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Pseudomonas syringae pv. *tomato* hijacks the *Arabidopsis* abscisic acid signalling pathway to cause disease

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We have found that a major target for effectors secreted by *Pseudomonas syringae* is the abscisic acid (ABA) signalling pathway. Microarray data identified a prominent group of effector-induced genes that were associated with ABA biosynthesis and also responses to this plant hormone. Genes upregulated by effector delivery share a 42% overlap with ABA-responsive genes and are also components of networks induced by osmotic stress and drought. Strongly induced were *NCED3*, encoding a key enzyme of ABA biosynthesis, and the abscisic acid insensitive 1 (*ABI1*) clade of genes encoding protein phosphatases type 2C (*PP2Cs*) involved in the regulation of ABA signalling. Modification of *PP2C* expression resulting in ABA insensitivity or hypersensitivity led to restriction or enhanced multiplication of bacteria, respectively. Levels of ABA increased rapidly during bacterial colonisation. Exogenous ABA application enhanced susceptibility, whereas colonisation was reduced in an ABA biosynthetic mutant. Expression of the bacterial effector AvrPtoB *in planta* modified host ABA signalling. Our data suggest that a major virulence strategy is effector-mediated manipulation of plant hormone homeostasis, which leads to the suppression of defence responses.

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Introduction

Plant–pathogen interactions involve a dynamic process of cut, thrust and counterthrust as the host attempts to contain the insurgent within the complex intracellular and extracellular landscape of the battlefield. Plant basal defences provide several strategic layers of constitutive and induced defences, the latter based upon the ability to detect and respond to conserved molecular patterns associated with the invading microbe. The various repertoires of predominately surface-expressed ligands, commonly referred to as PAMPs (pathogen-associated molecular patterns), engage different combinations of plant-surface receptors whose output is customised to reflect the degree of 'danger' posed by the particular pathogen. The basal defense response manifests itself in the form of biochemical and structural defences designed to restrict microbial multiplication and nutrition, including rapid ionic changes and phosphorylation cascades, ultimately leading to transcription of defence-related genes and the formation of defensive cell wall depositions, termed papillae (Nurnberger *et al*, 2004; Zipfel and Felix, 2005).

The frontline weapons of bacterial virulence comprise a collection of chemical virulence factors and ~40 proteinaceous 'effectors'. The latter are delivered into the plant cell via a type III protein secretion system (T3SS; Mudgett, 2005). Evidence is emerging that the type III effectors (T3Es) interfere with host signalling and metabolism to promote suppression of basal defence and pathogen nutrition. However, specific function has been assigned to very few T3Es, and our knowledge of the synergistic collaborations with other effectors that may be required for successful parasitism is rudimentary. Three phytohormones, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), are known to participate in regulating defence responses in plants. SA is predominately associated with resistance against biotrophic and hemi-biotrophic pathogens and the establishment of systemic acquired resistance (Grant and Lamb, 2006). By contrast, JA- and ET-dependent defence mechanisms generally contribute to resistance against necrotrophic pathogens, suggesting that the signalling network engaged by the host is dependent upon the nature of the pathogen and its mode of pathogenicity (Glazebrook, 2005). The plant hormone abscisic acid (ABA) is involved in plant responses to several abiotic stresses (drought, salt and cold) as well as seed germination and plant growth (Seo and Koshiba, 2002; Nambara and Marion-Poll, 2005). Additionally, exogeneous ABA treatment increases the susceptibility of various plant species to bacterial and fungal pathogens (Henfling *et al*, 1980; Ward *et al*, 1989; McDonald and Cahill, 1999; Mohr and Cahill, 2003; Thaler and Bostock, 2004). ABA-deficient mutants showed a reduction in susceptibility to the necrotroph *Botrytis cinerea* (Audenaert *et al*, 2002) and virulent isolates of *Pseudomonas syringae* pv *tomato* DC3000 in tomato (Thaler and Bostock,

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2004), and the oomycete *Hyaloperonospora parasitica* in *Arabidopsis* (Mohr and Cahill, 2003). Collectively, these data suggest that ABA behaves as a negative regulator of defence responses.

We are interested in how virulence factors (including T3Es) produced by DC3000 in *Arabidopsis* suppress plant basal defences. Building upon analysis of genome-wide transcriptome changes during early stages of the *A. thaliana*/DC3000 interaction (Truman *et al*, 2006) we now present results supporting effector-mediated manipulation of ABA biosynthetic and signalling pathways as a core virulence mechanism. The changes in plant hormones observed during infection by bacteria and fungi have traditionally been considered as 'side effects of successful parasitism'. By contrast, we now show that the successful manipulation of the ABA hormonal network has probably been a fundamental step in the evolution of a plant pathogenic bacterium.

Results

Microarrays reveal a role for ABA in virulence

We have analysed previously the temporal dynamics of host gene expression during bacterial infection of *Arabidopsis* leaves by global transcriptional profiling (Truman *et al*, 2006). Three challenges designed to report background inoculation effects (mock; 10 mM $MgCl_2$), PAMP responses (DC3000*hrpA*$^-$, a mutant compromised in T3SS) or disease (wild-type DC3000), and carefully chosen sampling times enabled the identification of differentially expressed genes associated with the activation of basal defence and establishment of pathogenesis. By removing the basal defence signature attributable to challenge with the *hrpA*$^-$ mutant, we captured the specific effects of bacterial virulence factors on host transcription. Hierarchical clustering identified transcripts coregulated by T3E delivery 12 h post-inoculation (12 hpi). These transcripts represent genes upregulated by T3Es, either due to the action of the effectors themselves or, alternatively, as a host response to T3E activities. We have now examined in detail one coregulated cluster consisting of 47 T3E-induced genes (Table I). The group of genes is characterised by an over-representation of transcripts encoding protein phosphatases of the 2C class (PP2C), which are implicated in responses to ABA, including *ABI1* (abscisic acid insensitive 1; Schweighofer *et al*, 2004). Remarkably, *NCED3* (*At3g14440*), which encodes the enzyme that catalyses the early limiting step in water stress-induced ABA biosynthesis (Qin and Zeevaart, 1999; Tan *et al*, 2003), was induced more than 15-fold by T3Es. Other ABA response-associated genes resident in this cluster include *AFP* (abscisic acid insensitive-5 binding protein), an ABA signalling regulator (At1g69260) (Lopez-Molina *et al*, 2003), and four *NAC* (for *NAM*, *ATAF1*, *2* and *CUC2*) transcription factors (At1g52890, At3g15500, At4g27410 [*RD26*] and At5g39610) previously shown to be ABA, drought and NaCl-inducible (Tran *et al*, 2004).

Analysis of the proximal 1 kb promoter regions of the coregulated genes using the PLACE Signal Scan programme (Higo *et al*, 1999) identified that more than 50% contain one or more ABA response elements (ABRE; ACGTG[GT]C), which could be further elaborated into three overlapping regions of increasing complexity. All three contained the core ACGTG motif, which itself was present in more than 75% of the promoters (Table I). The significant over-representation of these motifs was confirmed using POBO (Kankainen and Holm, 2004).

In summary, our analysis suggested that one mechanism of T3E action is to elevate components of the ABA signalling and biosynthesis pathways.

ABA levels in challenged tissues

In a compatible interaction leading to disease, the induction of *NCED3* between 4 and 12 hpi follows T3E delivery. As NCED is a key regulatory enzyme in ABA biosynthesis, we examined whether T3Es were directly manipulating levels of ABA . We measured ABA at 8, 12 and 18 h following mock, DC3000 or DC3000 *hrpA*$^-$ challenges. Increases in ABA occurred rapidly, and by 18 hpi ABA levels were $\sim$10-fold higher in DC3000 than in *hrpA*$^-$ or mock-challenged leaves (Figure 1A) and were directly correlated to the level of DC3000 inoculum (Figure 1B). Although DC3000 multiplies *in planta*, there was no significant difference in bacterial numbers (*hrpA*$^-$ versus DC3000) at 10 hpi despite increasing ABA levels (data not shown), and 15-fold less virulent bacteria produced more than six-fold more ABA than the non-pathogenic *hrpA*$^-$ mutant (Figure 1B). As the hormones SA and JA have already been demonstrated to play a role in plant defences, we compared SA, JA and ABA levels 18 hpi in uninoculated and pathogen-challenged leaves (Figure 1C). Foliar ABA and JA were significantly induced by DC3000, suggesting DC3000 induces both ABA and JA biosynthesis, whereas free SA did not differ significantly between *hrpA*$^-$ and DC3000.

NCED3, strongly induced by T3Es at 12 hpi, encodes the major stress-induced ABA regulatory enzyme, and is the most likely candidate to contribute to elevated ABA levels in the compatible interaction. In agreement with our microarray results *NCED3,* steady-state levels increased from 8 hpi only after challenge with DC3000 (Figure 1D). The transient increase in *NCED3* transcript at 2 hpi evident in both DC3000 and DC3000*hrpA*$^-$-challenged leaves resulted from the inoculation procedure, as it was also induced by mock infiltration (data not shown).

ABA application and pathogenicity responses share significant overlap

To identify common ABA signalling components induced by T3Es we interrogated experiments in the NASC arrays (http://affymetrix.arabidopsis.info) database indicative of host responses to hormone application or abiotic and biotic stresses. Hierarchical clustering of the 880 genes significantly differentially regulated between DC3000 and DC3000*hrpA*$^-$ challenges at 12 hpi closely associated abiotic stress experiments with compatibility. In addition to ABA treatments at 30 min, 1 and 3 h, genes modified by T3Es were also found to respond to salt and osmotic stress treatments (Figure 2A). A major inducible cluster (i) was shared between compatible responses (DC3000 challenge at 6, 12 and 24 hpi) and osmotic stress. This cluster contains a subcluster (ii) that comprises the majority of the genes represented in Table I, including seven *PP2C*-s, *NCED3* and three *NAM*-s (annotated in Supplementary Table I). Two other notable clusters represent genes commonly suppressed both by T3Es and osmotic stress (iii) and genes differing in response between osmotic stress and T3Es activity (iv).

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Table I A coregulated set of T3E-induced genes responsive to ABA identified by hierarchial clustering

AGI number	Function (name)	ABA/stress reference	ARE	FC
ABA biosynthetic genes				
At3g14440	ABA synthesis (*NCED3*)	Qin and Zeevaart (1999)	B	17.0
Protein phosphatase 2C clade A				
At5g59220	PP2C	Xin *et al* (2005)	2D	10.0
At1g72770	PP2C (*HAB1*)	Saez *et al* (2004)	2A2D	2.1
At1g07430	PP2C		AB4D	8.7
At3g11410	PP2C (*AtPP2CA*)	Kuhn *et al* (2006)	AC2D	4.2
At5g57050	PP2C (*ABI2*)	Merlot *et al* (2001)	B	3.2
At4g26080	PP2C (*ABI1*)	Merlot *et al* (2001)	C	2.9
NAC domain transcription factors				
At5g39610	NAC TF (*AtNAC6*)	He *et al* (2005)		3.6
At1g52890	NAC TF (*ANACO19*)	Tran *et al* (2004)	3C2D	2.9
At3g15500	NAC TF (*AtNAC3*)	Tran *et al* (2004)	C3D	7.9
At4g27410	NAC TF (*RD26*)	Fujita *et al* (2004)	A2CD	4.5
Transcription factors of various families				
At1g66550	WRKY TF (*WRKY67*)		D	10.2
At5g49450	bZIP family TF		2D	4.3
At1g24440	C3HC4 zinc finger?			3.1
Other signalling pathways				
At1g69260	ABI5 binding protein	Lopez *et al* (2003)	2C2D	3.1
At1g05100	MAP3K (*MAPKKK18*)		BC3D	10.9
At2g02710	PAC motif containing		BD	2.1
At5g62540	Ubiquitin conjugating		D	2.2
Assorted physiological processes				
At2g03760	Steroid sulphotransferase			7.0
At5g13750	Transporter (*ZIFL1*)		C2D	2.7
At3g11340	UDP glucosyl transferase			2.4
At3g46660	UDP glucosyl transferase		D	5.3
At3g06500	β-Fructofuranosidase		B3D	4.3
At4g21680	Oligopeptide transporter			2.5
At1g05560	UDP-glucose transferase	Tran *et al* (2004)	D	3.3
At1g26770	Expansin (*AtEXPA10*)			4.4
At5g13820	Telomeric DNA-binding-1	Nagaoka and Takano (2003)	C3D	3.2
At3g14660	Cytochrome P450		C	2.1
Genes of unknown function				
At3g01650	Copine related			3.3
At1g24600	Expressed protein		A2D	3.2
At3g48350	Cysteine proteinase like		3D	8.1
At3g28007	Nodulin MtN3 family		4D	2.7
At3g29575	Expressed protein		4C3D	3.3
At1g69480	ERD1/XPR1/SYG1		BC	3.4
At2g28400	Expressed protein			3.2
At4g33980	Expressed protein		3C	2.4
At5g04250	OTU cysteine protease?		C3D	5.1
At5g54730	WD40 repeat protein		3D	3.9
At5g42900	Expressed protein		C2D	11.5
At1g33110	MATE efflux protein			3.1
At5g64230	Expressed protein		D	6.2
At5g65660	HPRG protein		2D	2.8
At1g58270	MATH domain protein		2A4D	3.0
At3g07350	Expressed protein		ACD	6.5
At3g28210	Zinc-finger protein	Xin *et al* (2005)	D	2.1
At5g13360	Auxin responsive GH3		D	2.1
At5g64250	2-Nitropropane diox.			2.7

Each of the 880 genes, significantly differentially expressed in response to T3E at 12 hpi, were median-centred and logged before clustering. This table presents a cluster of 47 genes containing a significant over-representation of ABA response elements in their promoters (1 kb upstream). Most genes of known function had previously been associated with abiotic stress. ARE—presence or absence of an ABA response element as defined in the PLACE database (Higo *et al*, 1999). Scoring is non-redundant and the number of occurrences precedes the motif. (A) ABREMOTIFAOSOSEM—*TACGTGTC*; (B) ABREATCONSENSUS—*[CT]ACGTGGC*; (C) ACGTABREMOTIFA2OSEM—*ACGTG[GT]C*; (D) ABRELATEDD1—*ACGTG*. FC—fold change.

Given that T3Es increase foliar ABA and JA levels by 18 hpi (Figure 1) and the strong similarity between transcripts induced by ABA and T3Es (Figure 2A), we examined the overlap between responses to each hormone treatment and T3Es using experiments from the NASC database repository. The ABA response has a substantial overlap with T3E-upre-

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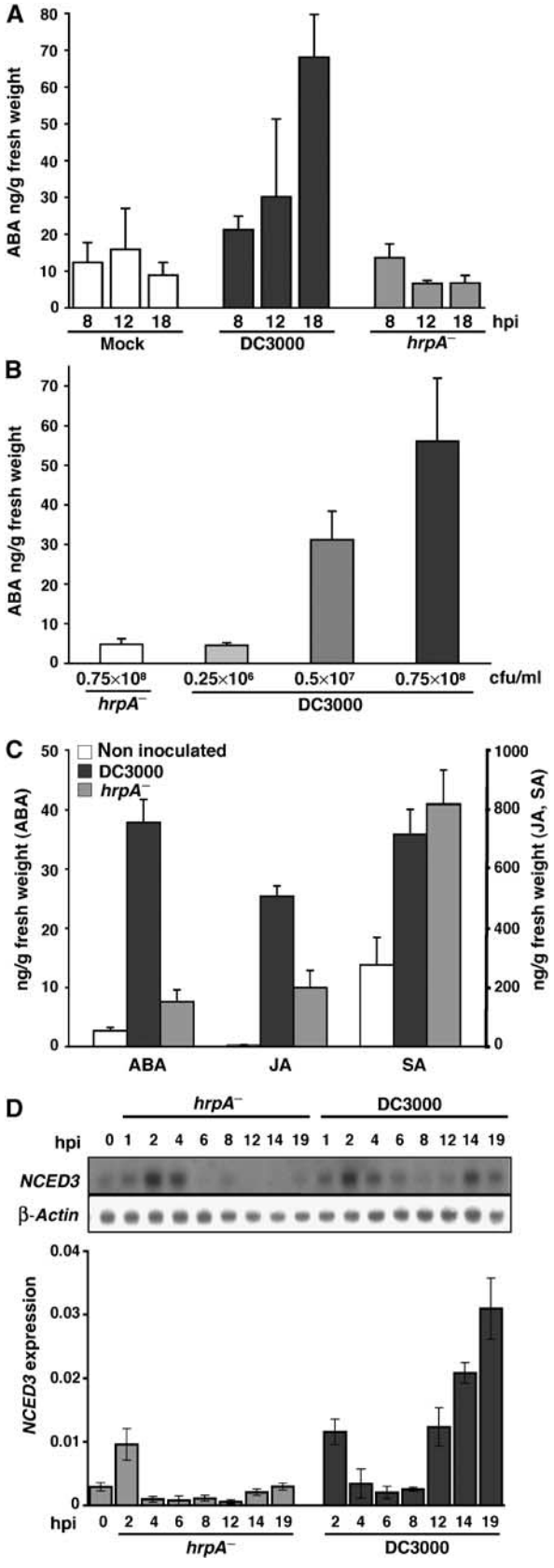
gulated transcripts (42%). Interestingly, 41% of the transcripts responsive to JA are also T3E induced (Figure 2B, Supplementary Table II). Collectively, these data suggest transcriptional reprogramming induced by T3E activity is highly similar to that induced through the ABA response pathway, and that ABA as well as JA signalling pathways contribute to pathogenicity.



Exogenous application of ABA enhances multiplication of wild-type DC3000 and a hrpA⁻ mutant

The effect of exogenous application of ABA on the multiplication of DC3000 and DC3000*hrpA*⁻ in challenged leaves was examined. If *de novo* ABA biosynthesis contributed to DC3000 virulence, we hypothesised that ABA application would allow the *hrpA*⁻ mutant to overcome host basal defences. ABA pretreatment 24 h before bacterial inoculation increased DC3000 virulence by an order of magnitude (Figure 3C). Furthermore, ABA application also enabled *hrpA*⁻ bacteria partially to overcome basal defences, consistently permitting a ~five-fold increase in growth (Figure 3A, statistical significance confirmed by '*t*'-tests). Thus, exogenously applied ABA promotes colonisation by both virulent and normally non-pathogenic *P. syringae*.

ABA signalling mutants in defence

To examine the potential role of ABA signalling in pathogenicity, we tested the T3E-induced Clade A *PP2C*s *ABI1*, *ABI2* and *HAB1* to determine their role in mediating the suppression of basal defense. The dominant mutations *abi1-1*, *abi2-1* as well as 35S::*HAB1* plants show reduced sensitivity to ABA compared with wild type (Kornneef *et al*, 1984; Saez *et al*, 2004). Conversely, intragenic revertants of the originally dominant *abi1-1* mutant, *abi1-1sup5* and *abi1-1sup7* behave as recessive alleles of *ABI1* (Gosti *et al*, 1999). These mutants were tested for their ability to support bacterial multiplication. Figure 3B shows that the ABA-hypersensitive lines, *abi1-1sup5* and *abi1-1sup7*, supported up to 30-fold more DC3000 multiplication. Conversely, the ABA-insensitive *abi1-1* and *abi2-1* mutants and the 35S::*HAB1* transgenic line were 20–80-fold more resistant than wild type (Figure 3C). In contrast to ABA application, no significant growth differences were obtained for DC3000*hrpA*⁻ (not shown). *Arabidopsis* ABA aldehyde oxidase 3 (At2g27150; AAO3), a cytosolically localised enzyme, catalyses the last step of ABA biosynthesis in response to drought stress (Seo and Koshiba, 2002). Multiplication of virulent DC3000 in an AAO3 T-DNA knockout line (SALK_072361) was significantly restricted compared

Figure 1 ABA levels increase during a compatible interaction in an inoculum concentration-dependent manner. (**A**) Time course of changes in ABA levels in Col-5 leaves after mock infiltration ($MgCl_2$, 10 mM) or challenge with DC3000 or DC3000 *hrpA*⁻. (**B**) ABA levels are dependent on DC3000 concentration. ABA levels in leaves challenged with DC3000 *hrpA*⁻ or increasing concentrations of DC3000 were measured 18 hpi. (**C**) Relative *in planta* levels of ABA, JA and SA during compatible (DC3000) and basal defence (DC3000*hrpA*⁻) responses. Hormones were measured in both non-inoculated and challenged (0.5×10^8 CFU/ml) Col-5 leaves at 18 hpi. (**D**) 9-*cis*-Epoxycarotenoid-dioxygenase3 (*NCED3*) is induced by T3Es concomitant with elevated foliar ABA. Time course of *NCED3* steady-state mRNA levels following DC3000 and DC3000*hrpA*⁻ challenge determined, in independent experiments, by RNA blot (upper panel) or RT–PCR. Bacterial titres and RT–PCR values (copies of target transcript/copy *actin 2*) are means of triplicate samples and error bars represent 1 s.d. All experiments were repeated at least twice with similar results.

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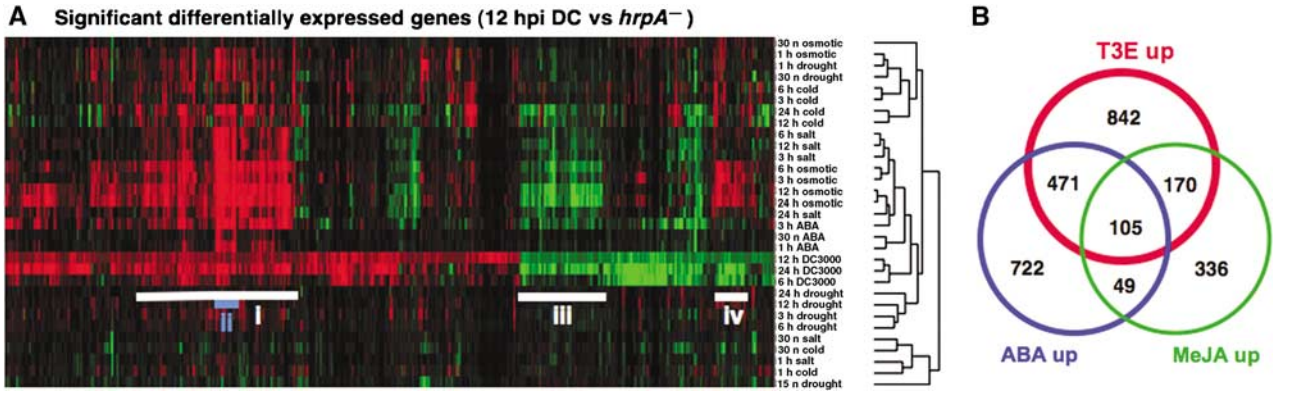

Figure 2 Hierarchal clustering of T3E-responsive transcripts shows a strong overlap with abiotic stresses. (**A**) The 880 probesets identified previously as significantly differentially expressed in response to T3Es at 12 hpi were clustered using GeneChip expression data from the AtGenExpress consortium. Experiments reporting the effects of cold, drought, salt stress, osmotic stress (mannitol) and ABA application were included as well as additional time points reporting T3E activity. All data sets were normalised and interpreted using the GCRMA function of the Bioconductor microarray analysis package (http://www.bioconductor.org/). Hierarchical clustering was applied using an uncentred correlation and complete linkage clustering. Genes induced relative to their control are coloured red, those suppressed are coloured green, whereas genes unchanged in their expression levels are coloured black. Cluster i, genes sharing strong similarity to regulatory networks induced by abiotic stresses, in particular, osmotic stress. Cluster ii, all the Clade A *PP2Cs* originally identified in Table I are highlighted in blue. Cluster iii, genes suppressed by both T3E and abiotic stresses. Cluster iv, genes suppressed by T3E but induced by abiotic stresses. (**B**) Venn diagram showing the commonality between transcripts differentially induced by T3Es and those strongly induced by ABA or MeJA application. SAM (Tusher *et al*, 2001) was used to identify genes induced by DC3000 relative to DC3000*hrpA*$^-$ or DC3000*hrcC* at 6, 12 and 24 hpi from two independent but similar experiments, with a minimum fold change of 2 and a false discovery rate of less than 5%. ABA- and MeJA-induced genes were identified solely on fold change taking the average of two replicates.

with wild type. We interpret these data to suggest that both *de novo* ABA biosynthesis induced by T3E delivery and PP2C activities collaborate to regulate pathogenicity.

ABA attenuates callose deposition associated with basal defence

The susceptible phenotype of the ABA hypersensitive *abi1*-suppressor mutants correlated with enhanced chlorosis and formation of water-soaked lesions on leaves 3–4 dpi (days post-infection) (Figure 4A). A hallmark of basal defence to attempted bacterial and fungal penetration is the deposition of callose in paramural deposits. We used aniline blue staining (Keshavarzi *et al*, 2004) to monitor callose deposition at the cellular level (Figure 4B). Compared with wild-type leaves challenged with DC3000 at 12 hpi (Figure 4Ba), both *abi1-sup5* (data not shown) and *abi1-sup7* (Figure 4Bb) were completely devoid of callose-associated fluorescence. By contrast, the ABA-insensitive mutants *abi1-1* (Figure 4Bc), *abi2-1* and the 35S::*HAB1* line (Figure 4Bd) showed augmented callose deposition, although no significant differences in the response were detected in mutant leaves following challenge with DC3000*hrpA*$^-$ (data not shown). Next, mock- (Figure 4Be) and ABA-treated (Figure 4Bf) leaves were compared following virulent DC3000 challenge. As predicted, less callose-associated fluorescence was detected in ABA-treated plants 12 hpi after DC3000 challenge than in control inoculated leaves (compare Figure 4Be with 4Bf).

ABA suppression of genes involved in basal defense

We have shown that effectors delivered intracellularly are able to downregulate certain PAMP-induced genes (PIGs; de Torres *et al*, 2006; Truman *et al*, 2006). To examine whether ABA itself contributes to suppression of these basal defence components, we used RNA blot and RT–PCR to examine the expression level of two *PIGs* suppressed by T3Es, *FRK1* (*flagellin-induced receptor kinase 1*) and the glycerol kinase encoding *NHO1* (Kang *et al*, 2003; Truman *et al*, 2006) in ABA-insensitive mutants challenged with DC3000 or DC3000*hrpA*$^-$. Figure 5 shows that suppression of both *FRK1* (Figure 5A and B) and *NHO1* (Figure 5A and C) is delayed in both *abi1-1* and *abi2-1* (ABA-insensitive) backgrounds. The effects of the *abi1-1* and *abi1-2* mutations appear to be to stabilise the relative amounts of defence transcripts. We are therefore unable to determine whether the delayed suppression of defence transcripts is due to enhanced innate immunity or an inability to activate suppression mechanisms. Whichever mechanism is operative, the elevated basal defence transcripts in the compatible interaction are consistent with the enhanced resistance to virulent DC3000 as shown in Figure 2.

Expression of the bacterial effector AvrPtoB in planta increases ABA levels

Conditional expression of the conserved *P. syringae* effector AvrPtoB increases susceptibility to DC3000*hrpA*$^-$, suppresses callose deposition and dramatically suppresses *PIG* transcripts (de Torres *et al*, 2006). Using transgenic plants carrying *avrPtoB* under the control of a dexamethasone (Dex)-activated promoter, we examined *NCED3*, *NHO1* and *FRK1* transcripts in response to $MgCl_2$ or DC3000*hrpA*$^-$ inoculations following Dex induction by RNA blot (Figure 6A) or RT–PCR (Figure 6B). As expected, Dex-treated leaves suppressed induction of *NHO1* and *FRK1* by DC3000*hrpA*$^-$. However, *avrPtoB* expression induced *NCED3* irrespective of the inoculation, suggesting that AvrPtoB alone can modify ABA signalling responses (Figure 6A and B). Notably, the levels of *NCED3* mRNA induced by Dex are ~5–6 times the levels obtained in a compatible interaction.

Consistent with these data, Dex treatment of Dex::*avrPtoB* transgenic plants resulted in a significant increase in ABA levels within 6 h of application (Figure 6C), confirming that AvrPtoB activity alone can induce ABA synthesis. In conjunc-

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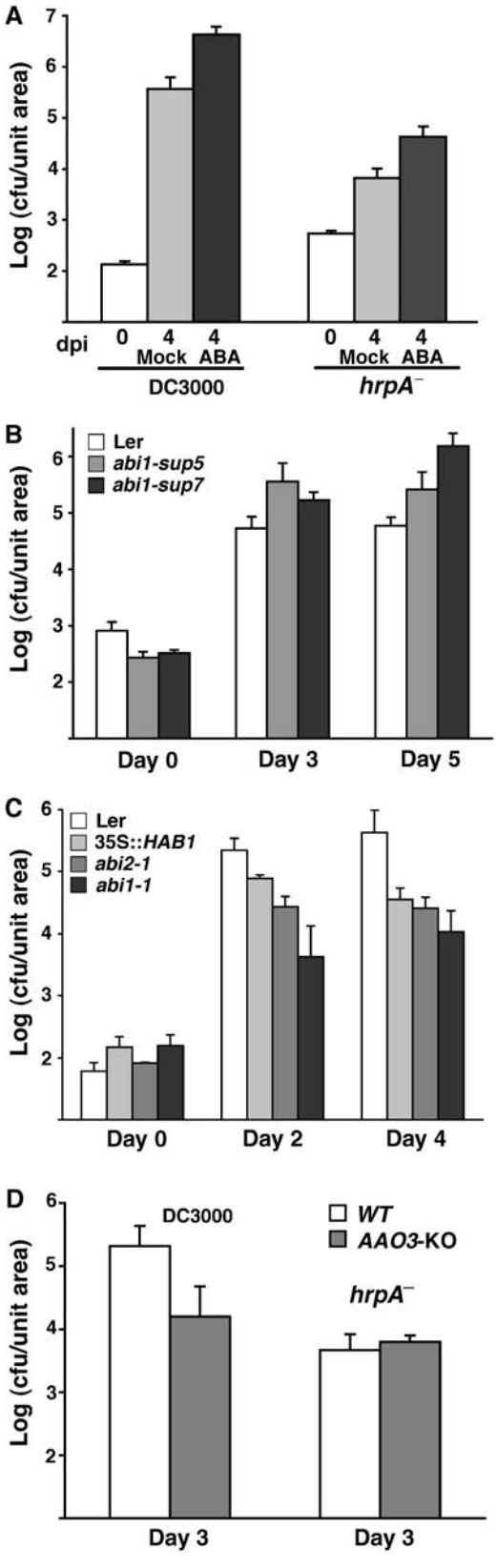
tion with analyses of the *PP2C* mutants, these data support the hypothesis that elevated ABA contributes to the modified basal defence phenotypes previously described by conditional induction of AvrPtoB (de Torres *et al*, 2006).



Discussion

Examples of signalling crosstalk between biotic and abiotic stress are emerging (Xiong and Yang, 2003; Anderson *et al*, 2004), but results are conflicting. Antagonism between biotic and abiotic stress responses has been reported for rice (Xiong and Yang, 2003). By contrast, other studies (Park *et al*, 2001; Mengiste *et al*, 2003; Chini *et al*, 2004) suggest that biotic and abiotic stress responses share common components, the eventual outcome being determined by the nature of the stress and the host genotype. Here we show that bacterial virulence factors specifically manipulate components of the ABA biosynthetic and response machinery, leading to an increase in ABA levels and signal responses in leaves of *Arabidopsis* plants. Application of ABA at physiologically relevant concentrations enhances the susceptibility of *A. thaliana* to two already virulent strains of *P. syringae*, DC3000 and M4 (data not shown), and also nonpathogenic DC3000*hrpA*$^-$. Collectively, these results suggest that ABA modifies general cellular metabolic homeostasis to facilitate bacterial growth, and can itself partially substitute for T3E activity. Moreover, conditional expression of a single bacterial effector, AvrPtoB, can enhance bacterial growth, elevate ABA levels and suppress PAMP-responsive genes. Our data suggest that *de novo* ABA biosynthesis is regulated by T3Es such as AvrPtoB through the induction of *NCED3*. These findings provide a mechanistic explanation for previous observations that ABA and/or drought stress increased susceptibility to the fungus *B. cinerea*, the oomycete *H. parasitica* and the avirulent *P. syringae* strain 1065 (Audenaert *et al*, 2002; Mohr and Cahill, 2003; Thaler and Bostock, 2004). Interestingly, apparently contradictory results demonstrate that ABA primes for callose accumulation and thereby enhances basal resistance in response to *Blumeria graminis* f sp *hordei* and activates induced resistance in response to the necrotrophic fungi *Alternaria brassicicola* and *Plectosphaerella cucumerina* (Ton and Mauch-Mani, 2004; Wiese *et al*, 2004). The apparently conflicting reports illustrate that ABA, like the other defence hormones JA/ET and SA, has effects which are dependent upon the mode of pathogenicity of the respective pathogen and possibly, also the nature of the PAMPs carried by each microbe.

Foliar ABA levels increased significantly within 12 hpi in a T3SS-dependent manner. DC3000 multiplied more rapidly and to greater titre in ABA hypersensitive plants compared with wild type. Restriction of virulent DC3000 growth was detected in the ABA-insensitive mutants *abi1-1* and *abi2-1*, in lines constitutively overexpressing *HAB1* and in the ABA biosynthetic mutant *aao3*. In *abi1-1* mutants, DC3000 multiplication only slightly outperformed the non-pathogenic *hrpA*$^-$ mutant, corroborating our observation that *hrpA*$^-$

Figure 3 Effect of exogenous application of ABA and ABA signalling mutants on the growth of compatible bacteria. (**A**) Growth of DC3000 and DC3000 *hrpA*$^-$ on Col-0 plants sprayed 24 h before inoculation with ABA (100 μM in 0.2% ethanol) or mock (0.2% ethanol). (**B**) Growth of DC3000 in the leaves of wild-type Ler and the ABA hypersensitive mutants *abi1-sup7* and *abi1-sup5*. (**C**) Growth of DC3000 in wild-type Ler and the ABA-insensitive mutants *abi1-1*, *abi2-1* or transgenic 35S::*HAB1* leaves. (**D**) Growth of DC3000 and DC3000 *hrpA*$^-$ in leaves of wild-type Col-0 and the ABA biosynthetic mutant *aao3*. Figures are representative of at least three replicate experiments. Error bars represent 1 s.d.

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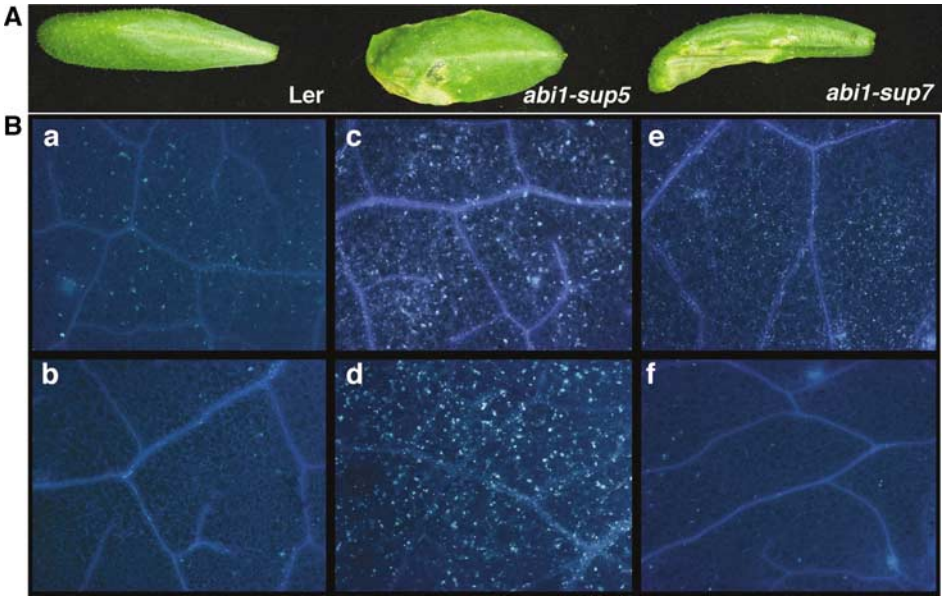

Figure 4 ABA signalling mutants have altered macroscopic and microscopic disease symptoms. (**A**) ABA-hypersensitive plants display enhanced chlorosis and necrosis 3 dpi following challenge with DC3000 (2.5×10^5 CFU/ml) compared with wild-type Ler. (**B**) Callose deposition in wild-type Ler (a) contrasts to reduced callose deposition in *abi1-sup7* (b) and enhanced callose deposition in *abi1-1* mutant and the transgenic 35S::*HAB1* line (c and d, respectively), 12 hpi with DC3000. Callose deposition in mock-treated (e) or ABA-treated (f) wild-type Col-0, 12 hpi with DC3000.

multiplication appears unaffected in *abi1-1* and *abi2-1* genetic backgrounds but is increased by ABA treatment in an ABI-1/ABI-2-dependent manner. ABI1 and ABI2 are PP2Cs that negatively regulate many aspects of ABA signalling. The reduced sensitivity to ABA observed in the *abi1-1* and *abi2-1* dominant mutants, and replicated in the *aao3* biosynthetic mutant, is probably owing to the formation of inactive complexes between the corresponding mutant proteins and master positive regulators of ABA signalling (Leung *et al*, 1997).

What is triggering ABA accumulation? Abiotic stress-induced ABA accumulation is a key factor in controlling downstream adaptive responses, including stomatal aperture, osmolyte accumulation and gene expression. However, the mechanism by which plants sense and signal the stress-induced changes in water status and initiate adaptive responses such as ABA accumulation and osmoregulation are unknown. The increased ABA levels observed under drought and salt stress are attained by the induction of genes that catalyse hormone synthesis. *NCED3* is the best candidate for direct regulation by upstream signalling and shows the strongest induction after water stress among all nine members of the gene family (Tan *et al*, 2003). *NECD3* was induced between 4 and 12 hpi following DC3000 challenge, consistent with the elevated foliar ABA levels within ~9 hpi (or ~6 h after *avrPtoB* Dex-induced expression). *NCED3* induction has therefore emerged as a possible target for effectors such as AvrPtoB.

What is the mechanism of ABA action? Manipulation of ABA biosynthesis and response pathways by T3Es represents a very powerful virulence strategy, as hormone homeostasis has a global impact on multiple cellular processes. One obvious physiological benefit for the pathogen would be ABA-mediated stomatal closure and consequent reduction in water loss. We examined the possibility that increases in ABA might lead to stomatal closure and, thereby, maintain potentially beneficial high water availability within the apoplast. Stomatal apertures were measured during the light periods 8 and 12 hpi with the *hrpA*$^-$ mutant or DC3000, but no significant difference in stomatal width/length ratios were found. Similarly, expression of AvrPtoB in the plant did not lead to stomatal closure 8, 12 or 27 h after Dex-mediated induction (see Supplementary data). We hypothesise that the effect of ABA accumulation on virulence is not simply through change in the physical conditions in the leaf, but through alteration of the signalling networks that coordinate plant defences. Suppression of the defensive transcriptional response requires a functional ABA signalling pathways and correlates positively with ABA levels.

It is fascinating that T3Es appear to have evolved to overcome basal defences, at least in part, through manipulating levels of a plant hormone. AvrPtoB, which is a member of the large HopAB family of effectors, singly appears to be able to suppress many components of basal defence, including alterations to the cell wall such as callose deposition (de Torres *et al*, 2006). Here we show that AvrPtoB induces *NCED3* and elevates foliar ABA levels when conditionally expressed *in planta*. Moreover, transcripts associated with key regulators of basal defence, *FRK1* and *NHO1*, are suppressed. Whether other individual effectors have evolved the ability to confer virulence through modifying ABA homeostasis remains to be determined.

ABA is normally associated with responses to abiotic stress and T3E-mediated increases in ABA may therefore exploit endogenous stress pathways to compensate for an enhanced metabolic load associated with pathogen nutrition in the apoplast. In addition to abiotic stress, ABA signal transduction is interlinked to the regulatory circuits of primary metabolism, cell growth and cell division. For instance, ABA plays a crucial role in the regulation of vegetative

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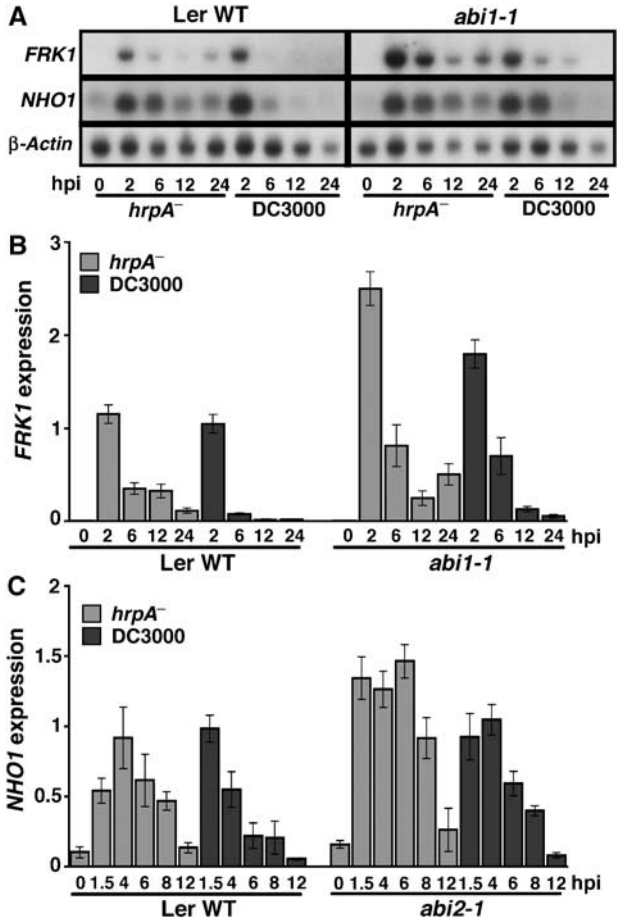

Figure 5 Suppression of defence gene transcripts is attenuated in ABA-insensitive mutants. (**A**) RNA blot of *FRK1* and *NHO1* transcript accumulation over time following DC3000*hrpA*$^-$ or DC3000 challenges of wild-type Ler (left hand panel) or the ABA-insensitive mutant, *abi1*-1. (**B**) Independent RT–PCR experiment showing suppression of *FRK1* accumulation in the *abi1-1* mutant is restricted after challenge with DC3000 compared with *hrpA*$^-$. (**C**) RT–PCR reveals that suppression of *NHO1* accumulation by DC3000 is also compromised in the *abi2-1* mutant. RT–PCR experiments measured copies of target transcript/copy *actin 2*, and were in triplicate. Error bars represent 1 s.d.

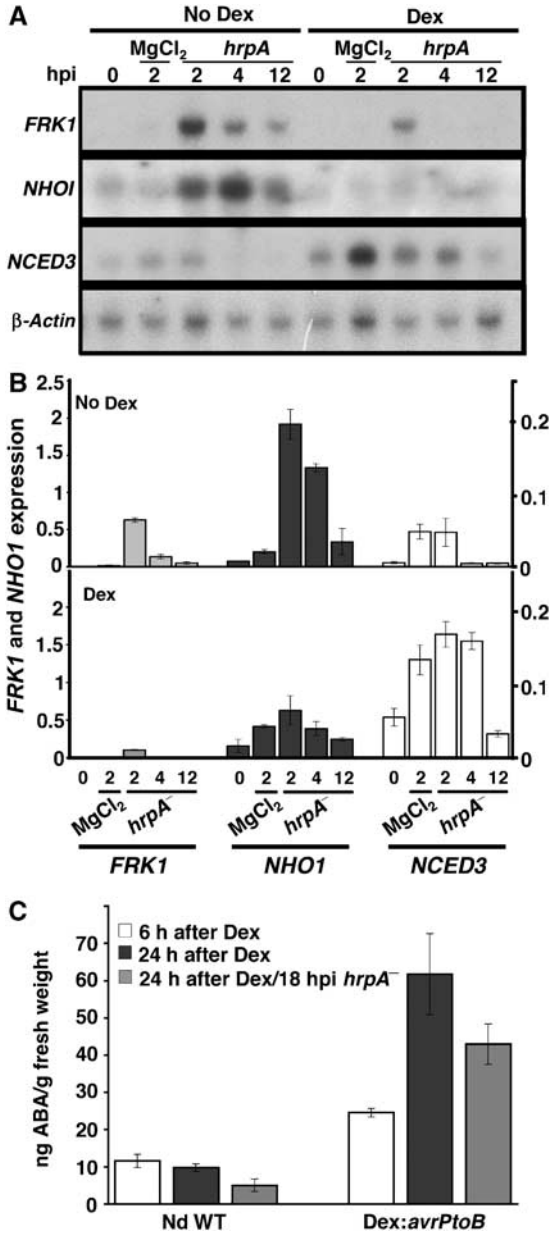

Figure 6 The bacteria effector, AvrPtoB, modifies basal defence transcripts and triggers elevated foliar ABA levels. (**A, B**) Six hours after Dex or mock treatment, Dex::*avrPtoB* transgenic plants were inoculated ($t = 0$) with $MgCl_2$ or DC3000*hrpA*$^-$ and harvested for RNA extraction at the times indicated. (**C**) Six hours after Dex treatment leaves from Nd-0 wild type or Dex::*avrPtoB* transgenic plants were inoculated with and without DC3000*hrpA*$^-$. ABA levels were determined at the times indicated after Dex treatment. (A) RNA blot showing time course of defence-related (*FRK1*, *NHO1*) or ABA biosynthesis (*NCED3*)-related transcript accumulation in control or *avrPtoB*-expressing plants after challenge with DC3000*hrpA*$^-$. The *actin 2* transcript was used as a loading control. (B) Independent experiment of (A), measuring transcript accumulation by RT–PCR with expression levels determined relative to the equivalent *actin 2* internal standard (copies of transcript/copy *actin 2*). Error bars represent 1 s.d. (C) Measurement of foliar ABA levels in *hrpA*$^-$-challenged and unchallenged Nd-0 (control) or Dex::*avrPtoB* plants 24 h following Dex induction. Error bars represent 1 s.d.

development by glucose (Cheng *et al*, 2002). The restriction of *Arabidopsis* growth, frequently associated with compatible *Pseudomonas/Arabidopsis* interactions (M de Torres, unpublished observation), may therefore represent a negative effect associated with elevated ABA levels. However, at the seed stage, ABA action is required for proper development, desiccation tolerance and long-term seed viability (Ooms *et al*, 1993), and therefore priming of ABA biosynthesis could confer enhanced survival to seed-borne Pseudomonads such as *P. syringae* pv. *tomato*.

Antagonism between ABA and the JA/ET signalling pathways was recently proposed as a strategy to regulate abiotic stress-related and biotic response pathways (Anderson *et al*, 2004). Given the large, independent overlap of ABA and JA responses with T3E-specific transcriptional reprogramming (Figure 3), our data suggest that both JA and ABA contribute to pathogenicity by the hemi-biotroph DC3000. These data are particularly interesting in light of recent results showing that ABA and SA-dependent PAMP-induced stomatal closure can be overcome by the *P. syringae* virulence factor coronatine, a JA mimic (Melotto *et al*, 2006). Thus, ABA appears to have a role in both pre-invasion innate immunity and post-

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invasion virulence. Analysis of relative ratios of these three hormones following challenge with coronatine-deficient strains of *P. syringae* pv. *tomato* (Brooks *et al*, 2004) should help resolve this issue.

Shen *et al* (2006) recently identified the H-subunit of Mg-chelatase as an ABA receptor (ABAR), which mediates seed germination, stomatal movement and post-germination growth. RNAi *ABAR* plants have corresponding ABA-insensitive phenotypes for these processes. The *ABAR* transcript is suppressed in plants challenged by DC3000 (Truman *et al*, 2006), suggesting that in addition to inducing negative regulators of ABA signalling (*PP2Cs*), T3Es also attenuate levels of the ABA receptor.

In summary, we have found that T3Es specifically target hormone homeostasis to promote virulence, and highlight a role for ABA alongside JA, SA and ET as phytohormone mediators of defence responses. How modification of these signalling processes contributes to pathogen multiplication remains to be elucidated. Whether or not ABA's role is simply in abrogating defensive cell wall alterations, or if it has additional roles in providing pathogen nutrition and the post-translational regulation of *PIGs* remains to be determined.

Materials and methods

Plants
Plants were grown under short days and 70% humidity, as previously described (de Torres *et al*, 2003). Source reference for the other *Arabidopsis* genotypes used are as follows: *abi1*-1 and *abi2*-1 (Kornneef *et al*, 1984), background La-*er*; 35S::*HAB1* (Saez *et al*, 2004), background Col-0; *abi1-1sup5* and *abi1-1sup7* (Gosti *et al*, 1999), background La-*er*; Dex::*avrPtoB* (de Torres *et al*, 2006), background Nd-0.

In planta bacterial population counts and time courses
Bacterial cultures were maintained, prepared and used for inoculation as described (de Torres *et al*, 2003, 2006). Final cell density was adjusted to OD_{600} 0.0002 ($\sim 1 \times 10^5$ CFU ml^{-1}) in 10 mM $MgCl_2$ for bacterial populations counts. For hormone determinations, RNA expression studies or phenotpye assays, bacterial cell densities were typically adjusted to OD_{600} 0.2 ($\sim 1 \times 10^8$ cfu ml^{-1}), unless otherwise indicated.

ABA treatment
ABA (Sigma, Dorset, UK) was solubilised in ethanol diluted in water. This ABA solution (100 μM, 0.2% ethanol) was sprayed on *Arabidopsis* plants. Control plants were treated identically with a solution of 0.2% ethanol. Assays were performed 24 h after ABA application.

Dex treatment
Conditional expression of *avrPtoB* was achieved by brushing leaves of Dex::*avrPtoB* plants with a Dex (Sigma, Dorset, UK) solution (6 μM in 0.02% Silwet). Control treatment was 0.02% Silwet alone (de Torres *et al*, 2006).

Hormone measurements
A detailed protocol for hormone measurements is included in Supplementary data.

Callose deposition
Epifluorescence microscopy was used to detect callose after staining with aqueous aniline blue as described in Keshavarzi *et al* (2004). Images were recorded using a Zeiss Axiophot camera and captured using Zeiss Axiovision software.

RNA extraction and RNA blots
Total RNA was isolated as described (de Torres *et al*, 2003). Probe templates were amplified by PCR from cDNAs or genomic DNA and labelled with [^{32}P]dCTP using the Prime-It®II labelling kit (Stratagene, California, USA). The genes, their AGI numbers and primers used and resultant amplicon size are detailed in Supplementary data.

RT–PCR
cDNA was generated from 1 μg of total RNA with SuperscriptIII (Invitrogen Corporation, California, USA) following the manufacturer's instructions. Quantitative PCR was performed on the cDNA using the QuantiTect SYBR Green PCR kit (Qiagen, West Sussex, UK) on a Rotor-Gene 3000 (Corbett Research, Cambridge, UK). Absolute quantification was determined by plotting standard curves using serial dilutions of the appropriate cDNA PCR products containing the target sequence. *Actin 2* was used as an internal standard to normalise cDNA content in the samples. Expression levels were calculated as number of copies of each particular mRNA per number of *Actin 2* copies. The primers used for RT–PCR and amplicon size are described in Supplementary data.

AAO3 T-DNA knockout
Seeds from SALK-072361 line were screened using the following gene-specific primers: F:5′-TTCTATTGGAAATGCATTGCC-3′; R:5′-CCATGTCTGCATGTTTCTGTG-3′ and the insertion-specific primer LBb1. Progeny from a homozygous plant were used for insertion subsequent experiments.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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