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Transcription factor ANAC032 modulates JA/SA signalling in response to *Pseudomonas syringae* infection

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Abstract

Responses to pathogens, including host transcriptional reprogramming, require partially antagonistic signalling pathways dependent on the phytohormones salicylic (SA) and jasmonic (JA) acids. However, upstream factors modulating the interplay of these pathways are not well characterized. Here, we identify the transcription factor ANAC032 from *Arabidopsis thaliana* as one such regulator in response to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). ANAC032 directly represses *MYC2* activation upon *Pst* attack, resulting in blockage of coronatine-mediated stomatal reopening which restricts entry of bacteria into plant tissue. Furthermore, ANAC032 activates SA signalling by repressing *NIMIN1*, a key negative regulator of SA-dependent defence. Finally, ANAC032 reduces expression of JA-responsive genes, including *PDF1.2A*. Thus, ANAC032 enhances resistance to *Pst* by generating an orchestrated transcriptional output towards key SA- and JA-signalling genes coordinated through direct binding of ANAC032 to the *MYC2*, *NIMIN1* and *PDF1.2A* promoters.

Keywords *Arabidopsis*; jasmonic acid; pathogens; salicylic acid; transcription factor

Subject Categories Microbiology, Virology & Host Pathogen Interaction; Plant Biology; Transcription

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Introduction

During evolution, both plants and pathogens have evolved complex strategies to overcome virulence or defence mechanisms. The final outcome of a plant–pathogen interaction depends on the competence of the host to recognize the pathogen and trigger an appropriate, rapid defence response, as well as on the ability of the pathogen to mask its attack and activate suppression of the plant's innate immune system. Fine-tuning of hormone signalling plays an essential role for governing the defence response of plants against pathogen challenge. The phytohormone salicylic acid (SA) is crucial for the defence against (hemi)biotrophic pathogens such as *Pseudomonas syringae*, while jasmonic acid (JA) and/or ethylene (ET) function as key signalling molecules in response to necrotrophic pathogens or insects [1–3]. Prioritizing one hormonal signalling pathway over the other in dependence of the pathogen encountered is crucial for attaining specificity and success of the overall plant's defence response [2].

Interestingly, as a counter strategy, pathogens manipulate phytohormone homeostasis or produce toxins that interfere with plant defence mechanisms [4–7]. For example, *Pseudomonas syringae*, a foliar bacterial pathogen, produces the phytotoxin coronatine (COR), which is structurally similar to bioactive jasmonate, that is JA-isoleucine (JA-Ile) [8,9]. COR functionally mimics JA and promotes susceptibility of the host by manipulating its defence hormone signalling [4,8–14]. The role of COR in combating the pathogen-associated molecular pattern (PAMP)-induced stomatal defence received much attention in recent years [15–21] and led to the identification of a COR-induced signalling cascade that involves the SCF^{COI1} (Skp1-Cdc53-F-box protein, CORONATINE1) ubiquitin-ligase complex, jasmonate ZIM domain (JAZ) repressor proteins, MYC2 (a bHLH domain-containing transcription factor) and NAC (NAM, ATAF and CUC) transcription factors (TFs) that jointly represent essential components in the suppression of the plant immune response by *Pst* [22]. Interestingly, the *myc2* mutant exhibits enhanced resistance to *Pst* with increased expression of *PATHOGENESIS-RELATED* (*PR*) genes and SA accumulation when compared to wild-type plants giving MYC2 an important position in the JA-SA antagonistic interplay [23,24]. Several reports clearly support the mutually antagonistic relation between the SA and JA pathways either mounted by plants as a defence response or manipulated by microbes for their own survival [25,26].

Activation of the plant's defence response involves complex transcriptional reprogramming. The central modulator of systemic acquired resistance (SAR), NPR1 (NONEXPRESSOR of PR GENE1), was identified as a key signalling node in the regulation of the SA-JA interplay [27,28]. By interacting with bZIP family TGA TFs, NPR1

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induces the expression of *PR* genes required for SAR establishment [29–33]. Moreover, interaction with TGA TFs was shown to be necessary for suppression of the JA-responsive gene *PDF1.2* (*PLANT DEFENSIN1.2*) by GRX480 (GLUTAREDOXIN480), an SA-responsive glutaredoxin gene whose SA inducibility itself is NPR1-dependent [34]. Additionally, NIMIN (NIM1 INTERACTING) proteins were identified to differentially interact with NPR1 and affect *PR1* gene expression at different stages of SAR, thus representing negative regulators of SAR [35,36]. Recently, a JA-responsive GCC-box motif was found to be enriched in the promoters of JA-responsive genes that are suppressed by SA [37]. Furthermore, COR-mediated MYC2-dependent activation of three NAC family TFs, namely *ANAC019*, *ANAC055* and *ANAC072*, and their tomato homologs *JA2* (*JASMONIC ACID2*) and *JA2L* (*JASMONIC ACID2 LIKE*), were reported to promote bacterial propagation by inhibiting SA accumulation [22,38]. Despite the identification of several components involved in plant defence responses, the molecular mechanisms involved in fine-tuning the hormonal pathways still remain largely unexplored. Here, we identified a novel TF of the NAC family, ANAC032, as a positive regulator of the defence response against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). ANAC032 promotes pathogen-induced defence responses by activating SA, but repressing JA signalling including COR-mediated stomatal reopening. ANAC032 acts upstream of the *MYC2-ANAC019/55/72* transcriptional cascade and also regulates the expression of the key pathogen-response genes *NIMIN1* and *PDF1.2A* by binding to their promoters. Our study provides new insights into the molecular mechanisms that underlie the antagonistic interaction between SA and JA signalling in response to *Pst*.

Results

Expression of *ANAC032* is induced by various defence signals

Pathogen-induced global transcriptome analysis and functional genomic studies have identified several members of NAC TF family as key components in transcriptional regulation of gene expression during pathogen attack [39]. We identified ANAC032 (AT1G77450), an uncharacterized member of the ATAF clade of the *Arabidopsis* NAC family, as a plausible candidate involved in the regulation of the plant's response to pathogens. Members of this clade exhibit strong expression responses to various types of stresses including those induced by pathogen attack. *ANAC032* expression is induced upon infection with the bacterial pathogens *P. syringae* pv. *tomato* DC3000 (*Pst*), *Pst* avrRPM1, *Pst* avrRpt2, *P. syringae* pv. *Maculicola* ES4326 (*Psm* ES4326) and with the leaf fungus *Alternaria brassicicola* (Genevestigator) which suggests an active role of the ANAC032 transcription factor in plant immune responses.

To confirm the induction of *ANAC032* by *Pst*, we monitored its expression in *Arabidopsis* Col-0 (wild type; WT) challenged by surface inoculation with *Pst* (1×10^8 c.f.u. ml^{-1}). *ANAC032* expression was highly induced 6 h post-inoculation (hpi) and showed a moderate increase at 24 hpi, suggesting that this NAC TF preferentially functions during the early pathogen-response phase (Fig 1A). As *Pst* can simultaneously trigger synthesis of both SA and JA [27], we next examined the effect of the two hormones on *ANAC032* expression. Treatment with methyl jasmonate (MeJA) and SA rapidly (3 h) induced *ANAC032* expression (Fig 1B). Ethylene has been shown to act synergistically with JA in response to pathogens and that it induces the expression of *ANAC032* in cooperation with JA [40]. Finally, we tested the effect of coronatine (COR) on *ANAC032* expression. COR, a phytotoxin produced by various strains of *Pseudomonas syringae* [8], is structurally similar to JA-isoleucine (JA-Ile) and can activate JA signalling [4,8–12]. *ANAC032* expression was induced in WT seedlings within 3 h and 6 h upon treatment with COR (Fig 1C). Taken together, these observations support a role for ANAC032 in response of *Arabidopsis* to pathogen (*Pst*) attack.

ANAC032 promotes plant disease resistance against *P. syringae* pv. *tomato* DC3000

To elucidate the possible involvement of ANAC032 in plant defence, we tested transgenic *Arabidopsis* plants with modified *ANAC032*

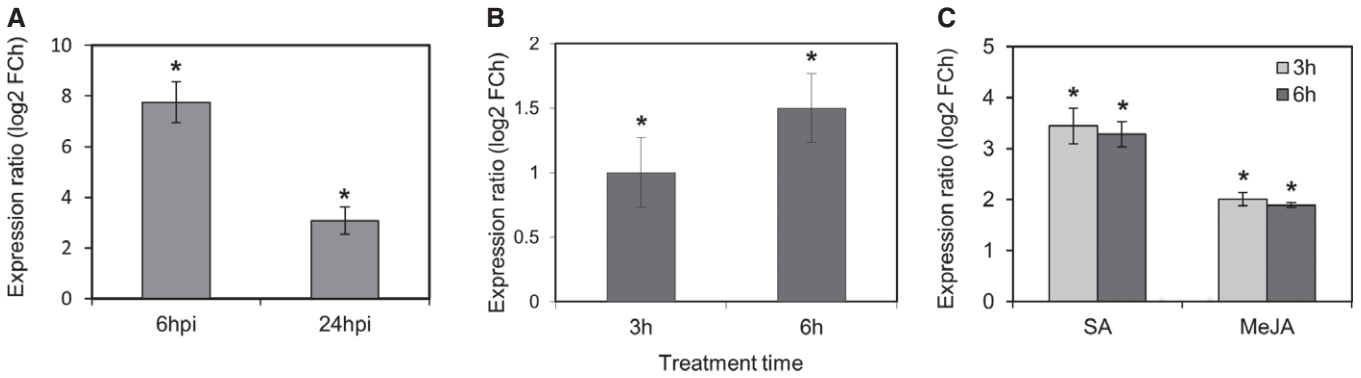

Figure 1. Enhanced expression of *ANAC032* by *Pst* DC3000-derived signals.

A *ANAC032* expression in WT plants sprayed with *Pst* DC3000, 6 and 24 hpi, compared to control (sprayed with 10 mM $MgCl_2$ (mock)). Bars represent means ± SD (n = 3 independently performed experiments, each including the rosette leaves of at least three plants grown in individual pots).

B, C *ANAC032* expression in WT treated with (B) SA or MeJA or (C) COR for 3 and 6 h compared to non-treated controls. Means ± SD are given (n = 3 independently performed experiments, each including at least 20 seedlings).

Data information: FCh, fold change. Asterisks indicate a significant difference from their respective controls (*$P < 0.01$; Student's t-test).

expression levels for their response to *Pst* infection. This we first tested by pressure-infiltrating leaves of *ANAC032* overexpressors (hereafter, *35S:ANAC032*) and *anac032-1* (a T-DNA insertion knockout mutant, SALK_012253; Fig EV1) with *Pst* (1×10^6 c.f.u. ml^{-1}). While *anac032-1* plants showed increased susceptibility towards *Pst* compared to WT, *35S:ANAC032* plants exhibited a strongly enhanced disease resistance (Fig 2A). We also observed greater *Pst* proliferation 3 days postinoculation (dpi) in *anac032-1* compared to WT (Fig 2B), while in *35S:ANAC032* plants, the bacterial titre was less compared to WT, suggesting that ANAC032 restricts bacterial growth. Since pressure infiltration bypasses the normal mode of pathogen entry through natural openings (stomata), we next sprayed plants with inocula containing *Pst* (1×10^8 c.f.u. ml^{-1}). While severe chlorotic lesions occurred in *anac032-1* (Fig 2C), *35S:ANAC032* plants showed only minimal disease symptoms 4–5 dpi (Fig 2C and D).

To verify that the observed phenotypes of *anac032-1* plants resulted from a loss of ANAC032 function, we generated complementation lines of the mutant (hereafter *comp*) (Fig EV1D). To this end, the *ANAC032* coding sequence was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter and introduced into *anac032-1* plants. Upon challenge with *Pst* (both by pressure infiltration and by surface inoculation), *comp* plants clearly displayed a recovery from the phenotype of *anac032-1* plants with reduced progression of disease symptoms and bacterial load compared to WT (Fig EV2), proving that the lack of ANAC032 function is responsible for the phenotypes observed. Taken together, our data clearly indicate that ANAC032 functions as a positive regulator of the plant defence response against *Pst*.

ANAC032 activates the SA-mediated defence response, but antagonizes JA-responsive gene expression

To elucidate the molecular mechanisms underlying ANAC032-mediated defence, we tested the expression of 123 defence-/stress-related genes and 15 genes encoding UDP-glucosyl transferases (UGTs) in 6-week-old *ANAC032* transgenic and WT plants after *Pst* infection (at 6 hpi) by qRT–PCR (quantitative real-time polymerase chain reaction). Genes included in the platform (Table EV1) were extracted from reports on previous studies on plant defence responses against invading pathogens, in particular *Pseudomonas syringae* [25,26,37,41,42]. All genes chosen are induced by *Pst* infection. Our data revealed that 37 genes, including JA and SA defence responsive genes, biotic stress-induced TFs, and several UGTs, were differentially expressed after *Pst* treatment in *ANAC032* transgenic plants compared to the WT (Fig 2E). Upon *Pst* treatment, expression of 12 *UGTs* (*UGT85A1*, *UGT73C1*, *UGT73B3*, *UGT84A3*, *UGT76E11*, *UGT76B1*, *UGT74F2*, *UGT74D1*, *UGT84A2*, *UGT75B1*, *UGT72B1* and *At2g36770)* was significantly reduced in *anac032-1* plants compared to WT, whereas their transcript levels were enhanced in *35S:ANAC032* plants. Among the JA-related genes, expression of *JAZ3*, *JAZ7*, *JAZ8* and *JAZ10,* which encode JAZ proteins, was downregulated in *anac032-1,* but upregulated in *35S:ANAC032* upon *Pst* treatment (compared to WT). In contrast, expression of *MYC2*, a key transcription factor in JA signalling [43–45], was upregulated in *anac032-1,* but downregulated in *35S:ANAC032* plants upon *Pst* treatment (compared to WT). Expression of other JA-responsive genes including *PDF1.2A*, *THI2.1*, *VSP1* and *VSP2* was also upregulated in *anac032-1*, but downregulated in *35S:ANAC032* plants 6 h after *Pst* treatment (Fig 2E). JAZ proteins act as repressors of JA signalling under basal conditions, while the accumulation of JA leads to the release of their repressive effect, for example on JA activator TFs such as *MYC2*, which results in induced JA responses [44–48]. In addition to the negative regulation of JA signalling, JAZ10 was reported to attenuate the development of disease symptoms upon *Pst* infection [49]. The elevated expression of *MYC2* and other JA-responsive genes in *anac032-1* suggests a role for ANAC032 as a negative regulator of JA signalling in response to *Pst* attack.

Among the SA-related genes, significant downregulation in the *anac032-1* mutant was observed for *PAD4* (*PHYTOALEXIN DEFICIENT4*) and *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY1*), both of which are involved in SA accumulation [50–52]. Transcript levels of these genes were increased in *35S:ANAC032* plants compared to WT upon *Pst* treatment. In contrast, the transcript level of *NIMIN1*, a central but negative regulator of SA-dependent pathogen defence [35,36], was significantly increased in *anac032-1* but reduced in *35S:ANAC032* plants compared to WT. Consistent with this observation, the transcript level of *PR1*, the SA-dependent defence marker gene negatively affected by NIMIN1 [36], was significantly reduced in *anac032-1* and increased in *35S:ANAC032* compared to WT upon *Pst* treatment (Fig 2E). Activation of SA-responsive defence genes and the concomitant suppression of JA-responsive genes suggest that ANAC032 modulates the interplay between SA- and JA-dependent defence signalling in response to *Pst*.

ANAC032 directly regulates *MYC2*, *PDF1.2A* and *NIMIN1* expression *in vivo*

To further elucidate the regulatory function of ANAC032 in the SA/JA interplay and defence against *Pst*, we aimed to identify genes directly regulated by ANAC032. To this end, we first identified *cis*-elements recognized by ANAC032 (ANAC032 binding sites), using an *in vitro* binding site selection experiment (BSSE) employing the CELD-fusion method [53]. *In vitro* binding site selection revealed two consensus sequences bound by ANAC032, that is RgWann-CAAnnnnnnYACGnMWCY (24 bp) and RgWKnCGTRnnnnnnYACG tMWcY (23 bp). The nucleotides underlined are fully conserved in all 18 and 8, respectively, sequenced clones suggesting that ANAC032 binds to a bipartite *cis*-regulatory site with two core motifs separated by a flexible linker region (Table EV2). Mutation analysis identified several nucleotides essential for high binding affinity (Table EV3).

We then analysed the promoters of genes expressed differentially between *ANAC032* transgenic and WT plants upon *Pst* treatment and identified *NIMIN1*, *MYC2* and *PDF1.2A* harbouring ANAC032 binding sites in their 500-bp upstream promoter regions as putative direct targets of ANAC032. All three genes showed reduced and increased expression, respectively, in *35S:ANAC032* and *anac032-1* plants (Fig 3A), suggesting that they are directly and negatively controlled by ANAC032.

We next employed electrophoretic mobility shift assays (EMSAs) to test for physical interaction of ANAC032 with the respective promoter sequences of *MYC2*, *PDF1.2A* and *NIMIN1*. ANAC032 protein fused to glutathione S-transferase (GST) was incubated with 5′-DY-682-labelled 40-bp double-stranded DNA fragments containing the ANAC032 binding sites (Table EV1). As evident from the

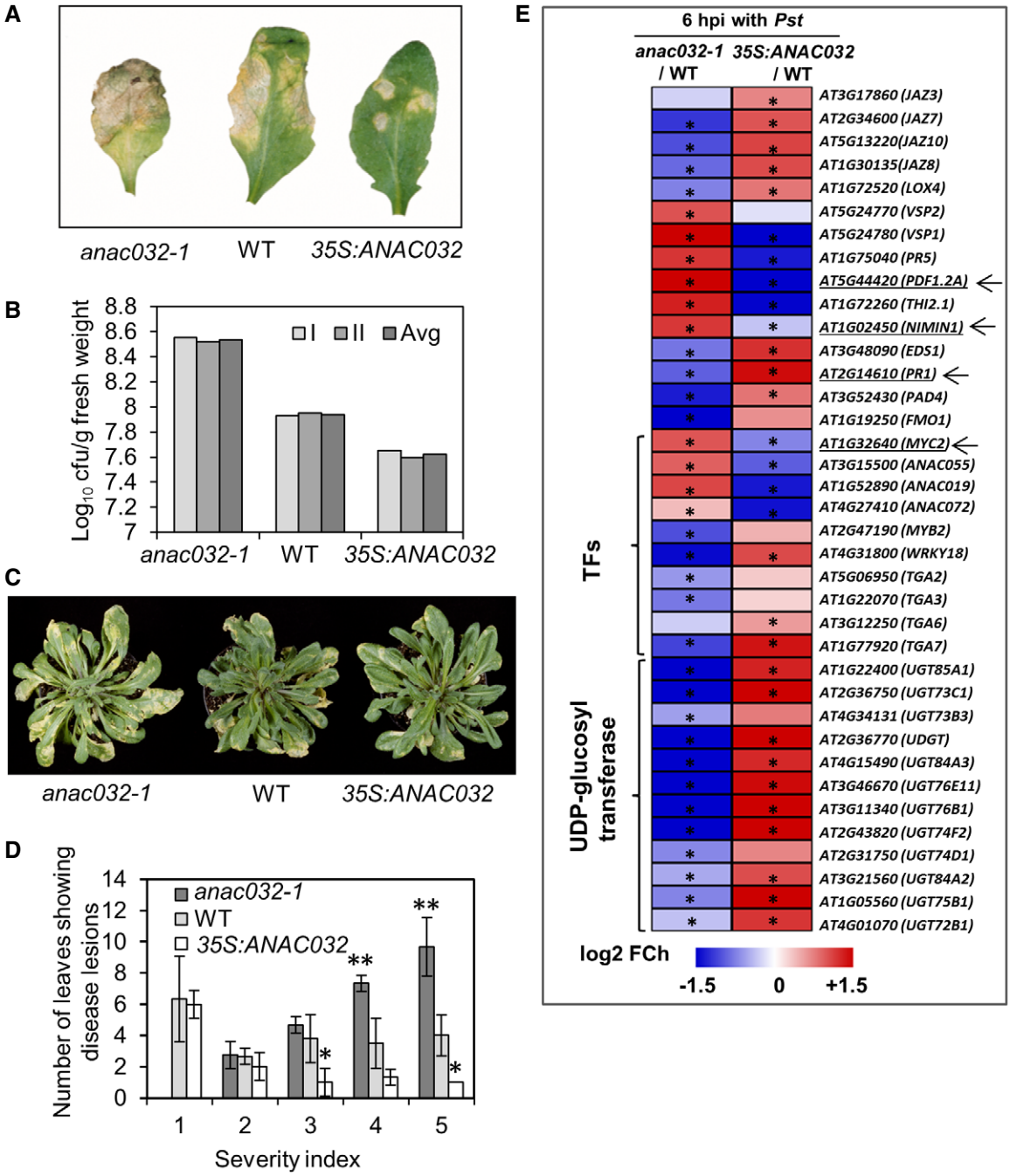

Figure 2. Role of *ANAC032* in resistance against *Pst* DC3000.

A Plants after pressure infiltration with *Pst* DC3000, at 4 dpi. The experiment was repeated three times (each time 9–12 plants) with similar results.

B *Pst* DC3000 growth in *ANAC032* transgenics and WT plants 3 dpi after pressure infiltration. Two independent experiments were performed with three replications per experiment, each replicate consisting of three plants grown in individual pots (six measurements in total). The graph shows data points of the two individual experiments (I and II) along with their mean (Avg).

C Disease status of plants sprayed with *Pst* DC3000, 5 dpi. The experiment was repeated four times with similar results.

D Disease severity index (1, small chlorotic lesions; 5, large lesions) scored 5 dpi after spraying with *Pst* DC3000. Data are from three independent experiments with at least six plants per genotype in each. Means ± SD are shown. Asterisks indicate significant (*$P < 0.05$ and **$P < 0.005$) differences between transgenic and WT plants in chi-square test analysis.

E Heat map showing the fold change (log2 basis) in the expression ratio of defence-/stress-related differentially expressed genes in *anac032-1 and 35S:ANAC032* compared to WT after spraying with *Pst* DC3000 (6 hpi) normalized to their respective controls. Blue, downregulated; red, upregulated. Data represent means of three independent experiments, each including the rosettes leaves of at least three plants grown in individual pots. Asterisks indicate significant differences from WT plants (Student's *t*-test, $P \leq 0.05$).

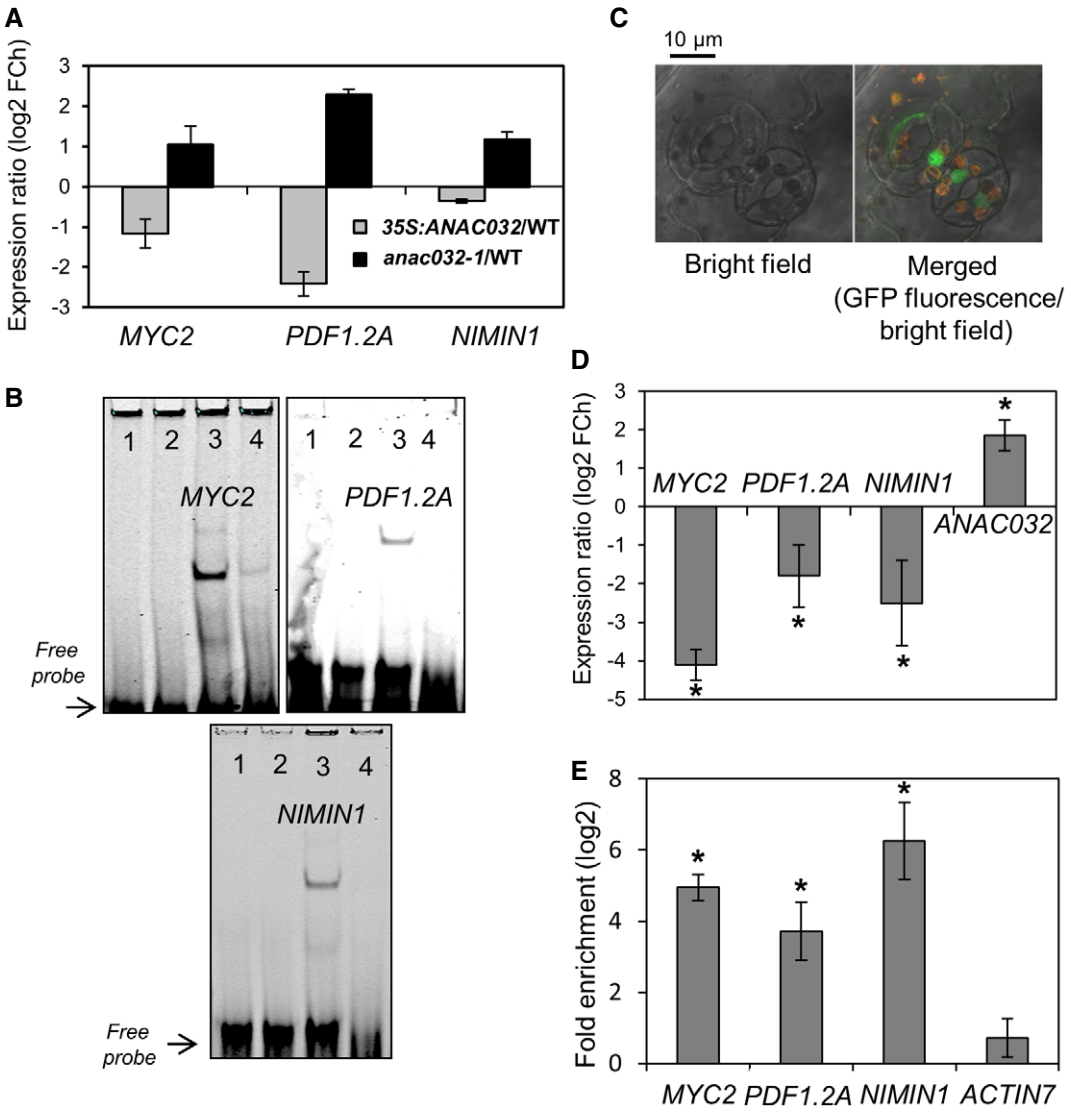

Figure 3. ANAC032 directly regulates *MYC2*, *PDF1.2A* and *NIMIN1*.

A *MYC2*, *PDF1.2A* and *NIMIN1* expressions in *ANAC032* transgenics compared to WT after spraying with *Pst* DC3000 (24 hpi) normalized to their respective controls. FCh, fold change. Means ± SD (n = 3 independently performed experiments, each including the rosette leaves of at least three plants grown in individual pots).

B EMSA showing binding of ANAC032 to *MYC2*, *PDF1.2A* and *NIMIN1* promoter regions (in 5′-DY682-labelled double-stranded oligonucleotides) harbouring ANAC032 binding sites. 1, labelled probe only; 2, labelled probe plus GST protein; 3, labelled probe plus ANAC032-GST protein; 4, labelled probe, ANAC032-GST protein and 100× competitor (unlabelled oligonucleotide containing ANAC032 binding site).

C Confocal microscope image showing nuclear localization of ANAC032-GFP fusion protein expressed from the *ANAC032* promoter in *ANAC032*$_{prom}$*:ANAC032-GFP/ anac032-1* seedlings treated with *Pst* at 6 hpi. Left, bright field; right, chlorophyll auto-fluorescence (red) and GFP fluorescence (green) under bright field.

D Expression of *MYC2*, *PDF1.2A*, *NIMIN1* and *ANAC032* in 5-week-old *ANAC032*$_{prom}$*:ANAC032-GFP/anac032-1* plants compared to WT at 6 hpi with *Pst*, normalized to their respective controls. FCh, fold change. Means ± SD (n = 3 independently performed experiments, each including the rosette leaves of at least three plants grown in individual pots). Asterisks indicate a significant difference from WT, normalized to their respective controls (*P < 0.05; Student's t-test).

E ChIP-qPCR shows enrichment of *MYC2*, *PDF1.2A* and *NIMIN1* promoter regions containing ANAC032 binding site compared to a promoter region lacking the ANAC032 binding site (*AT5G09810*; *ACTIN7*). Means ± SD (n = 3 independently performed experiments, each including the rosette leaves of at least three plants grown in individual pots). Asterisks indicate a significant difference from negative control (*P < 0.01; Student's t-test).

retarded bands seen in Fig 3B, ANAC032 interacts with the promoter sequences of all three genes. This interaction is significantly reduced when unlabelled promoter fragments (competitor) are added in excess, indicating the specificity of the interaction.

To test whether ANAC032 also interacts *in vivo* with the *MYC2*, *PDF1.2A* and *NIMIN1* promoters, we performed chromatin immunoprecipitation coupled with qRT–PCR (ChIP-qPCR) using a transgenic line that expresses an ANAC032-GFP fusion protein from the native *ANAC032* promoter in *anac032-1* plants (Figs EV1E and 3C). ANAC032 is a nuclear localized protein (Fig 3C) and *ANAC032*$_{\text{prom}}$*:ANAC032-GFP/anac032-1* plants showed reduced expression of *MYC2*, *PDF1.2A* and *NIMIN1* upon *Pst* treatment (Fig 3D), demonstrating that fusion to GFP did not impair the biological function of ANAC032. Significant ChIP enrichment was observed for the three

promoter regions harbouring ANAC032 binding sites compared to a promoter lacking the ANAC032 binding site (negative control) (Fig 3E). Thus, the ChIP-qPCR results confirmed direct transcriptional regulation of *MYC2*, *PDF1.2A* and *NIMIN1* by ANAC032 *in vivo*.

SA-induced *NIMIN1* expression is suppressed by ANAC032

NIMIN1 is a key regulator of SA-dependent systemic acquired resistance (SAR) [36]. At the protein level, it targets the NPR1-TGA complex and negatively regulates expression of NPR1-dependent SAR genes such as *PR1*. *NIMIN1* expression has been shown to be transiently enhanced soon after SA application, before induction of *PR1*, thereby preventing premature activation of the latter [36]. To investigate the SA-ANAC032-NIMIN1-PR1 regulatory network further, we analysed *NIMIN1* and *PR1* transcript abundance in *ANAC032* transgenic and WT plants upon SA treatment compared to control condition. As shown in Fig 4A, *NIMIN1* expression increased markedly (~fourfold) within 3 h of SA treatment in WT plants, while its expression was attenuated at 6 h (up ~twofold compared to the initial level), consistent with previous reports showing early and transient induction of *NIMIN1* upon SA treatment [36]. *PR1* transcript level was not increased 3 h after SA treatment (possibly due to a high level of the *PR1* suppressor, NIMIN1, at this time point), whereas it increased significantly after 6 h (Fig 4B). Induction of *NIMIN1* by SA was further enhanced at both time points in *anac032-1*, while it was greatly diminished in *35S:ANAC032* plants, suggesting that ANAC032 is a negative regulator of *NIMIN1* expression in response to SA. In accordance with this model, *PR1* induction by SA is completely suppressed in *anac032-1*. Furthermore, *PR1* expression is induced at 3 h after SA treatment in *35S:ANAC032* plants, possibly due to reduced *NIMIN1* expression (Fig 4B). Collectively, our data convincingly implicate ANAC032 as a regulator of SA-dependent defence gene expression. It acts upstream of *NIMIN1* and modulates time-specific SA-dependent *PR1* regulation by *NIMIN1*.

ANAC032 is required for SA-mediated suppression of *PDF1.2A*

SA strongly antagonizes JA signalling, resulting in the downregulation of JA-responsive genes [25,34,37]. Our study shows that ANAC032 negatively regulates the transcript levels of various JA-responsive genes (such as *PDF1.2A*) upon *Pst* infection (Figs 2 and 3). To test whether ANAC032 is required for SA-mediated suppression of JA signalling, we treated 2-week-old WT and *anac032-1* and *35S:ANAC032* seedlings with SA for 3 h and 6 h and analysed (compared to untreated control) expression of *PDF1.2A*, the direct target of ANAC032, by qRT–PCR. In WT, SA treatment reduced *PDF1.2A* transcript level at both time points (~twofold and 3.5-fold after 3 and 6 h SA treatment, respectively), consistent with previous reports [27,37,54]. *PDF1.2* expression upon SA treatment was slightly more reduced at both time points (~2.6- and fourfold after 3 and 6 h SA treatment, respectively), in *35S:ANAC032* seedlings compared with WT. However, repression of *PDF1.2A* by SA was completely abolished in *anac032-1* (Fig 4C), clearly indicating ANAC032 as a regulator of SA-mediated *PDF1.2A* suppression.

ANAC032 inhibits *Pst*-induced stomatal reopening

Stomata play an active role in restricting bacterial invasion [16–18]; within ~1 h after *Pst* DC3000 infection, stomata close in *Arabidopsis* Col-0, a process dependent on ABA (abscisic acid)-mediated signalling and PAMPs such as the flg22 (Flagellin 22) or lipopolysaccharide [16]. However, stomata reopen at ~4 hpi, a process which requires COR signalling [16]. ANAC032 is induced by COR (Fig 1C), ABA [55] and flg22 [56], and it enhances resistance towards *Pst* as shown here, which provoked us to check whether ANAC032 is involved in regulating stomata movements during bacterial infection.

We found that stomatal closing upon *Pst* treatment was normal in all genotypes analysed (Figs 5A and C, and EV3A), showing that ANAC032 is not needed for ABA/PAMP-triggered stomatal closure. However, at 4 hpi, stomata of WT, *anac032-1* and *ANAC032*-complemented plants reopened, whereas stomata of *35S:ANAC032* plants remained closed, suggesting an inhibitory role for ANAC032 in COR-dependent stomatal reopening or signalling (Figs 5B and C, and EV3B). To test this hypothesis further, we treated all genotypes for 1 h with ABA or COR. While ABA closed stomata in all genotypes, COR alone did not affect stomatal closure (Figs 5D and EV3C). However, when COR was applied together with

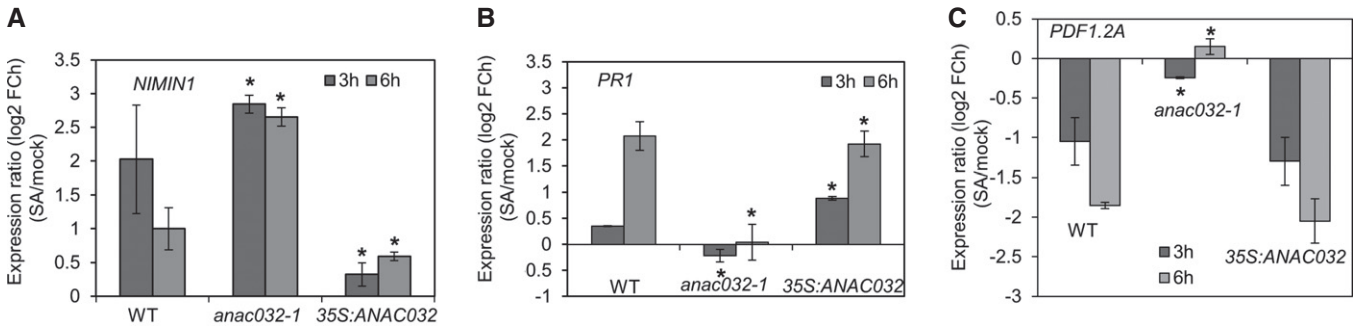

Figure 4. ANAC032 facilitates the SA-mediated transcriptional repression of *NIMIN1* and *PDF1.2A*.

A, B Transcript levels of (A) *NIMIN1* and (B) *PR1* in WT, *anac032-1* and *35S:ANAC032* plants after 3 and 6 h of treatment with SA compared to their respective controls.

C Expression of *PDF1.2A* in WT and *anac032-1* plants after 3 and 6 h of treatment with SA compared to their respective controls.

Data information: Transcript levels were measured using qRT–PCR and numbers on the *y*-axis indicate fold change (FCh; log2 basis). Means ± SD are shown ($n = 3$ independently performed experiments). Asterisks indicate a significant difference from wild type (*$P < 0.05$; Student's *t*-test).

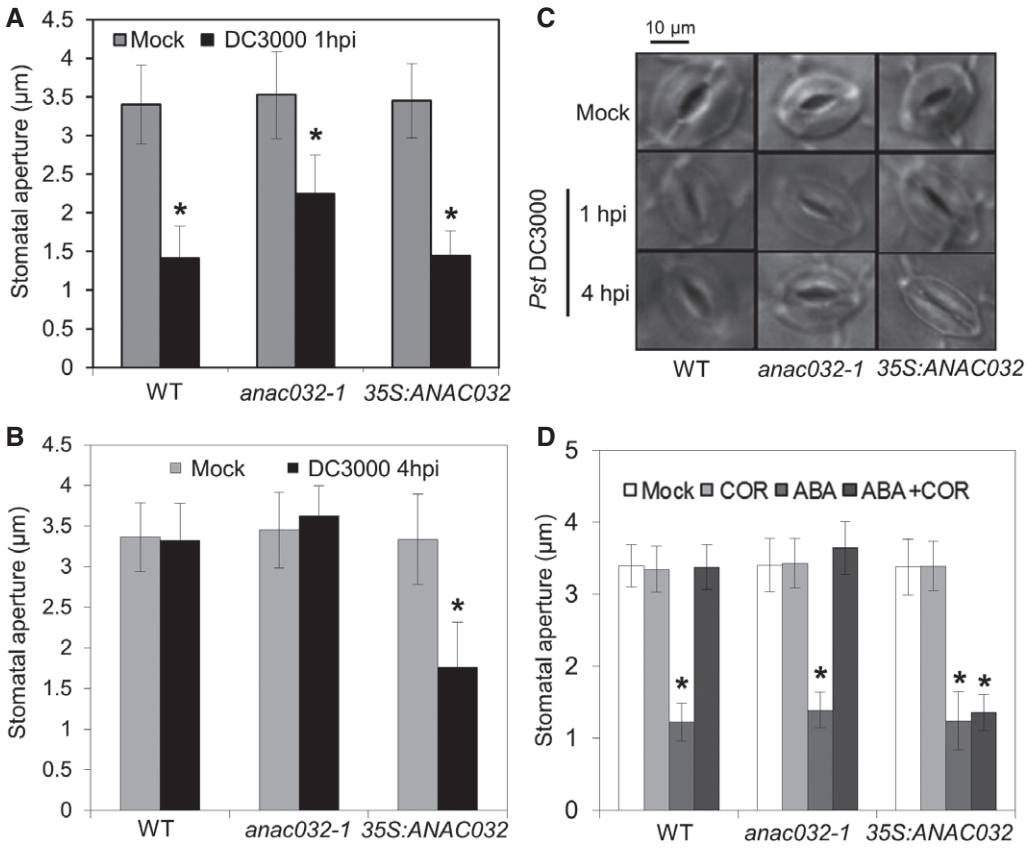

Figure 5. ANAC032 inhibits *Pseudomonas*-induced reopening of stomata.

A, B Stomatal aperture 1 h (A) or 4 h (B) after spraying with *Pst* DC3000 or mock treatment.

C Microscope images of stomata at 1 and 4 hpi with *Pst* DC3000 (mock at 4 h).

D Stomatal aperture 1 h after treatment with COR, ABA or COR plus ABA, compared to mock.

Data information: In (A), (B) and (D), data are means ± SD of 12–16 measurements; in each measurement, the rosette leaves from at least 6-8 plants were used. Asterisks indicate a significant difference from mock treatment (*$P < 0.0001$; Student's *t*-test).

ABA, stomata remained open in WT, *ANAC032*-complemented and *anac032-1* plants, but they closed in *35S:ANAC032* plants (Figs 5D and EV3C), suggesting that ANAC032 suppresses the inhibitory effect of COR on ABA-mediated stomatal closure.

Finally, we surface inoculated *anac032-1* and WT plants with a COR-deficient (COR^-) *Pst* DC3000 strain and used *Pst* DC3000 in control experiments. Compared to WT, the *anac032-1* mutant exhibited increased susceptibility to both strains of DC3000; however, the severity of the disease symptoms upon infection with DC3000 COR^- was more strongly diminished in *anac032-1* than in WT (Fig EV4), suggesting that ANAC032 regulation of disease response may, at least in part, be mediated through inhibition of COR-induced stomatal reopening.

ANAC032 directly suppresses *MYC2* expression (Fig 3), and MYC2 positively regulates expression of the three closely related NAC genes *ANAC019*, *ANAC055* and *ANAC072*, which mediate COR-induced stomatal reopening after *Pst* infection [22]. Interestingly, transcript abundance of all three *NAC*s was enhanced in *anac032-1*, but repressed in *ANAC032* overexpressors upon *Pst* infection (Fig 2E), further supporting the role of ANAC032 as a negative regulator of COR signalling.

Discussion

Interplay between SA- and JA-dependent pathways is essential for generating an appropriate physiological output upon pathogen attack [2,26]. Here, we identified transcription factor ANAC032 from *Arabidopsis thaliana* as a central regulator of the plant's defence response against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*); while overexpression of *ANAC032* resulted in enhanced resistance to *Pst*, the *ANAC032* loss-of-function mutant (*anac032-1*) exhibited severe disease symptoms (Figs 2A–D and EV2). ANAC032 directly controls the expression of key elements of SA signalling (*NIMIN1*) and JA signalling (*MYC2* and *PDF1.2A*) (Fig 3), suggesting that it exerts its physiological function by simultaneously enhancing SA, but decreasing JA signalling thereby shifting the plant's response activity towards biotrophic over necrotrophic invaders.

NIMIN1 is one of the central components involved in the differential regulation of the transcriptional outputs of *PR* gene expression at different stages of SAR [35,36]. At an early stage of SAR, NIMIN1 interacts with NPR1 (resulting in the formation of the NIMIN1-NPR1-TGA complex) and negatively affects expression of the late SAR gene, *PR1*. At a later stage of SAR, accumulation of SA results in the

removal of repressing NIMIN1 from the NPR1 complex, which is a prerequisite for induction of *PR1* gene expression [36]. Although tightly controlled, transcriptional control of *NIMIN1* by upstream regulators is not well understood at present. A previous study demonstrated that a TGA2-binding motif located in the *NIMIN1* promoter is necessary for SA inducibility of *NIMIN1* expression [57,58]. Here, we identified ANAC032 as a direct negative transcriptional regulator of *NIMIN1* (Figs 2–4), which to our knowledge is the first TF reported to directly control *NIMIN1* expression *in planta*.

Suppression of *NIMIN1* transcription by ANAC032 may be considered as one of the mechanisms to relieve *PR* gene expression in the course of SAR. Additionally, analysis of *ANAC032* transgenics and WT plants at different time points upon SA treatment revealed differential expression levels of *NIMIN1* and *PR1*. In WT plants, the transcript levels of both genes increased upon SA treatment. However, while *NIMIN1* expression was more pronounced at the earlier time point (3 h), *PR1* was higher at the later time point (6 h), which is in agreement with previous reports [36]. Interestingly, SA-induced expression of *NIMIN1* was further enhanced in *anac032-1* plants compared to WT, at the earlier time point and remained high even at the later time point, resulting in the suppression of *PR1* expression at both time points tested. In contrast, *NIMIN1* induction by SA was significantly suppressed in transgenic plants overexpressing *ANAC032*, which correlated with increased *PR1* expression (Fig 4A and B). These data provide strong evidence that ANAC032 regulates *PR1* gene expression through its negative effect on SA-mediated *NIMIN1* induction and therefore suggest ANAC032 as a key component regulating the SAR response.

MYC2 is a major regulator of JA signalling and functions as a negative regulator of SA biosynthesis and metabolism [8,22,23,45]. *myc2/jin1* mutants exhibit reduced sensitivity to *Pst* DC3000 as well as to COR treatment due to higher accumulation of SA and enhanced expression of *PR1* [23,24]. MYC2 mediates COR signalling by directly activating three NAC factors involved SA biosynthesis and metabolism (*ANAC019, ANAC055* and *ANAC072*). The three NACs repress expression of the SA synthesis gene *ICS1* and activate expression of the SA metabolism gene *BSMT1* [22]. Expression of the three NACs was enhanced in *anac032-1* but lowered in *ANAC032* overexpressors upon *Pst* infection, further supporting the role of ANAC032 as a negative regulator of COR signalling and COR-mediated stomatal reopening (Fig 2E), in accordance with our observation that *ANAC032* overexpressors are impaired in stomatal reopening 4 h after surface inoculation with *Pst* and are less sensitive to COR (Figs 5 and EV3). Furthermore, *anac032-1* mutants exhibit an increased susceptibility to both *Pst* strains (DC3000 and DC3000 COR$^-$) compared to WT, although disease symptoms induced by COR-deficient DC3000 were more reduced in *anac032-1* than in WT plants indicating that ANAC032 contributes to the disease response by restricting COR-induced stomatal reopening (Fig EV4). Stomatal closure establishes a physical barrier to restrict pathogen entry and is regulated by both, SA and ABA signalling [16–18]. To counteract the plant's defence response, *Pseudomonas syringae* produces the polyketide toxin COR to reopen stomata [16,21,22]. In addition to overcoming stomatal defence, COR also aids to apoplastic bacterial propagation and promotes systemic susceptibility by inducing cell death leading to the formation of disease-associated chlorosis and necrosis [4,8,13,14]. Interestingly, COR has been shown to more actively trigger the COI1-JAZ interaction and the consecutive JAZ degradation than the plant hormone JA-Ile [9,11]. It has recently been demonstrated that COR exerts its virulence by executing MYC2 signalling to suppress SA-mediated defence response [22].

MYC2 has also been identified to act as a key defence response regulator against herbivores [59]. The repressive effect of ANAC032 on *MYC2* transcription suggests that it negatively regulates the response to herbivory. However, as *ANAC032* expression is induced by MeJA, this observation indicates the presence of a negative feedback loop in herbivore defence/JA signalling, an interesting aspect to study in the future.

We furthermore observed that expression of *PAD4*, *EDS1*, *PBS3*, several *TGA* TFs and *FMO1* is upregulated in *35S:ANAC032*, but downregulated in *anac032-1* plants upon pathogen infection (Fig 2E). The *eds1* and *pad4* mutants in *Arabidopsis* are defective in SA production and exhibit enhanced disease susceptibility [50–52], and FMO1 has been shown to be critical for the establishment of SAR against necrotizing bacteria; in addition, it is required for the establishment of the EDS1/PAD4-induced defence response [60].

Loss of *ANAC032* also leads to transcriptional repression of *JAZ* genes (*JAZ3*, *JAZ7*, *JAZ8* and *JAZ10*) while it enhances expression of *MYC2* and several JA-responsive genes including *PDF1.2A*, *THI2.1*, *VSP1* and *VSP2* (Fig 2E). JAZ proteins are major repressors of JA signalling under basal conditions [48]. These gene expression profiles support the conclusion that ANAC032 acts as a negative regulator of JA responses upon infection by *Pst*.

Although previous studies had demonstrated the involvement of other TFs from diverse families including WRKYs (e.g. WRKY70, WRKY50, WRKY51), NACs (e.g. ANAC055, ANAC019, ANAC072) and bZIPs (e.g. TGA2, TGA5, TGA6) in the regulation of the SA/JA interplay [2,22,25,26,34,61,62], information about their direct target genes is currently limited.

Model for ANAC032 action

Collectively, our study identifies ANAC032 as a negative and positive regulator of JA and SA signalling, respectively, thereby channelling defence towards the SA pathway for a more efficient immune response against *Pst.* Our data suggest the following working model for the action of ANAC032 in mediating the interplay between SA- and JA-dependent defence signalling (Fig 6). Expression of *ANAC032* is induced by a number of pathogen-derived signals and ANAC032 regulates the plant's response to *Pst* infection through a network that involves genes associated with multiple layers of defence: (i) ANAC032 negatively regulates JA signalling through the suppression of JA-responsive defence genes (*MYC2*, *PDF1.2A*, *THI2.1*, *VSP1* and *VSP2*). (ii) ANAC032 positively affects SA signalling and expression of an SAR marker gene (*PR1*) by direct repression of SA-induced *NIMIN1* expression. (iii) Inhibition of pathogen-triggered stomatal reopening by ANAC032 may be achieved by direct repression of the MYC2-ANAC019/55/72 transcriptional cascade in the JA/COR signalling pathway, thereby restricting pathogen invasion.

In summary, ANAC032—and likely its orthologs in crops (http://bioinformatics.psb.ugent.be/plaza/) —plays a decisive role in regulating the plant's immune response against *Pst* DC3000 and possibly other biotrophic pathogens. This involves orchestrating the expression of multiple genes that are key to the SA/JA interplay, leading to

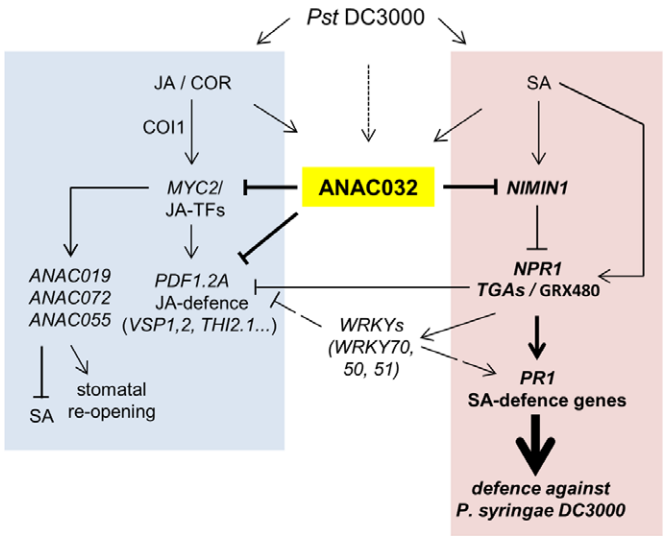

Figure 6. Working model for the role of ANAC032 in mediating the interplay between SA- and JA-dependent defence signalling in response to *Pst* DC3000.

ANAC032 expression is induced by pathogen-derived signals. ANAC032 represses *MYC2* and *PDF1.2A*, but stimulates *JAZ* repressors thereby reducing JA/COR signalling. COR-mediated stomatal reopening and thus bacterial re-entry into host cells is negatively affected by ANAC032 possibly through the *MYC2-ANAC019/55/72* transcriptional cascade. Simultaneously, ANAC032 activates *PR1* gene expression and SA signalling by direct transcriptional repression of *NIMIN1*. Lines ending in arrows and bars indicate positive and negative interactions, respectively.

enhanced SA, but reduced JA signalling. The upstream elements that regulate *ANAC032* transcription in response to *Pst* infection remain to be identified.

Materials and Methods

General

Oligonucleotides (Table EV1) were obtained from Eurofins MWG Operon (Ebersberg, Germany). Tools provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), the Arabidopsis Information Resource (http://www.arabidopsis.org/), the Plant Transcription Factor Database (http://plntfdb.bio.uni-potsdam.de/v3.0/) and Genevestigator (http://genevestigator.com) were used for computational analyses.

Growth conditions

Arabidopsis thaliana (L.) Heynh. (Col-0) was used. For experiments with seedlings, surface sterilized seeds were sown on 0.5× Murashige and Skoog (MS) agar medium containing 1% (w/v) sucrose. Plants were grown at 22°C under a 16-h day (140 μmol m^{-2} s^{-1})/8-h night regime. For growth under short-day conditions, seedlings were grown in soil (Einheitserde GS90; Gebrüder Patzer) in a climate-controlled chamber with an 8-h day length at 120 μmol m^{-2} s^{-1} and a day/night temperature regime of 20/16°C, relative humidity 60/75%.

The T-DNA insertion line SALK_012253 (*anac032-1*) was obtained from the European Arabidopsis Stock Centre (http://arabidopsis.info/). Homozygous plants were identified by PCR using a T-DNA left border primer and the gene-specific primers LP and RP.

Constructs

Constructs were generated by PCR and restriction enzyme-mediated or directional cloning. PCR-generated amplicons were checked by DNA sequence analysis (MWG or Seqlab). Constructs were transformed into *Arabidopsis* using *Agrobacterium tumefaciens*.

For *35S:ANAC032*, the *ANAC032* open-reading frame was amplified by PCR from *Arabidopsis* leaf cDNA, inserted into pUni/V5-His-TOPO (Invitrogen), and then cloned, via added *Pme*I-*Pac*I sites, into a modified pGreen0229-35S plant transformation vector. For *35S:ANAC032-GFP* and *ANAC032-GST*, the *ANAC032* open-reading frame was PCR-amplified without the stop codon and cloned into the pENTR/D-TOPO vector using the pENTR Directional TOPO Cloning kit (Invitrogen). The verified entry clones were then transferred to the pK7FWG2 vector (Ghent University) and the Gateway pDEST24 vector (Invitrogen), respectively, by LR recombination. For *$ANAC032_{prom}$-ANAC032-GFP*, the 35S promoter in the *35S:ANAC032-GFP* construct was replaced with the *ANAC032* upstream promoter (1 kb) using SLiCE (Seamless Ligation *Cloning* Extract) [63]. *$ANAC032_{prom}$-ANAC032-GFP/anac032-1* plants were produced by transforming the *$ANAC032_{prom}$-ANAC032-GFP* construct into *anac032-1* plants. For *anac032-1* complementation, the above-described *35S:ANAC032* construct was transformed into *anac032-1* plants. For *ANAC032-CELD*, the *ANAC032* CDS was inserted into pCR2.1-TOPO (Invitrogen) and then cloned into the plasmid pTacLCELD6XHis [53] to create an in-frame fusion construct, pTacANAC032CELD6XHis.

Treatments

For hormone treatments, 2-week-old seedlings grown on solid MS medium were transferred to flasks containing liquid MS medium (1% sucrose) and 1 mM SA or 100 μM MeJA or 1 ng/ml COR (with 0.015% [v/v] Silwet 77); flasks containing the seedlings were kept on a rotary shaker at 75–80 rpm. Medium with only 0.015% [v/v] Silwet 77 served as controls. Samples (whole seedlings) were harvested by flash-freezing them in liquid nitrogen, and expression analysis was performed using quantitative real-time PCR (qRT–PCR). Three independent experiments were performed for hormone treatments and gene expression analyses. Each replicate consisted of at least 20 seedlings.

Pseudomonas syringae pv. *tomato* DC3000 (*Pst*) or *Pst* DC3000 COR^- (DB29, kindly provided by Dr. Barbara Kunkel, USA) was grown on King's B medium plates with appropriate antibiotics at 28°C for 2 days [13]. *Pseudomonas* infection was performed as described [64]. Briefly, bacterial cells were collected by centrifugation (2,500 *g*) and resuspended in 10 mM $MgCl_2$. Pressure infiltration of *Pst* (c.f.u. 1×10^6 ml^{-1}; $OD_{600\ nm} = 0.002$) was carried out using a needleless syringe. For inoculation by spraying, a *Pst* suspension with 1×10^8 c.f.u. ml^{-1} ($OD_{600\ nm} = 0.2$) or 10 mM $MgCl_2$ mock inoculum solution containing 0.02% [v/v] Silwet L-77 was used; the boxes containing the plants were covered with plastic lids. Whole rosette leaves were harvested at the indicated time points after bacterial challenge, frozen immediately in liquid

nitrogen and used for ChIP and gene expression analysis. Three independent experiments were performed for gene expression analysis and ChIP. Each replicate consisted of rosettes leaves of at least three plants grown in individual pots.

Bacterial growth count and disease severity index

Bacterial growth was quantified by assessing the *Pst* population [c.f.u. (g fresh weight)$^{-1}$] 3 days postbacterial challenge (by pressure infiltration) as described [64]. The disease severity index was scored as described [64].

Stomatal assay

Pst DC3000 infection was performed by evenly spraying bacterial suspension (1×10^8 c.f.u. ml^{-1}, containing 0.02% [v/v] Silwet L-77) onto leaves of 6-week-old, soil-grown plants. Leaves were fixed using formaldehyde fixative solution [16,22] at the indicated time points and used for microscopic measurements [16,22]. Plants treated with 10 mM $MgCl_2$ were used as mock infection controls. For stomatal studies after ABA, COR, or ABA + COR treatment, detached leaves were transferred to solutions containing either 15 μM ABA, 1 ng/μl COR, or 15 μM ABA + 1 ng/μl COR and placed on a rotary shaker (75–80 rpm) under constant light for 1 hour. Leaves were fixed using formaldehyde fixative solution [65]; an epifluorescence microscope was used to take images at random of at least 30 stomata for each sample and time point. Measurement of stomatal apertures was performed using Leica Cell software (http://www.leica-microsystems.com/).

Expression profiling by qRT–PCR

Total RNA extraction, synthesis of cDNA and qRT–PCR were performed as described [66]. *ACTIN2* served as reference gene. PCRs were run on an ABI PRISM 7900HT sequence detection system (Applied Biosystems Applera), and amplification products were visualized using SYBR Green (Life Technologies).

In vitro binding site selection assay

In vitro binding site selection was performed using the CELD method with the pTacANAC032CELD6XHis protein, employing biotin-labelled double-stranded oligonucleotides (Bio-RS-Oligo 3), which contained 30-nucleotide random sequences [53]. ANAC032-selected oligonucleotides were cloned and sequenced. The DNA binding activity of ANAC032-CELD was measured using methylumbelliferyl-beta-D-cellobioside as substrate [53]. DNA binding assays with a biotin-labelled single-stranded oligonucleotide or a biotin-labelled double-stranded oligonucleotide without a target binding site were used as controls.

EMSA

ANAC032-GST fusion protein was purified from *Escherichia coli* expression strain BL21 Star (DE3) pRARE, which was generated by transforming the pRARE plasmid isolated from Rosetta (DE3) pRARE cells (Merck) into *E. coli* BL21 Star (DE3) (Invitrogen). Protein was purified using a GSTrap HP column (GE Healthcare) coupled to the ÄKTA purifier FPLC system (GE Healthcare). EMSA was performed as described [67] using an Odyssey Infrared EMSA kit (LI-COR). 5′-DY682-labelled DNA fragments (Table EV1) were purchased from Eurofins MWG Operon.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was carried out on chromatin extracted from 5-week-old *Arabidopsis* plants expressing the ANAC032-GFP fusion protein in *anac032-1* background (*ANAC032prom-ANAC032-GFP/anac032-1*), 6 hpi with *Pst* DC3000. Anti-GFP antibody was employed to immunoprecipitate protein–DNA complexes [68]. Wild-type (Col-0) plants treated with *Pst* DC3000 for 6 h served as negative control. Three independent experiments of the ChIP experiment were run. The qPCR primers (Table EV1) for the target promoters were designed to flank the ANAC032 binding sites. As a negative control, we used primers annealing to a promoter region of an *Arabidopsis* gene (*ACTIN7; AT5G09810*) lacking an ANAC032 binding site. ChIP-qPCR data were analysed as described [68].

Expanded View for this article is available online.

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Author contributions
SB conceived the study and wrote the manuscript. ADA performed the experiments. YB contributed to the *Pst* infection experiments and performed the chi-square test analysis. G-PX identified the ANAC032 binding sites.

Conflict of interest
The authors declare that they have no conflict of interest.

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