-regulated genes from Table S3 with published microarray datasets of genes activated by benzothiadiazole or salicylic acid, various pathogens or the PAMP flagellin flg22.

Table S5. Comparison of down-regulated genes from Table S3 with published microarray datasets.

Table S6. Gene AT numbers and sequences of the primers used in this study.

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