Priming of plant innate immunity by rhizobacteria and β-aminobutyric acid: differences and similarities in regulation

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Summary

• *Pseudomonas fluorescens* WCS417r bacteria and β-aminobutyric acid can induce disease resistance in Arabidopsis, which is based on priming of defence.

• In this study, we examined the differences and similarities of WCS417r- and β-aminobutyric acid-induced priming.

• Both WCS417r and β-aminobutyric acid prime for enhanced deposition of callose-rich papillae after infection by the oomycete *Hyaloperonospora arabidopsis*. This priming is regulated by convergent pathways, which depend on phosphoinositide- and ABA-dependent signalling components. Conversely, induced resistance by WCS417r and β-aminobutyric acid against the bacterial pathogen *Pseudomonas syringae* are controlled by distinct NPR1-dependent signalling pathways. As WCS417r and β-aminobutyric acid prime jasmonate- and salicylate-inducible genes, respectively, we subsequently investigated the role of transcription factors. A quantitative PCR-based genome-wide screen for putative WCS417r- and β-aminobutyric acid-responsive transcription factor genes revealed distinct sets of priming-responsive genes. Transcriptional analysis of a selection of these genes showed that they can serve as specific markers for priming. Promoter analysis of *WRKY* genes identified a putative *cis*-element that is strongly over-represented in promoters of 21 NPR1-dependent, β-aminobutyric acid-inducible *WRKY* genes.

• Our study shows that priming of defence is regulated by different pathways, depending on the inducing agent and the challenging pathogen. Furthermore, we demonstrated that priming is associated with the enhanced expression of transcription factors.

Abbreviations: BABA, β-aminobutyric acid; BTH, benzothiadiazole; ET, ethylene; IR, induced resistance; ISR, induced systemic resistance; JA, jasmonic acid; MeJA, methyl jasmonate; PR, pathogenesis-related; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; SA, salicylic acid; SAR, systemic acquired resistance; TAIR, The Arabidopsis Information Resource; TF, transcription factor.

Introduction

Many plant pathogens have evolved the capacity to circumvent or suppress host defence mechanisms that are controlled by the plant's innate immune system. As an evolutionary response, plants have evolved additional defence strategies that allow them to detect and halt pathogen invasion at an early stage of infection. Resistance (*R*) gene-mediated resistance

is a well-known example of a counter-evolved defence system that enables plants to respond quickly to pathogens that suppress immune responses (McDowell & Woffenden, 2003; Jones & Dangl, 2006). In addition, plants have evolved the ability to enhance their basal resistance after perception of specific biotic or abiotic stimuli, such as pathogen attack, root colonization by selective rhizobacteria or selected chemicals. This so-called induced resistance (IR) does not necessarily require direct activation of defence mechanisms, but can also result from a sensitization of the tissue to express basal defence mechanisms more rapidly and more strongly after subsequent pathogen attack. This sensitization of the plant's innate immune system is commonly referred to as 'priming' (Conrath *et al.*, 2006). As demonstrated recently, the induction of priming yields broad-spectrum resistance with minimal reductions in plant growth and seed set (Van Hulten *et al.*, 2006). Hence, priming is a cost-efficient resistance strategy that increases the plant's ability to cope with environmental stress.

The classic form of IR develops on attempted or limited infection by a pathogen, and results in a systemic acquired resistance (SAR) that protects against various types of pathogen (Ryals *et al.*, 1996). The signalling pathway controlling SAR depends on the endogenous accumulation of the phytohormone salicylic acid (SA) and on the presence of the defence regulatory protein NPR1 (Durrant & Dong, 2004). NPR1 functions in both SA-dependent basal resistance and SAR by controlling the expression of many stress-related genes, including those that encode pathogenesis-related (PR) proteins and proteins involved in the secretory pathway (Dong, 2004; Wang *et al.*, 2005). Colonization of plant roots by the nonpathogenic rhizobacterial strain *Pseudomonas fluorescens* WCS417r also triggers a systemic resistance against a wide range of pathogens, including the downy mildew-causing oomycete *Hyaloperonospora arabidopsis*, formerly known as (*Hyalo*)*peronospora parasitica* (Göker *et al.*, 2004), and the bacterial speck pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Pieterse *et al.*, 1996; Ton *et al.*, 2002). In contrast with pathogen-induced SAR, this so-called rhizobacteria-induced systemic resistance (ISR) functions independently of SA, but requires components of response pathways that depend on jasmonic acid (JA) and ethylene (ET) (Pieterse *et al.*, 1998; Van Wees *et al.*, 2008). ISR elicited by WCS417r bacteria (WCS417r-ISR) is not accompanied by direct activation of *PR* genes (Pieterse *et al.*, 1996), but leads to priming of predominantly JA- and ET-responsive genes (Van Wees *et al.*, 1999; Verhagen *et al.*, 2004). Both WCS417r-ISR and SAR require NPR1 (Pieterse *et al.*, 1998), suggesting that this protein is important in regulating and connecting different hormone-dependent defence pathways. Recently, the transcription factors (TFs) MYB72 and MYC2 have been demonstrated to be crucial for WCS417r-ISR (Pozo *et al.*, 2008; Van der Ent *et al.*, 2008). *MYB72* gene expression is specifically activated in the roots of WCS417r-colonized plants (Van der Ent *et al.*, 2008), whereas MYC2 is thought to regulate ISR expression in systemic plant parts (Pozo *et al.*, 2008).

In addition to biological resistance-inducing agents, many chemicals have been reported to trigger IR. Most of these agents trigger the SAR pathway, as they activate a similar set of *PR* genes and fail to induce resistance in SAR-impaired mutants (Lawton *et al.*, 1996; Dong *et al.*, 1999). However, the non-protein amino acid β-aminobutyric acid (BABA) has been shown to trigger a partially different IR response. Like WCS417r-ISR, BABA-IR is not associated with a major transcriptional activation of defence-related genes (Zimmerli *et al.*, 2000). Instead, BABA primes the plant tissue for enhanced activation of SA-responsive genes, such as *PR-1* (Zimmerli *et al.*, 2000, 2001). BABA-IR in Arabidopsis against *P. syringae* DC3000 resembles pathogen-induced SAR in its requirement for SA and NPR1 (Zimmerli *et al.*, 2000). Yet, BABA-IR against *H. arabidopsis* is fully expressed in Arabidopsis genotypes that are impaired in SAR signalling (Zimmerli *et al.*, 2000). This SA- and NPR1-independent form of BABA-IR is based on a priming of cell-based defences, which manifests as an augmented deposition of callose-rich papillae at the sites of attempted tissue penetration by the attacking pathogen (Zimmerli *et al.*, 2000; Jakab *et al.*, 2001; Ton & Mauch-Mani, 2004). Screening for Arabidopsis mutants that are impaired in BABA-induced sterility (*ibs*) resulted in the isolation of two mutants, *ibs2* and *ibs3*, which are affected in BABA-induced priming of papillae deposition. The *ibs2* mutant carries a mutation in the *SAC1b* gene that encodes a polyphosphoinositide phosphatase (Despres *et al.*, 2003), suggesting involvement of a phosphoinositide-dependent signalling pathway in BABA-induced priming of cell wall defence (Ton *et al.*, 2005). The *ibs3* mutant, however, is impaired in the regulation of the zeaxanthin epoxidase gene *ABA1/NPQ2*, which links ABA signalling to BABA-induced priming of cell wall defence (Ton *et al.*, 2005). The latter finding is supported by previous observations that the ABA biosynthetic mutant *aba1-5* and the ABA response mutant *abi4-1* are impaired in BABA-induced priming of callose deposition after inoculation with the necrotrophic fungi *Alternaria brassicicola* and *Plectosphaerella cucumerina* (Ton & Mauch-Mani, 2004). Hence, BABA-induced priming of pathogen-inducible papillae deposition involves regulation by a phosphoinositide- and ABA-dependent signalling pathway.

Despite the differences in signalling pathways between WCS417r-ISR and BABA-IR, both forms of IR are characterized by primed resistance mechanisms (Van Wees *et al.*, 1999; Zimmerli *et al.*, 2000; Ton & Mauch-Mani, 2004; Verhagen *et al.*, 2004). Although priming has been known for years, the current understanding of its underlying mechanisms remains rudimentary. It has been hypothesized that the induction of priming leads to a subtle increase in the level of signalling components that play a role in basal resistance (Conrath *et al.*, 2006). After perception of a second,

pathogen-derived signal, the enhanced signalling capacity would facilitate a faster and stronger basal defence reaction. As primed plants display a faster and stronger transcription of defence-related genes after pathogen challenge (Zimmerli *et al.*, 2000; Kohler *et al.*, 2002; Verhagen *et al.*, 2004; Ton *et al.*, 2007), TFs may well play a role in the onset of priming. We hypothesize that the induction of priming causes a subtle increase in the expression of defence regulatory TFs, which is sufficient to prime defence genes, but too weak to activate them directly. However, primed deposition of papillae is a relatively rapid defence reaction that is unlikely to be controlled at the transcriptional level, suggesting that transcription-independent mechanisms are involved as well.

In this study, we compared the involvement of two cellular responses in WCS417r-ISR and BABA-IR: (1) the formation of callose-rich papillae and (2) the expression of TF genes. To study the mechanisms behind priming of papillae deposition, we focused on the roles of IBS2 and IBS3 in WCS417r-ISR and BABA-IR against *H. arabidopsis* WACO9. As WCS417r-ISR and BABA-IR are both associated with priming for enhanced transcription of defence-related genes (Zimmerli *et al.*, 2000; Verhagen *et al.*, 2004), we also examined the involvement of TFs during the onset of the WCS417r- and BABA-induced defence priming. These analyses revealed sets of TF genes that are directly responsive to priming treatment with WCS417r bacteria or BABA, and identified a putative *cis*-element in promoters of NPR1-dependent, BABA-inducible *WRKY* genes.

Materials and Methods

Plants and microorganisms

Seeds of *Arabidopsis thaliana* (L.) Heynh. accession Col-0 and its mutants *ibs2-2*, which carries a T-DNA insertion in the 5′-untranslated region of *SAC1b* (At5g66020; this mutant is also referred to as s-031243; Ton *et al.*, 2005), *ibs3-2*, which harbours an ethyl methanesulfonate (EMS)-induced mutation in the *ABA1*/*NPQ2* gene (also referred to as *npq2-1*; Niyogi *et al.*, 1998; Ton *et al.*, 2005), *myb72-1* (Van de Mortel *et al.*, 2008) and *npr1-1* (Cao *et al.*, 1994) were cultivated as described previously (Pieterse *et al.*, 1996). *Pseudomonas fluorescens* strain WCS417r and *P. syringae* pv. *tomato* DC3000 (Whalen *et al.*, 1991) were cultivated as described previously by Pieterse *et al.* (1996). *Hyaloperonospora arabidopsis* strain WACO9 was cultivated as described by Ton *et al.* (2002).

Induction treatments and disease assays

ISR was elicited by transplanting 2-wk-old Arabidopsis seedlings into a sand–potting soil mixture containing 5×10^7 colony-forming units WCS417r bacteria per gram of soil. Control soil was supplemented with an equal volume of 10 mM $MgSO_4$. BABA-IR was triggered by applying BABA (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) as a soil drench at the indicated concentrations. Treatment with methyl jasmonate (MeJA) was performed by dipping 5-wk-old Col-0 plants in an aqueous solution containing 50 μM MeJA (Serva, Brunschwig Chemie, Amsterdam, The Netherlands) and 0.015% Silwet L-77 (Van Meeuwen Chemicals B.V., Weesp, The Netherlands), as described previously (Pieterse *et al.*, 1998). Benzothiadiazole (BTH; CIBA-GEIGY GmbH, Frankfurt, Germany) was administered by spraying 5-wk-old plants with a solution containing 19 mg l^{-1} wettable powder and BTH at the indicated concentrations. Leaf rosettes were harvested at the indicated intervals after application and immediately frozen in liquid nitrogen. IR assays with *P. syringae* DC3000 and *H. arabidopsis* WACO9 were performed as described by Pieterse *et al.* (1996) and Ton *et al.* (2005), respectively. To visualize colonization by *H. arabidopsis* WACO9, infected leaves were stained with lactophenol–trypan blue and examined microscopically at 5 d after inoculation, as described by Koch & Slusarenko (1990). Quantification of callose deposition was performed as described by Ton & Mauch-Mani (2004).

Northern blot analysis and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from pooled shoot samples, as described by Van Wees *et al.* (1999). For northern blot analysis, 10 μg RNA was denatured using glyoxal and dimethylsulphoxide (Sambrook *et al.*, 1989), electrophoretically separated on a 1.5% agarose gel, and blotted onto Hybond-N^+ membranes (Amersham, 's-Hertogenbosch, The Netherlands) by capillary transfer. The electrophoresis and blotting buffer consisted of 10 and 25 mM sodium phosphate (pH 7.0), respectively. Northern blots were hybridized with gene-specific probes for *PR-1* and *LOX2*, as described previously (Pieterse *et al.*, 1998). To check for equal loading, RNA gel blots were stripped and hybridized with a gene-specific probe for 18S rRNA. qRT-PCR analysis was performed basically as described by Czechowski *et al.* (2004) (for further details, see Supporting Information Notes S1).

Statistical analysis of expression data

Cluster analysis (Euclidean distance) and principal component analysis of the transcriptional patterns of the selected TF genes were based on the expression values from three independent biological samples per treatment, using TIGR Multi-experiment Viewer software (Saeed *et al.*, 2003). Both analyses were performed with the $\log_e$-transformed values of the fold induction ratio of each gene, which was defined as the expression value in each replicate sample divided by the mean expression value of the three corresponding control samples.

Log-transformed fold induction values of TF-encoding genes from independent experiments were analysed using a paired Student's *t*-test or a nonparametric Wilcoxon Mann–Whitney test.

Promoter analysis

Promoter analyses were based on the 1000-bp sequences preceding the 5′-end of each transcription unit, which were obtained from the Sequence Bulk Download and Analysis tool of The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/tools/bulk/sequences/index.jsp). The promoter sequences of WCS417r- and BABA-inducible TFs were examined for the over-representation of *cis*-acting elements, using the POBO bootstrapping program (Kankainen & Holm, 2004) and SCOPE for parameter-free *de novo* computational motif discovery. POBO and SCOPE analyses were performed under the program settings recommended at http://ekhidna.biocenter.helsinki.fi/poxo/pobo/help#p2 and http://genie.dartmouth.edu/scope/, respectively. Differences in DNA element frequency between selected groups of promoters were analysed statistically with SPSS 11.5 software, using a chi-squared test to compare proportions between: (1) the total number of promoters in the selected group; (2) the number of promoters containing the DNA element; and (3) the total number of DNA elements. The TAG[TA]CT motif in the promoter regions of the BABA-responsive *WRKY* genes was identified with the Statistical Motif Analysis tool of TAIR (http://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp). SCOPE analysis was used to verify the analysis and identify variations to the TAG[TA]CT motif.

Results

WCS417r- and BABA-induced priming of callose deposition after *H. arabidopsis* infection differs in the requirement for NPR1

The effectiveness of BABA-IR against *H. arabidopsis* is almost entirely based on NPR1-independent priming of callose-containing cell wall papillae (Zimmerli *et al.*, 2000; Ton *et al.*, 2005). Primed callose deposition in SAR-expressing plants, however, requires NPR1 (Kohler *et al.*, 2002). Furthermore, we have recently found that augmented callose deposition also occurs during the expression of WCS417-ISR against *H. arabidopsis* (Van der Ent *et al.*, 2008). To further elucidate the role of NPR1 in the priming of cell wall defence, we quantified levels of *H. arabidopsis*-induced callose in Col-0 and *npr1-1* plants after treatment with BABA and WCS417r. Both priming agents reduced downy mildew disease and colonization by *H. arabidopsis* in wild-type plants, although BABA protected the leaves more effectively than did WCS417r (Fig. 1a,b, respectively). WCS417r and BABA both mediated an augmented deposition of callose-rich papillae at 2 d after inoculation with *H. arabidopsis* spores (Fig. 1c), which was proportional to the level of protection (Fig. 1a,b). Despite these similarities, WCS417r- and BABA-induced priming of cell wall defence differed in the requirement for NPR1: although WCS417r-treated *npr1* plants failed to deposit primed levels of callose, BABA-treated *npr1* plants displayed a similar augmentation in callose deposition as BABA-treated wild-type plants (Fig. 1). In addition, the level of callose deposition correlated with the level of protection: WCS417r-colonized *npr1* plants failed to express ISR, whereas BABA-treated *npr1* was protected to similar levels as BABA-treated wild-type plants (Fig. 1c). Hence, WCS417r-induced priming of cell wall defence depends on NPR1, whereas the same priming during BABA-IR is regulated independently of NPR1.

WCS417r- and BABA-induced priming of cell wall defence requires IBS2 and IBS3

BABA-induced priming of cell wall defence is dependent on *IBS2* and *IBS3*, which encode a polyphosphoinositide phosphatase and a zeaxanthin epoxidase, respectively (Ton *et al.*, 2005). To test whether *IBS2* and *IBS3* also contributed to WCS417r-induced priming of cell wall defence, we quantified levels of ISR and callose deposition in wild-type, *ibs2* and *ibs3* plants. In contrast with wild-type plants, *ibs2* and *ibs3* failed to express WCS417r-ISR against *H. arabidopsis* (Fig. 2a). Furthermore, WCS417r-treated *ibs2* and *ibs3* plants did not deposit primed levels of callose at 2 d after pathogen inoculation (Fig. 2b). This demonstrates that WCS417r-induced priming of cell wall defence requires IBS2 and IBS3. Conversely, the ISR-impaired mutants *npr1* and *myb72* expressed wild-type levels of BABA-IR against *H. arabidopsis* WACO9 and deposited primed amounts of callose on BABA treatment (Fig. 3). Hence, WCS417r-ISR and BABA-IR against *H. arabidopsis* differ in their requirement for NPR1 and MYB72, but both pathways converge in their requirement for IBS2 and IBS3 to mediate primed papillae deposition and IR against *H. arabidopsis*.

WCS417r-ISR and BABA-IR against *P. syringae* DC3000 function independently of IBS2 and IBS3

To test the involvement of IBS2 and IBS3 in WCS417r-ISR and BABA-IR against the bacterial pathogen *P. syringae* DC3000, we quantified levels of bacterial speck disease in wild-type, *ibs2* and *ibs3* plants after treatment with WCS417r or BABA. Since as WCS417r-ISR and BABA-IR against *P. syringae* DC3000 both require NPR1 (Pieterse *et al.*, 1998; Zimmerli *et al.*, 2000), the *npr1* mutant was included as a negative control genotype. As shown in Fig. 4, both WCS417r and BABA conferred statistically significant levels of disease protection in wild-type, *ibs2* and *ibs3* plants, but not in *npr1* plants. This demonstrates that WCS417r-ISR and BABA-IR against *P. syringae* DC3000 do not require IBS2 and IBS3, but

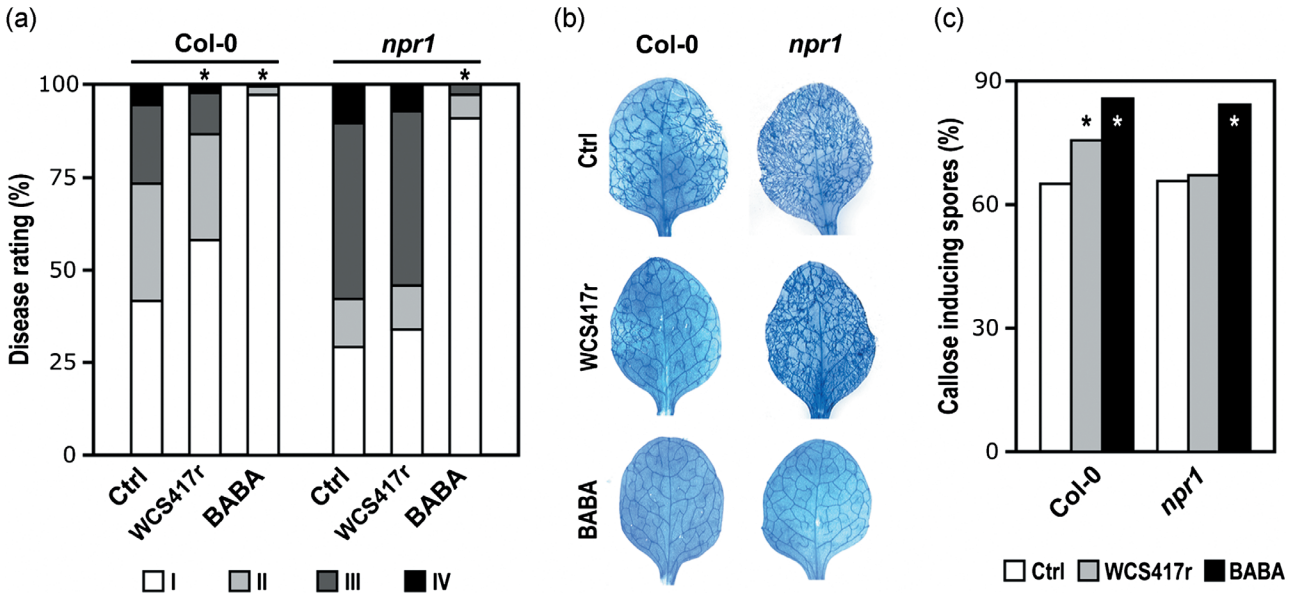

Fig. 1 Primed expression of cell wall defence against *Hyaloperonospora arabidopsis* WACO9 during expression of WCS417r-induced systemic resistance (WCS417r-ISR) and β-aminobutyric acid-induced resistance (BABA-IR) in Arabidopsis wild-type (Col-0) and *npr1* plants. (a) Quantification of WCS417r-ISR and BABA-IR against *H. arabidopsis* WACO9 at 8 d after inoculation. ISR was triggered by transferring 2-wk-old seedlings to potting soil containing *Pseudomonas fluorescens* WCS417r bacteria. BABA was applied to 3-wk-old plants by soil drenching to a final concentration of 80 μM BABA. One week after transplanting and 1 d after soil-drench treatment with BABA, leaves were inoculated with *H. arabidopsis* spores. Disease ratings are expressed as the percentages of leaves in disease classes I (no sporulation), II (trailing necrosis), III (< 50% of the leaf area covered by sporangia) and IV (heavily covered with sporangia, with additional chlorosis and leaf collapse). Asterisks indicate statistically significantly different distributions of leaves in disease severity classes compared with the water control (χ^2 test; $\alpha = 0.05$). (b) Colonization by the pathogen at 8 d after inoculation was visualized by lactophenol–trypan blue staining and light microscopy. (c) Pathogen-induced papillae deposition at 2 d after inoculation was quantified by the percentage of callose-inducing spores in the epidermal cell layer of calcofluor–aniline blue stained leaves using epifluorescence microscopy (UV). The data presented are from a representative experiment that was repeated twice with similar results.

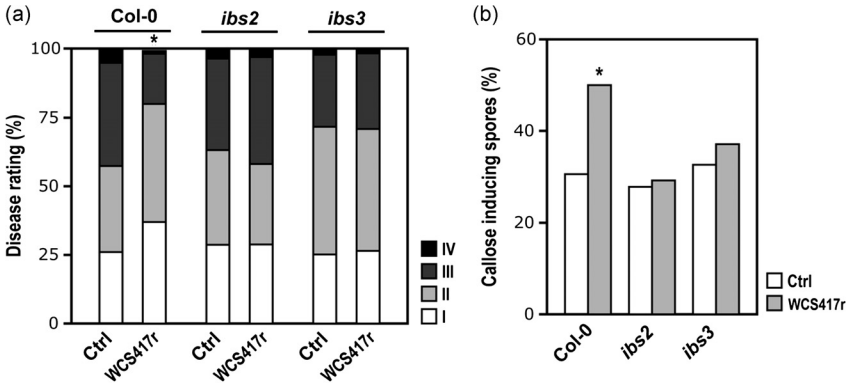

Fig. 2 Primed expression of cell wall defence against *Hyaloperonospora arabidopsis* WACO9 during WCS417r-induced systemic resistance (WCS417r-ISR) in leaves of Arabidopsis wild-type (Col-0), *ibs2* and *ibs3* plants. (a) Quantification of WCS417r-ISR against *H. arabidopsis*. For details, see legend to Fig. 1a. (b) Quantification of papillae deposition. For details, see legend to Fig. 1c.

depend on NPR1. Furthermore, these results illustrate that WCS417r-ISR and BABA-IR against *P. syringae* DC3000 are regulated differently from WCS417r- and BABA-induced priming of cell wall defence against *H. arabidopsis* WACO9.

WCS417r and BABA prime defence gene induction in response to exogenously applied JA and SA, respectively

The expression of WCS417r-ISR against *P. syringae* DC3000 is associated with a faster and stronger induction of JA-inducible genes after pathogen infection (Van Wees *et al.*, 1999; Hase *et al.*, 2003; Verhagen *et al.*, 2004). Recently, Pozo *et al.* (2008) reported that colonization by WCS417r enhances the plant's transcriptional response to JA. In agreement with this, we found that WCS417r-treated plants display an augmented induction of the JA-responsive marker gene *LOX2* at different time points after treatment of the leaves with 50 μM MeJA (Fig. 5a). Hence, WCS417r bacteria prime the responsiveness to JA, suggesting that WCS417r-induced priming targets signal transduction steps downstream of JA

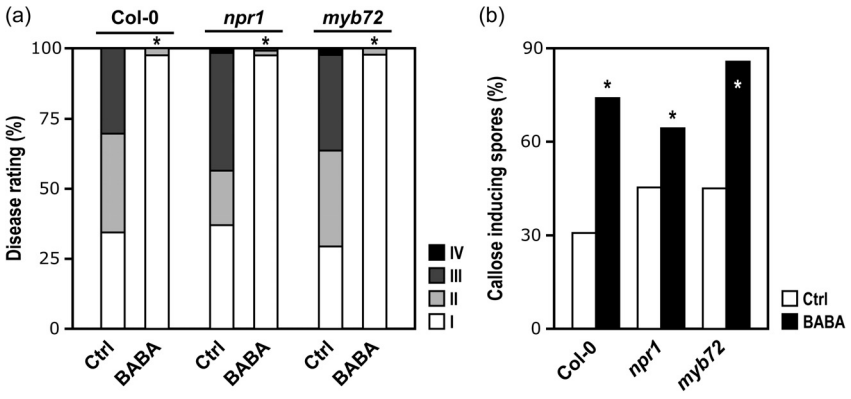

Fig. 3 Primed expression of cell wall defence during β-aminobutyric acid-induced resistance (BABA-IR) against *Hyaloperonospora arabidopsis* WACO9 in leaves of Arabidopsis wild-type (Col-0), *npr1* and *myb72* plants. (a) Quantification of BABA-IR against *H. arabidopsis*. For details, see legend to Fig. 1a. (b) Quantification of papillae deposition. For details, see legend to Fig. 1c.

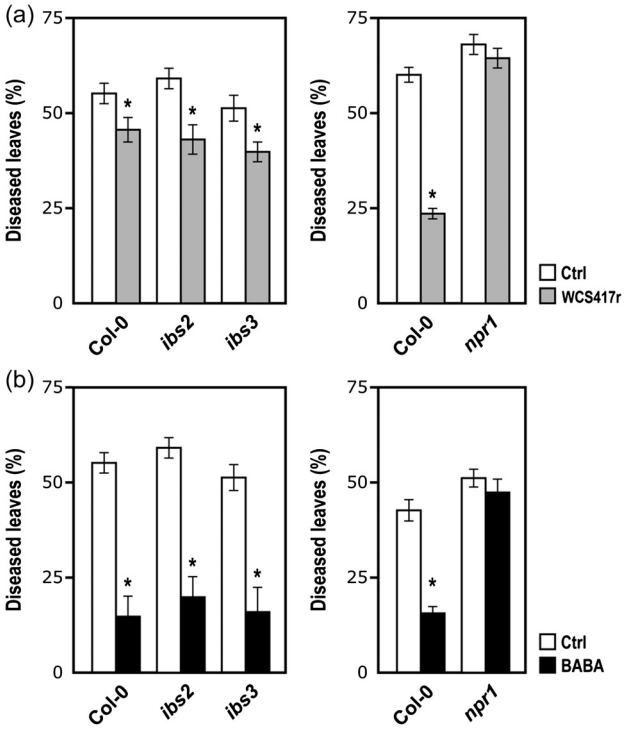

Fig. 4 WCS417r-induced systemic resistance (WCS417r-ISR) and β-aminobutyric acid-induced resistance (BABA-IR) against virulent *Pseudomonas syringae* DC3000 in leaves of Arabidopsis wild-type (Col-0), *ibs2*, *ibs3* and *npr1*. (a) Quantification of WCS417r-ISR at 4 d after inoculation of the leaves with a suspension of virulent *P. syringae* DC3000. Data presented are the means of the average percentage of diseased leaves per plant (± SD). Asterisks indicate statistically significant differences compared with noninduced control plants (Student's *t*-test; $\alpha = 0.05$; $n = 20–25$). (b) Quantification of BABA-IR against *P. syringae* DC3000. Inoculation and disease scoring were performed as described above.

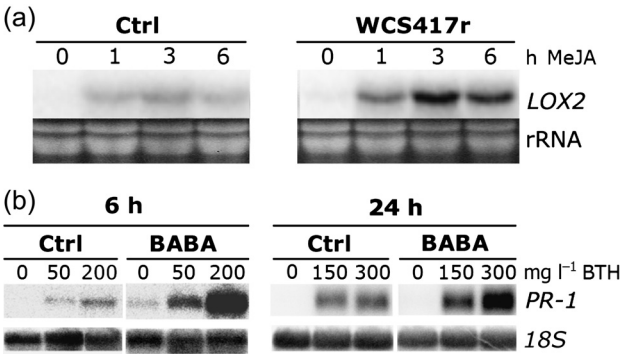

Fig. 5 Priming for augmented induction of defence genes after exogenously applied jasmonic acid (JA) and salicylic acid (SA). (a) WCS417r-induced priming for accelerated induction of the JA-inducible *LOX2* gene. Shoots of 5-wk-old Arabidopsis plants (Col-0) were dipped in a solution containing 50 μM methyl jasmonate (MeJA). Leaf rosettes were harvested at the indicated time points after MeJA treatment. (b) β-Aminobutyric acid (BABA)-induced priming for enhanced transcription of the SA-inducible *PR-1* gene after treatment with the SA analogue benzothiadiazole (BTH). Five-week-old plants (Col-0) were soil-drenched with 250 μM BABA and, 1 d later, sprayed with the indicated concentrations of BTH on the leaves. Leaf rosettes for RNA blot analysis were collected at 6 or 24 h after BTH treatment.

accumulation. In contrast with WCS417r-ISR, BABA-IR against *P. syringae* DC3000 is marked by primed induction of SA-responsive genes after pathogen infection (Zimmerli *et al.*, 2000; Van Hulten *et al.*, 2006). To assess whether BABA-induced priming acts through increased responsiveness to SA, leaves of water- and BABA-treated plants were sprayed with increasing concentrations of the SA analogue BTH and examined for induced transcription of the SA-inducible marker gene *PR-1*. At 6 h after treatment with either 50 or 200 mg l^{-1} BTH, BABA-treated plants showed strongly augmented induction of *PR-1* in comparison with nonprimed control plants (Fig. 5b). At this time point, BABA-treated plants displayed a faint induction of *PR-1* in comparison with control-treated plants. In a separate experiment, similar priming effects were observed at 24 h after treatment with 150 and 300 mg l^{-1} BTH, whereas the direct effect of BABA on *PR-1* expression was no longer detectable (Fig. 5b). Together, these results demonstrate that BABA boosts the plant's responsiveness to SA, suggesting that signal transduction steps downstream of SA are targeted by priming.

WCS417r and BABA influence expression of TF genes systemically

To examine whether transcriptional priming by WCS417r and BABA is based on enhanced expression of TFs, we performed a genome-wide screen for putative WCS417r- and/or BABA-inducible TF genes. RNA was extracted from leaves of control-, WCS417r- and BABA-treated plants and analysed by qRT-PCR using the previously described set of 2248 primer pairs (Czechowski *et al.*, 2004; McGrath *et al.*, 2005; Libault *et al.*, 2007). Samples from water- and BABA-treated *npr1* plants were included in the analysis to distinguish between NPR1-dependent and NPR1-independent priming effects by BABA. The screen identified 90 TF genes with more than two-fold induction after WCS417r treatment, whereas 31 TF genes were more than two-fold repressed by WCS417r (Fig. S1A; Table S1; see Supporting Information). Furthermore, soil drenching of wild-type plants with BABA resulted in more than two-fold induction of 186 TF genes and more than two-fold repression of 44 TF genes, whereas BABA treatment of *npr1* plants resulted in more than two-fold induction of 135 TF genes and more than two-fold repression of 141 TF genes (Fig. S1A; Table S1). Of all putative BABA-inducible genes, only 32 (10%) were induced in both wild-type and *npr1* plants (Fig. S1B), indicating a major role of NPR1 in BABA-induced expression of TF genes. Furthermore, of all the 247 WCS417r- and/or BABA-inducible TF genes, only 27 genes (10.9%) were induced by both WCS417r and BABA. This suggests that WCS417r and BABA target largely different groups of TF genes. Analysis of the 1-kB promoter sequences of the WCS417r- and BABA-inducible TF genes revealed a statistically significant over-representation of G-box and PLGT1-box elements, which are associated with pathogen- and salt stress-responsive genes (Dröge-Laser *et al.*, 1997; Faktor *et al.*, 1997; Boter *et al.*, 2004; Park *et al.*, 2004). Promoters of BABA-inducible TF genes in *npr1* displayed a significantly stronger enrichment in G-box elements than those of BABA-inducible TF genes in the wild-type (Fig. S2, see Supporting Information), suggesting a function of G-box elements in the NPR1-independent induction of TF genes by BABA. Furthermore, the group of BABA-inducible promoters in wild-type plants displayed a statistically significant enrichment of WRKY-binding W-box elements, which was absent in promoters of WCS417r-inducible genes and in the set of BABA-inducible genes in *npr1* (Fig. S2). This suggests a role of WRKYs in the NPR1-dependent activation of TF genes by BABA.

To validate the responsiveness of the putative WCS417r- and BABA-inducible genes, we quantified the expression levels of a selection of 37 TF genes in three biological replicate samples by qRT-PCR (Table S2, see Supporting Information). This selection was based on the genome-wide screen and contained five different categories of TF genes: (1) responsive to BABA in wild-type only (10 genes); (2) responsive to BABA in wild-type and *npr1* (six genes); (3) responsive to WCS417r and BABA in wild-type only (four genes); (4) responsive to both BABA and WCSC417r in wild-type and responsive to BABA in *npr1* (nine genes); and (5) only responsive to WCS417r in wild-type (eight genes). Although the *MYC2/JIN1* gene showed less than two-fold induction by WCS417r in the genome-wide screen, this gene was included as well, as MYC2/JIN1 has been identified recently as critical for WCS417r-ISR (Pozo *et al.*, 2008). Finally, the selection of 37 TF genes was supplemented with seven phytohormone-responsive genes (*PR-1*, *PR-5*, *RAB18*, *PDF1.2*, *LOX2*, *VSP2* and *EBF2*) and four constitutively expressed genes (*GAPDH*, *UBQ10*, At1g13320 and At1g62930) (Czechowski *et al.*, 2005). To test the specificity of the transcriptional response, the ISR-noninducing rhizobacterial strain *P. fluorescens* WCS374r (Van Wees *et al.*, 1997) and the inactive BABA isomer α-aminobutyric acid (Jakab *et al.*, 2001) were included as negative control treatments. In a third experiment, a comparison was made between the BABA responses of wild-type and *npr1* plants. Hierarchical cluster analysis (Fig. 6a) and principal component analysis (Fig. 6b) of the transcriptional profiles clearly distinguished the WCS417r-induced profiles from the control- and WCS374r-induced profiles. Similarly, the transcriptional profiles of BABA-treated plants formed a separate cluster from those of control- and α-aminobutyric acid-treated plants (Fig. 6a,b), whereas the profiles of BABA-treated wild-type plants clustered from those of BABA-treated *npr1* plants and corresponding control treatments (Fig. 6a,b). These results not only validate the outcome of the genome-wide screen, but also illustrate that the transcriptional behaviour of the selected TF genes can mark the onset of WCS417-ISR and BABA-IR.

Identification of a novel promoter element in BABA-inducible *WRKY* TF genes

Various WRKYs have been shown to regulate SA-inducible defence genes (Eulgem & Somssich, 2007). Moreover, some WRKYs are directly regulated by NPR1 (Wang *et al.*, 2006). In this context, WRKYs may play a significant role in the NPR1-dependent priming of SA-inducible defences by BABA. The genome-wide screen for putative BABA-inducible TF genes identified 21 *WRKY* genes that were more than two-fold induced by BABA (Table S1). To verify these results, we quantified all 71 *WRKY* genes for BABA responsiveness in samples from three independent experiments. In wild-type plants, 22 of the 71 *WRKY* genes were, on average, more than two-fold induced by BABA at a statistical significance level of $\alpha < 0.05$ (paired Student's *t*-test or Wilcoxon Mann–Whitney test). By contrast, only three *WRKY* genes, *WRKY30*, *WRKY33* and *WRKY54*, met these criteria in the *npr1* mutant. However, all BABA-responsive *WRKY* genes, except *WRKY54*, showed fold induction values that were more than two-fold reduced by the *npr1* mutation (Fig. 7a; Table S3, see Supporting

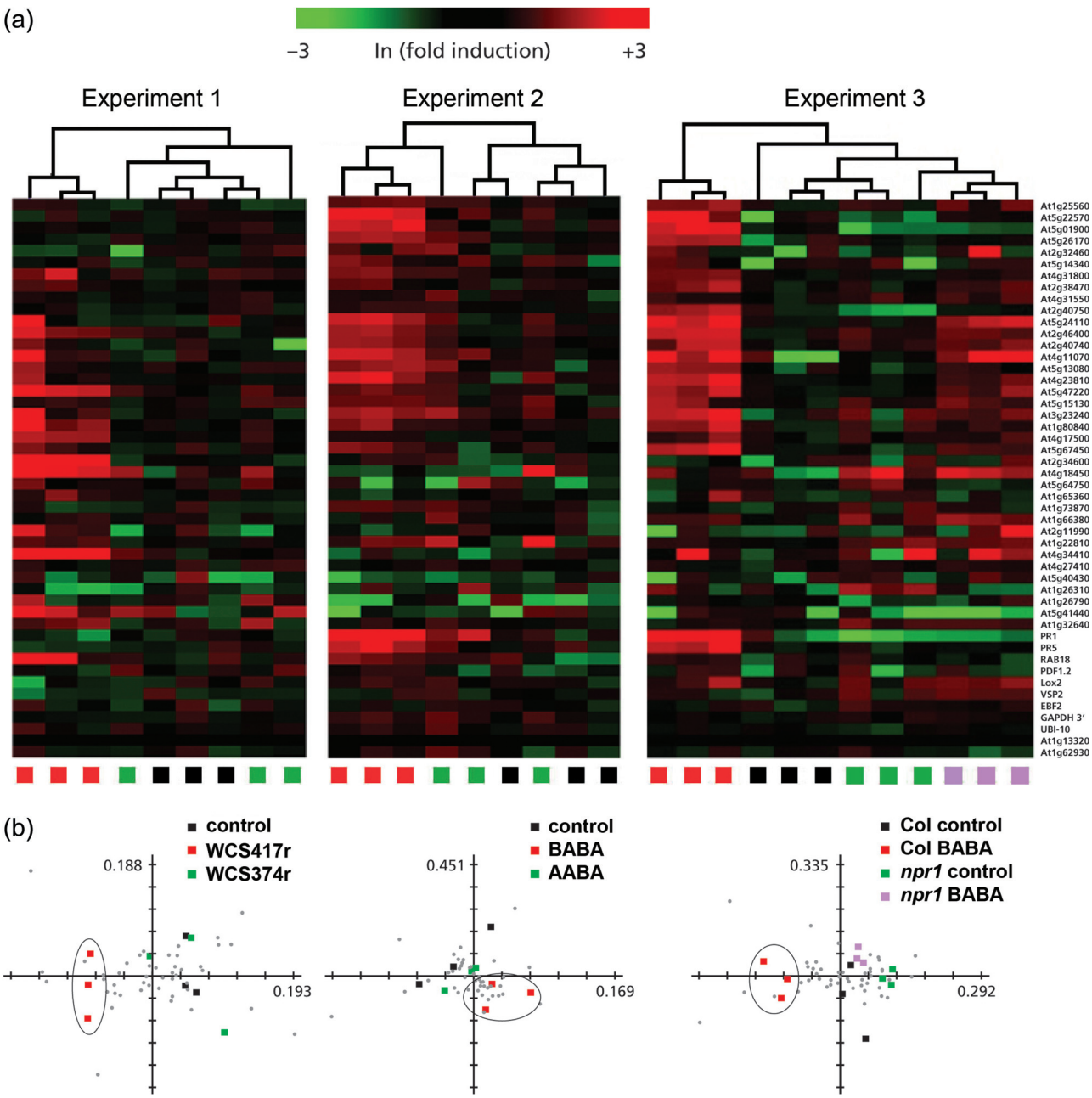

Fig. 6 Systemic expression of selected transcription factor (TF) genes in Arabidopsis after treatment of the roots with WCS417r or β-aminobutyric acid (BABA). Root colonization with the induced systemic resistance (ISR)-noninducing *Pseudomonas fluorescens* strain WCS374r (Experiment 1) and application of the inactive BABA isomer α-aminobutyric acid (Experiment 2) were included as negative control treatments. The *npr1* mutant was included to examine the contribution of NPR1 in BABA-responsive TF gene expression (Experiment 3). (a) Cluster analysis of WCS417r- and BABA-responsive transcription profiles. The colour intensity of induced (red) or repressed (green) genes is proportional to the fold induction values of each gene. Fold induction was defined as the expression value in each replicate sample divided by the mean expression value of the three corresponding control samples (water or $MgSO_4$). Log_e-transformed fold inductions were subjected to average linkage clustering (Euclidean distance). (b) Principal component analysis of WCS417r- and BABA-responsive gene expression. The analysis was based on the log_e-transformed fold induction values, as described above. Grey dots represent genes; coloured squares represent treatments.

Information). Hence, 21 of the 22 BABA-inducible *WRKY* genes require NPR1 for full responsiveness to BABA. To investigate the regulation of these BABA-inducible *WRKY* genes, we analysed their promoter regions for over-representation of DNA elements. Using the Statistical Motif Analysis tool of TAIR, we found a significant over-representation of two nearly identical motifs, TAGTCT and TAGACT (binomial distribution; $P = 7.28e^{-05}$ and

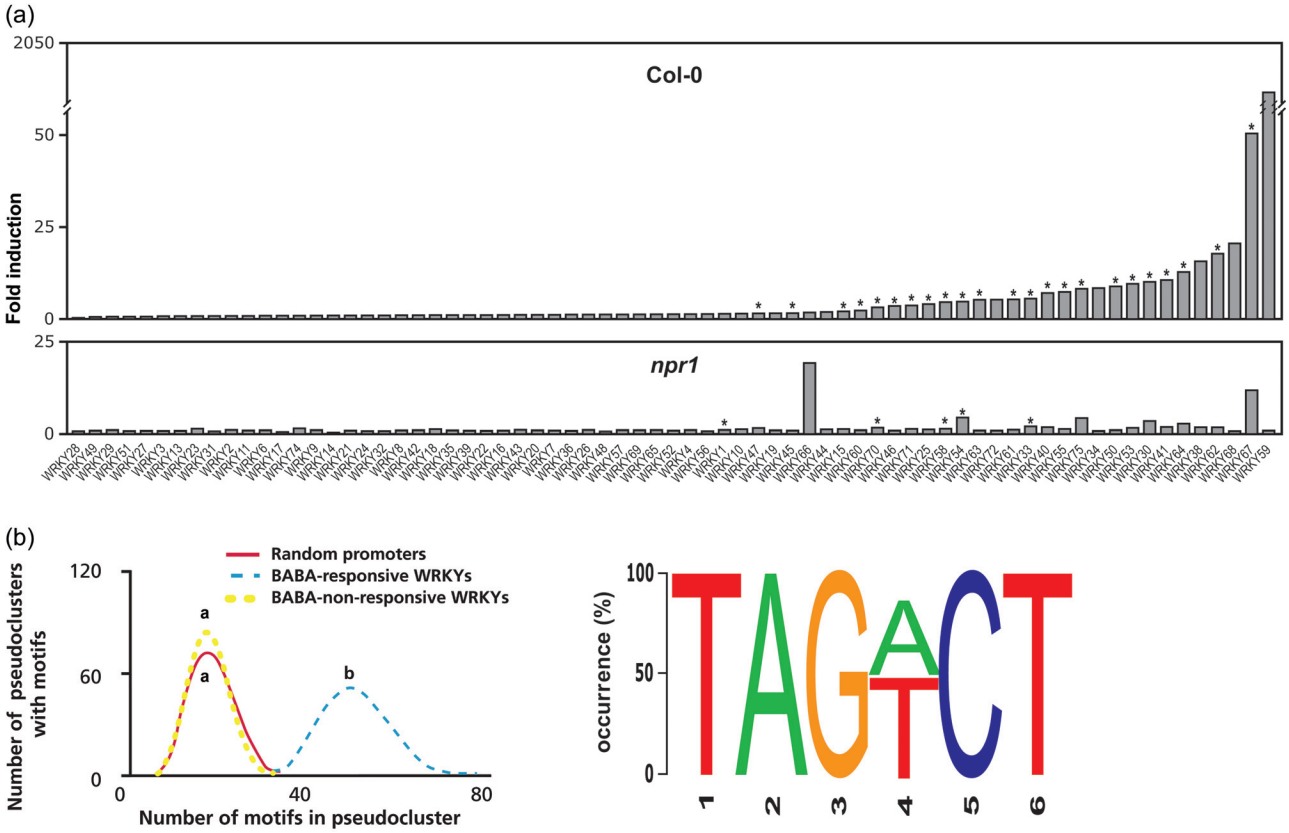

Fig. 7 Identification of a putative *cis*-element in promoter regions of NPR1-dependent, β-aminobutyric acid (BABA)-inducible *WRKY* genes. (a) Fold inductions of 71 Arabidopsis *WRKY* transcription factor (TF) genes in Arabidopsis wild-type (Col-0) and *npr1* plants after treatment of the roots with BABA. Asterisks indicate statistically significant levels of induction based on three independent experiments (paired Student's *t*-test or Wilcoxon Mann–Whitney test; $P < 0.05$). (b) Occurrences of the TAG[TA]CT motif in promoter regions of NPR1-dependent BABA-inducible *WRKY* genes (blue broken line), BABA nonresponsive *WRKY* genes (yellow broken line), and random Arabidopsis promoters (red full line). Different letters indicate statistically significant differences in occurrence of the TAG[TA]CT motif (χ^2 test; $\alpha = 0.05$).

$P = 9.02e^{-05}$, respectively). Subsequent comparison between promoters of BABA-inducible *WRKY* genes, promoters of BABA-noninducible *WRKY* genes and a set of random Arabidopsis promoters revealed clear differences in occurrence. Although TAGTCT and TAGACT were both significantly over-represented in NPR1-dependent BABA-inducible *WRKY* promoters (data not shown), the most contrasting differences were found for the combined TAG[TA]CT motif (Fig. 7b). The fact that this motif is strongly over-represented in NPR1-dependent, BABA-inducible *WRKY* promoters ($\chi^2 = 15.93$; $P < 0.001$), and not in BABA-nonresponsive *WRKY* promoters ($\chi^2 = 0.025$; $P = 0.9$), points to the existence of a TAG[TA]CT-binding factor to regulate NPR1-dependent activation of *WRKY* genes by BABA.

Discussion

WCS417r-ISR and BABA-IR are associated with priming of various pathogen-inducible defence mechanisms (Conrath *et al.*, 2006). As both forms of IR are effective against overlapping spectra of pathogens (Zimmerli *et al.*, 2000; Ton *et al.*, 2002; Ton & Mauch-Mani, 2004), it is plausible to assume that WCS417r bacteria and BABA prime at least partially similar defence reactions. In this study, we investigated the similarities and differences between WCS417r- and BABA-induced priming. A clear difference between the two priming responses is that they target distinct classes of defence-related genes. WCS417r bacteria prime the induction of JA-dependent genes, whereas BABA primes the induction of SA-dependent genes (Verhagen *et al.*, 2004; Ton *et al.*, 2005; Pozo *et al.*, 2008) (Fig. 5). However, both WCS417r and BABA mediate the augmented deposition of callose-containing papillae after infection by the oomycete pathogen *H. arabidopsis* WACO9 (Figs 1–3). In both cases, priming is impaired in *ibs2* and *ibs3* mutants (Fig. 2), indicating that WCS417r-ISR and BABA-IR against *H. arabidopsis* share similar ABA- and phosphoinositide-dependent signalling components (Fig. 8). Mutants *ibs2* and *ibs3* are not affected in expression of

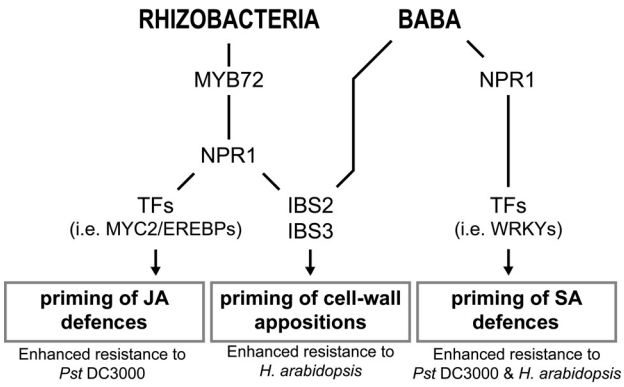

Fig. 8 Differences and similarities in the regulation of WCS417r- and β-aminobutyric acid (BABA)-induced priming of defence. Root colonization by the rhizobacterial strain *Pseudomonas fluorescens* WCS417r triggers induced systemic resistance (ISR) against the bacterial pathogen *Pseudomonas syringae* DC3000 and oomycete pathogen *Hyaloperonospora arabidopsis* WACO9. This ISR depends on MYB72- and NPR1-dependent signalling pathways. WCS417r bacteria enhance the systemic expression of regulatory transcription factors (TFs) in the jasmonic acid (JA) response, such as *MYC2* and *ERF/EREBP*, which leads to primed expression of JA-dependent defences after infection by *P. syringae* DC3000. In addition, WCS417r bacteria trigger IBS2- and IBS3-dependent priming of cell wall defence, which leads to an augmented deposition of callose-rich papillae after infection by *H. arabidopsis*. Root treatment with BABA also induces resistance against *P. syringae* and *H. arabidopsis*. BABA-induced resistance (IR) against *P. syringae* DC3000 depends on salicylic acid (SA)- and NPR1-dependent signalling pathways. Downstream of NPR1, the BABA-induced resistance pathway leads to the enhanced expression of regulatory TFs in the SA response, such as WRKYs, which mediate the primed activation of SA-dependent defences on infection by *P. syringae* DC3000 and *H. arabidopsis*. In addition, BABA triggers IBS2- and IBS3-dependent priming for augmented deposition of papillae after infection by *H. arabidopsis*. This BABA-induced priming of cell wall defence functions independently of NPR1.

WCS417r-mediated ISR and BABA-IR against the bacterial pathogen *P. syringae* DC3000 (Fig. 4). Hence, primed defence against *P. syringae* requires different signalling mechanisms than primed defence against the oomycete *H. arabidopsis*. This dissimilarity could be related to differences in infection strategy by the two pathogens. For instance, *P. syringae* bacteria enter leaves through natural openings (Melotto *et al.*, 2006), and are therefore less restricted by cell wall barriers, whereas *H. arabidopsis* hyphae actively penetrate the epidermal cell layer (Slusarenko & Schlaich, 2003).

As WCS417r and BABA prime the transcriptional induction of JA- and SA-inducible genes, respectively (Fig. 5), we examined whether this enhanced transcriptional capacity is related to a direct increase in TFs. To this end, we screened the level of expression of nearly all TF genes in the Arabidopsis genome after priming treatment with WCS417r or BABA. In contrast with earlier findings reported by Verhagen *et al.* (2004), we found consistent effects by WCS417r on TF gene expression in leaves (Figs 6, 7, S1). This discrepancy can be explained by the different methodologies used to quantify gene expression. Verhagen *et al.* (2004) used microarrays to quantify gene expression, whereas the transcriptional profiling in this study was based on qRT-PCR. This technique is substantially more sensitive than hybridization-based techniques and, consequently, more reliable for the detection of low-abundant mRNAs that are characteristic for the expression of TF genes (Czechowski *et al.*, 2004).

Many of the priming-related TF genes in this study have previously been reported to be responsive to pathogen attack, during which they promote defence gene expression directly (McGrath *et al.*, 2005; Wang *et al.*, 2006). However, the reported fold induction values on pathogen infection are typically an order of magnitude higher than those observed in our study on priming treatment (Tables S1–S3). This difference supports our model, which predicts that priming causes a subtle increase in the expression of defence regulatory TFs that is sufficient to prime defence genes, but too weak to strongly activate them directly. Conversely, a relatively strong induction of TFs during pathogen infection is consistent with direct activation of defence genes. It is, nevertheless, plausible that pathogen-induced TF expression also contributes to a longer lasting priming of defence genes after the invading pathogen has been constrained. As most defence regulatory TFs act as amplifiers in defence signalling cascades, even a modest induction during priming can be sufficient to enhance the defence signalling capacity, thereby giving the primed plant a 'head start' during the early stages of pathogen infection.

Our screen for priming-related TF genes revealed that WCS417r and BABA influence largely different sets of TF genes (Figs 6, 7, S1). In combination with our previous findings that WCS417r and BABA prime for different sets of defence-related genes (Verhagen *et al.*, 2004; Ton *et al.*, 2005), we propose that WCS417r-induced TF genes contribute to the priming of JA-inducible genes, whereas BABA-induced TF genes contribute to the priming of SA-inducible genes (Fig. 8). In support of this, WCS417r bacteria induce TF genes that have been related to the regulation of JA- and ET-dependent defence reactions, such as *AP2/EREBP* genes (Table S1). Amongst these, the *ERF1* gene encodes a key regulator in the integration of JA- and ET-dependent signalling pathways (Lorenzo *et al.*, 2003). In addition to ERF1, JA-induced expression of defence genes is co-regulated by the MYC2 TF (Lorenzo *et al.*, 2004). Interestingly, also, the *MYC2* gene is weakly, yet consistently, induced by WCS417r (Fig. 6a; Table S2). With respect to the BABA-inducible group of TF genes, the majority of genes are substantially less responsive to BABA in the *npr1* mutant, indicating that NPR1 plays an important role in BABA-induced expression of TF genes (Figs 6, 7). These NPR1-dependent, BABA-inducible TF genes include 21 members of the *WRKY* family. Some of these, such as *WRKY38*, *WRKY53*, *WRKY58* and *WRKY70*, play a role in the fine-tuning of SA-inducible defences and are directly

targeted by NPR1 (Eulgem, 2005; Wang *et al.*, 2005). Therefore, we conclude that NPR1 plays an important role in the onset of BABA-induced priming of SA-inducible defences (Fig. 8).

It is tempting to speculate that the relatively small number of TFs that are induced by both WCS417r and BABA regulate defence mechanisms that are primed by both treatments, such as the expression of callose-rich papillae. Priming of this cell wall defence is dependent on IBS2 and IBS3, regardless of the nature of the inducing priming agent (Fig. 2). Yet, this priming is regulated differently at the level of NPR1 and MYB72. Our bioassays indicated that NPR1 is necessary for WCS417r-induced priming of callose, whereas it is dispensable for BABA-induced priming of callose (Figs 1, 3). Similarly, MYB72 is important for WCS417r-induced callose priming, whereas it has no contribution to BABA-induced callose priming (Fig. 3). As MYB72 is thought to operate at a relatively early stage in the WCS417r-induced signalling pathway (Van der Ent *et al.*, 2008), we propose that MYB72 and NPR1 act upstream of IBS2 and IBS3 in the WCS417r pathway of cell wall defence (Fig. 8).

The role of NPR1 in the priming signalling network is complex. Our data suggest that NPR1 not only controls WCS417r-induced priming of cell wall defence, but also regulates WCS417r-ISR against *P. syringae* DC3000, which is based on priming of JA-dependent defences (Verhagen *et al.*, 2004; Pozo *et al.*, 2008). In addition, NPR1 is necessary for BABA-IR against different pathogens (Zimmerli *et al.*, 2000, 2001), and plays a major role in BABA-induced expression of TF genes (Figs 6, 7, S1). Finally, NPR1 contributes to SAR-related priming of pathogen-induced expression of the *PHENYL AMMONIA LYASE* gene (Kohler *et al.*, 2002). Hence, the function of NPR1 in priming depends on the signalling pathway that is activated upstream (Fig. 8). In this context, it would be interesting to investigate the role of NPR1-interacting signalling proteins, such as TGA TFs (Dong, 2004). Another potentially interesting component is the SNI1 protein, which functions as a negative regulator of NPR1-dependent gene expression. Interestingly, SNI1 has been reported to influence chromatin structure around the SA-inducible *PR-1* promoter (Mosher *et al.*, 2006). Therefore, it is tempting to speculate that priming antagonizes SNI1-dependent chromatin remodelling, which would enhance the TF-binding capacity to the gene promoters and successively facilitate a faster and stronger transcriptional induction of NPR1-dependent defence genes after pathogen attack.

The direct effects of WCS417r and BABA on TF gene expression may point to earlier signalling steps in the priming of defence genes. These transcriptional activations are controlled by other TFs, whose activity is not necessarily regulated at the transcriptional level. Such 'early-acting' TFs in the pathway may function as key regulators in the onset of priming. In a first step in the identification of such factors, we analysed the promoter regions of WCS417r- and BABA-inducible TFs for the over-representation of common *cis*-acting elements. The promoter regions of both WCS417r- and BABA-inducible TF genes were significantly enriched in G- and PLGT1-boxes (Fig. S2). Both elements have been related to transcriptional responses to pathogen infection, salt stress, JA and ABA (Dröge-Laser *et al.*, 1997; Faktor *et al.*, 1997; Boter *et al.*, 2004; Park *et al.*, 2004). Conversely, we found a statistically significant over-representation of W-boxes in BABA-inducible genes in the wild-type, but not in the *npr1* mutant (Fig. S2). This result points to the involvement of WRKYs in the NPR1-dependent induction of TF genes by BABA. In support of this, we identified 21 *WRKY* genes that were directly induced by BABA in wild-type plants, but not, or significantly less, in the *npr1* mutant (Fig. 7a). Promoter analysis of the 21 NPR1-dependent *WRKY* genes revealed a significant over-representation of an unknown promoter element (Fig. 7b). We hypothesize that this TAG[TA]CT element functions as an important *cis*-acting element in the NPR1-dependent activation of TF genes by BABA. Future studies will focus on the identification of TF proteins that bind to the TAG[TA]CT element, aiming at the discovery of a novel key regulator in priming of SA-inducible defence mechanisms.

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Supporting Information

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

Fig. S1 Genome-wide screen for putative WCS417r- and β-aminobutyric acid (BABA)-responsive transcription factor (TF) genes in Arabidopsis wild-type (Col-0) and *npr1* plants.

Fig. S2 Occurrences of *cis*-acting elements in the promoter regions of WCS417r- and β-aminobutyric acid (BABA)-inducible transcription factor (TF) genes in Arabidopsis wild-type (Col-0) and *npr1* plants.

Notes S1 Experimental details of quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

Table S1 Genome-wide screen for putative WCS417r- and β-aminobutyric acid (BABA)-responsive transcription factor (TF) genes

Table S2 Fold induction values of a selection of 37 transcription factor (TF) genes in three biological replicate samples on treatment of the roots with WCS417r or β-aminobutyric acid (BABA) in leaves of Arabidopsis wild-type (Col-0) or *npr1* plants

Table S3 Fold induction values of 71 *WRKY* genes in three independent experiments on treatment of the roots with β-aminobutyric acid (BABA) in leaves of Arabidopsis wild-type (Col-0) or *npr1* plants

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