*The Plant Journal* (2006) **47**, 368–382 doi: 10.1111/j.1365-313X.2006.02798.x

# *Pseudomonas syringae* effector AvrPtoB suppresses basal defence in Arabidopsis

**Marta de Torres[1], John W. Mansfield[1,*], Nina Grabov[1], Ian R. Brown[1], Hassan Ammouneh[1,2], George Tsiamis[1,3], Alec Forsyth[1], Silke Robatzek[4], Murray Grant[1] and Jens Boch[5]**

[1]*Division of Biology, Imperial College London, Wye Campus, Ashford, Kent TN25 5AH, UK,*
[2]*Atomic Energy Commission of Syria, PO Box 6091, Damascus, Syria,*
[3]*Department of Environmental and Natural Resources Management, University of Ioannina, 2 Seferi str., 30100 Agrinio, Greece,*
[4]*Max-Planck-Institute for Plant Breeding Research, Carl-von-Linne-Weg 10, 50829 Cologne, Germany, and*
[5]*Institute of Genetics, Martin-Luther-University, Halle-Wittenberg, Weinbergweg 10, 06099 Halle (Saale), Germany*

Received 9 January 2006; revised 30 March 2006; accepted 3 April 2006.
*For correspondence: (fax: +02 075 9426 40; e-mail j.mansfield@imperial.ac.uk).

**Summary**

**The virulence and avirulence activities of members of the *Pseudomonas syringae* HopAB family of effectors and AvrPto were examined in bean, tomato and Arabidopsis. Proteins were delivered by the RW60 strain of *P. syringae* pv. *phaseolicola*. RW60 causes a hypersensitive reaction (HR) in bean and tomato but is restricted without the HR in Arabidopsis. Dual avirulence and virulence functions in tomato and bean, respectively, were identified in *virPphA* homologues but only *avrPtoB* strongly enhanced virulence to Arabidopsis, overcoming basal defences operating against RW60. Virulence activity in both bean and Arabidopsis required regions of the C-terminus of the AvrPtoB protein, whereas elicitation of the rapid HR in tomato, with the matching *Pto* resistance gene, did not. The effect of AvrPtoB on Arabidopsis was accession-specific; most obvious in Wassilewskija (Ws-3), intermediate in Columbia and not detectable in Niedersenz (Nd-1) after inoculation with RW60 + *avrPtoB*. Analysis of crosses between Ws-3 and Nd-1 indicated co-segregation for the AvrPtoB virulence function with the absence of the Nd-1 *FLS2* gene which mediates recognition of bacterial flagellin. *In planta* expression of AvrPtoB did not prevent the HR activated by *P. syringae* pv. *tomato* DC3000 + *avrB, avrRpm1, avrRps4* or *avrRpt2*, but suppressed cell wall alterations, including callose deposition, characteristic of basal defence and was associated with reprogramming of the plant's transcriptional response. The success or failure of AvrPtoB in suppressing basal defences in Nd-1 depended on the timing of exposure of plant cells to the effector and the flagellin flg22 peptide.**

**Keywords: innate immunity, effector proteins, plant disease resistance, bacterial pathogenicity.**

## Introduction

Recent advances in our understanding of the molecular mechanisms of bacterial pathogenicity have highlighted the role of effector proteins delivered into plant cells by the type III secretion system (TTSS; Alfano and Collmer, 2004; Büttner and Bonas, 2003; Nomura *et al.*, 2005). Certain effectors have been shown to suppress plant defences such as the hypersensitive resistance reaction (HR) and also what has been termed 'basal resistance' involving the localized reinforcement of plant cell walls at challenge sites (Abramovitch *et al.*, 2003; Brown *et al.*, 1995; Hauck *et al.*, 2003; Jamir *et al.*, 2004; Kim *et al.*, 2005). It has been estimated that pathovars of *Pseudomonas syringae* may utilize as many as 40 effector proteins (Alfano and Collmer, 2004; Greenberg and Vinatzer, 2003). The identification of effectors has been through functional screening and bioinformatics analyses based on the detection of promoter motifs or N-terminal structure favouring delivery through the TTSS (Chang *et al.*, 2005; Guttman *et al.*, 2002; Lindeberg *et al.*, 2005). Paradoxically, function has usually been demonstrated by the effector acting as an avirulence (*avr*) determinant, triggering the HR in plants with the cognate resistance (*R*) gene product (Espinosa and Alfano, 2004; Tsiamis *et al.*, 2000). The potentially dual role of effectors, as suppressors or inducers of resistance, may reflect the evolution of plant–pathogen

interactions occurring in the following sequence: (i) Effectors initially evolved within prototype pathogens to overcome basal defences in eukaryotes. (ii) In plants, recognition systems based on *R* genes allowed specific detection of injected effector proteins, triggering the HR and establishing gene-for-gene interactions. (iii) Loss of the recognized effector has often allowed pathogens to continue parasitizing resistant varieties of their hosts (to break gene-for-gene mediated resistance) because other effectors are able to suppress basal defences. The evidence for such a sequence is, however, based on limited functional analysis of very few effectors. Where enzymatic activities have been demonstrated, for example AvrPphB and AvrRpt2 have been identified as cysteine proteases (Kim *et al.*, 2005; Zhu *et al.*, 2004) and HopPtoD2 found to possess protein tyrosine phosphatase activity (Espinosa *et al.*, 2003), the full significance of such activity for suppression of defence is not clear. In some interactions results suggest that the mode of action of effector proteins operating against basal defences may also lead to interference with the HR (Jackson *et al.*, 1999; Jamir *et al.*, 2004; Tsiamis *et al.*, 2000).

Comparative bioinformatics has grouped effectors into families of structurally related proteins (Lindeberg *et al.*, 2005). For example, the HopAB family contains homologues of VirPphA (Jackson *et al.*, 1999). This effector was the first to be identified based on virulence function because of its ability to restore pathogenicity to RW60, an attenuated, plasmid-cured strain of *P. syringae* pv. *phaseolicola* (hereafter, *Pph*). The HopAB1 subfamily contains effectors very similar to VirPphA from *P. syringae* pvs *glycinea* and *savastanoi* (Jackson *et al.*, 2002). A second subfamily, HopAB2, includes AvrPtoB which was first identified as an Avr protein triggering the HR in tomato through its direct interaction with the cognate *R* gene product Pto (Abramovitch and Martin, 2005). A second effector, AvrPto, also interacts with Pto and was the first bacterial protein to be shown to suppress defensive cell wall alterations, particularly callose deposition, in Arabidopsis. Expression of AvrPto *in planta* allowed colonization by a non-pathogenic mutant of the Arabidopsis pathogen *P. syringae* pv. *tomato* DC3000 with a deletion in *hrcC* which encodes a core component of the TTSS (Hauck *et al.*, 2003).

Deposition of callose is one of the most striking responses of Arabidopsis to the bacterial flagellin peptide, flg22 (Gomez-Gomez and Boller, 2000, 2002). Core microbial molecules such as flg22 which elicit defences have been termed 'pathogen-associated molecular patterns' (PAMPs). Ausubel (2005) has, however, rightly criticized the use of PAMP, preferring the term 'microbe-associated molecular pattern' because the elicitors are also present in non-pathogenic microbes. The perception of PAMPs provides the basis for innate immunity in animals and invertebrates (Janeway and Medzhitov, 2002). Recognition of flg22 in Arabidopsis requires FLS2, which is a leucine-rich receptor protein with some similarity to the mammalian TOLL family of receptors (Asai *et al.*, 2002; Beutler, 2005; Gomez-Gomez and Boller, 2002). The first articles on FLS2 did not indicate a clear role for the flagellin perception system in basal resistance in Arabidopsis because *fls2* mutants were found to be as resistant as wild-type plants to *hrp* mutant bacteria or pvs of *P. syringae*, such as *Pph*, which are unable to cause symptoms or multiply in Arabidopsis. Increased susceptibility was, however, demonstrated to inocula of DC3000 sprayed onto the leaf surface (Zipfel *et al.*, 2004). More recently a clear role in non-host resistance has emerged from examination of the pathogenicity of flagellin-deficient mutants of *P. syringae* pv. *tabaci* to Arabidopsis (Li *et al.*, 2005). Suppression of flg22-induced expression of the *NONHOST1* (*NHO1*) gene in protoplasts has also been shown for several effectors including AvrPto, but members of the HopAB family were not tested in this assay (Li *et al.*, 2005). The Arabidopsis *NHO1* gene encodes a glycerol kinase which is required for resistance to many strains of *P. syringae* that are not pathogens of *Arabidopsis thaliana*, but is ineffective against DC3000 (Kang *et al.*, 2003).

Here, we focus on the interaction between the effector AvrPtoB and defence systems in Arabidopsis. Unlike other HopAB family members, or AvrPto, AvrPtoB promoted lesion formation by the plasmid-cured RW60 strain of *Pph.* The virulence-enhancing effect of AvrPtoB was most clearly observed in accessions such as Wassilewskija (Ws-3) lacking *FLS2* whereas Niedersenz (Nd-1) remained resistant. In mapping populations of Nd-1 and Ws-3, the virulence effect of AvrPtoB segregated with the lack of a single dominant *R* gene which was identified as the Nd-1 allele of *FLS2*. Symptomless resistance to RW60 $\pm$ *avrPtoB* was associated with striking alterations to the plant cell wall. Although delivery of AvrPtoB by RW60 was unable to suppress FLS2-based resistance in Nd-1, suppression of defence was observed if the effector protein was expressed *in planta*, The promotion of bacterial colonization in Arabidopsis expressing AvrPtoB was linked to specific suppression of genes involved in basal defence, including *NHO1*. We propose that the outcome of the interaction between effectors and basal defences depends not only on the intrinsic activity of the effector but also the speed with which it is delivered and the time taken to reach an effective dose in the plant cell.

## Results

### *Differential virulence activities of HopAB family members in Arabidopsis, bean and tomato*

Our earlier experiments using *Pph* strain RW60 to deliver VirPphA demonstrated its dual activity, acting not only as a gene for virulence promoting colonization in French bean but also as an avirulence gene leading to induction of the HR

 

in soybean (Jackson *et al.*, 1999, 2002). We extended the screening experiment to compare the activities of other HopAB family members on Arabidopsis in which RW60 caused no macroscopic symptoms (Soylu *et al.*, 2005), and on bean and tomato, both of which respond to RW60 with the HR. AvrPto is not closely related to AvrPtoB in structure (Abramovitch *et al.*, 2003; Wu *et al.*, 2004), but interacts with the same resistance protein, Pto, in tomato. Because of the possibility of further functional homologies between the two effectors we therefore included AvrPto in the comparative study. In order to avoid differences caused by levels of expression, all genes were cloned into pDSK600 under regulation of the same constitutively active promoter in the vector.

Completion of the interaction matrix summarized in Table 1 showed that *hopPmaL* and *hopPmaN*, both of which appear to be truncated forms of the *hopAB* backbone (Lindeberg *et al.*, 2005), lacked activity in any assay. Virulence was found in bean as indicated by the production of water-soaked lesions in pods of cv. Tendergreen rather than the brown HR lesion typical of RW60 (Figure 1). In Arabidopsis, the development of symptoms was enhanced by *avrPtoB* but not by other HopAB homologues. The effects caused by *avrPtoB* were accession-dependent. In Ws-3, infiltration sites collapsed 3–4 days after inoculation whereas an increase in yellowing with occasional patchy collapse was found in Col-5, as shown in Figure 1(a). We constructed deletion derivatives of *avrPtoB* to determine the protein domains involved in these activities. Successive deletion of amino acids from the C-terminus of AvrPtoB led to loss of virulence to bean, then to Arabidopsis and finally failure to elicit the HR in tomato (Figure 1b and c, and Table 1). Avirulence activities were detected in tomato Rio Grande (R) containing the resistance gene *Pto*, in which the speed of the HR collapse caused by RW60 containing the empty vector was greatly increased by several HopAB family members, as shown in Table 1. The HR caused by RW60 in Rio Grande (S), lacking *Pto*, was not modified.

### *Promotion of colonization by AvrPtoB in Arabidopsis*

The enhanced symptom development caused by RW60 expressing *avrPtoB* was examined more closely in a range of accessions after development of a five-point symptom index based on increasing yellowing and collapse of inoculated tissues. The semi-quantitative system allowed clear differentiation of reactions between accessions and highlighted the activity of *avrPtoB*. Fifty accessions were examined for their response to RW60 ± *avrPtoB*, and grouped into (i)

**Table 1** Use of *Pseudomonas syringae* pv. *phaseolicola* strain RW60 as a delivery vehicle to examine the activities of VirPphA homologues in different plants

| | | Activity detected if delivered by RW60[a], virulence in bean or Arabidopsis, or avirulence in tomato | | |
|---|---|---|---|---|
| Homologue | Predicted size (amino acids) | Bean cv. Tendergreen | Arabidopsis Ws-3 | Tomato cv. Rio Grande (R) |
| VirPphA | 540 | Vir | × | Hypersensitive reaction (HR) |
| $VirPphA_{pgy}$ | 523 | Vir | × | HR |
| $VirPphA_{psv}$ | 541 | Vir | × | HR |
| AvrPtoB | 553 | Vir | Vir | HR |
| AvrPtoBΔ1 | 532 | Vir– – | Vir– | HR |
| AvrPtoBΔ2 | 508 | × | Vir– – | HR |
| AvrPtoBΔ3 | 483 | × | Vir– – | HR |
| AvrPtoBΔ4 | 432 | × | × | HR |
| AvrPtoBΔ5 | 372 | × | × | HR |
| AvrPtoBΔ6 | 344 | × | × | × |
| AvrPtoBΔ7 | 299 | × | × | × |
| AvrPto | 164 | × | × | HR* |
| HopPmaL | 385 | × | × | × |
| HopPmaN | 155 | × | × | × |

[a]In bean cv. Tendergreen, RW60 alone causes an HR-like reaction, Vir indicates formation of a water-soaked susceptible lesion. In Arabidopsis, RW60 fails to cause symptoms, virulence activity was indicated by lesion formation. In tomato Rio Grande (R), RW60 causes the HR after about 9 h, avirulence activity of homologues was identified by more rapid HR development within 5 h of inoculation. The minus (–) sign(s) indicate reduced effects and '×' indicates no change in the phenotype produced by RW60 + empty vector controls. The asterisk (*) indicates that RW60 + *avrPto* consistently caused a slightly more rapid HR than RW60 + *avrPtoB* constructs. Inoculum concentrations used in bean and Arabidopsis were about 1.5 or $3 \times 10^8$ and in tomato 0.5 or $1 \times 10^8$ cells $ml^{-1}$. The same reactions were observed in at least three repeated experiments.

 

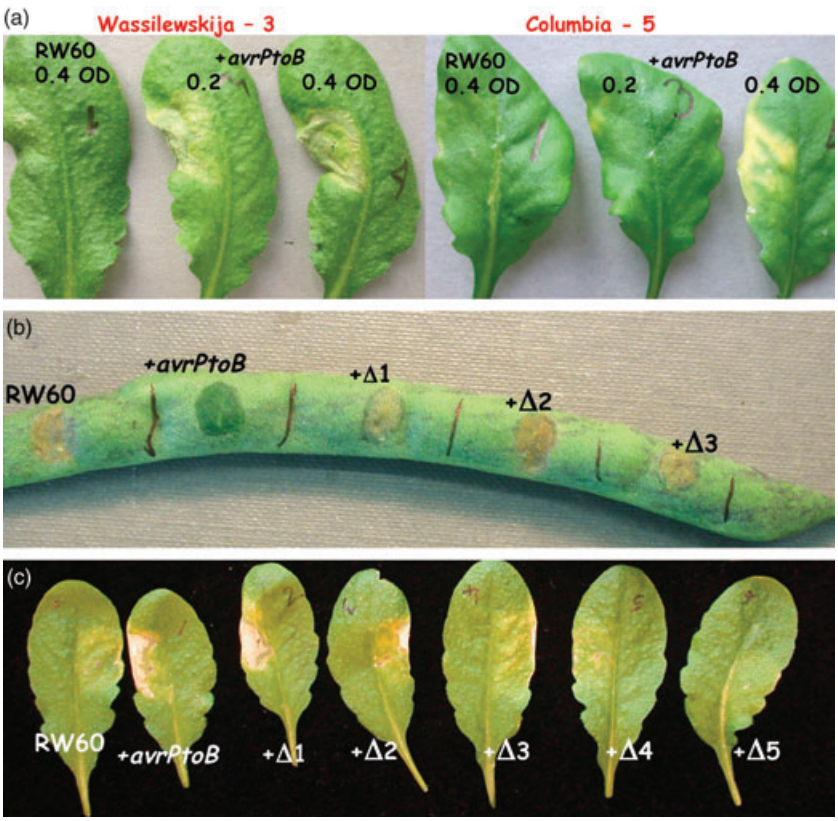


**Figure 1.** AvrPtoB promotes the virulence of *Pseudomonas syringae* pv. *phaseolicola* RW60 to Arabidopsis and bean.
(a) Reactions of accessions Ws-3 and Col-5 to RW60 + blank vector pDSK600 controls (RW60) or RW60 + *avrPtoB*. AvrPtoB has stronger effects in Ws-3 than in Col-5 at inoculum densities of 0.2 or 0.4 $OD_{600\ nm}$.
(b), (c) Truncation of AvrPtoB from the C-terminus of the protein leads to loss of virulence function in bean and Arabidopsis. In (b) a pod of bean cv. Tendergreen was inoculated with strains of RW60 containing pDSK600 alone, the full length AvrPtoB ORF construct (553 amino acids) or deletions to Δ1 (532), Δ2 (508) and Δ3 (483 amino acids).
(c) Symptom development in Arabidopsis Ws-3 leaves inoculated in one half with RW60 + *avrPtoB* deletions as in (b) with the addition of Δ4 (432) and Δ5 (372 amino acids).

those forming distinct lesions, (ii) those with some increase in symptoms or (iii) those with no change in response. Accessions Ws-3 (or Ws-0) and Ws-*eds* displayed the clearest differentiation between RW60 (resistant) and RW60 + *avrPtoB* (susceptible). The commonly studied accession Landsberg erecta (La-er) was grouped with Columbia (Col-0 or Col-5) in category (ii), whereas Nd-1 remained virtually symptomless (Figure 2).

Electron microscopy showed that in Col-5 (or-0), Nd-1 and Ws-3 the restriction of multiplication of RW60 was associated with striking alterations to the plant cell wall, including deposition of callose-rich papillae (Figure 3a). Bacteria appeared to be agglutinated within the apoplast. In Nd-1 deposits were characterized by the inclusion of regions of electron density indicative of the incorporation of phenolics at reaction sites (Bestwick *et al.*, 1998). No changes in reaction were observed in Nd-1 challenged with RW60 + *avrPtoB* (Figure 3b), but in the other accessions, particularly in Ws-3, certain colonies continued to multiply. In the more susceptible accessions, although deposition of papillae was observed after inoculation with RW60 + *avrPtoB*, alterations to the cell wall typically lacked electron dense inclusions and the response did not appear to lead to the restriction of bacterial multiplication. The colonization-promoting effect of *avrPtoB* was most pronounced at infection sites located next to vascular parenchyma in Ws-3 (Figure 3c). Such cells had often collapsed by 24 h after inoculation with RW60 + *avrPtoB*. In the Arabidopsis accession Ws-3, *avrPtoB* clearly allowed RW60 to overcome basal

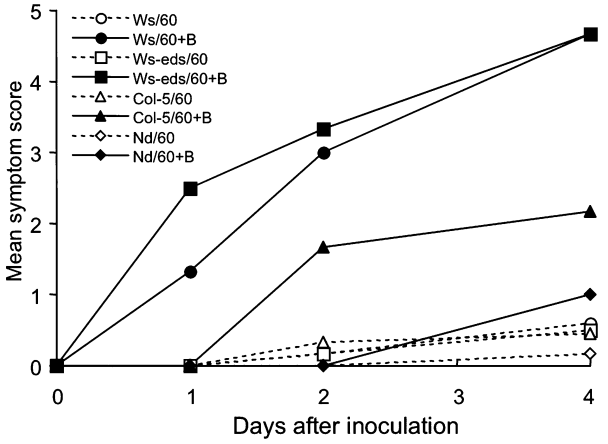


**Figure 2.** Differential activity of AvrPtoB in accessions of Arabidopsis.
Symptom development at inoculation sites was assessed using a five-point scale with 5 reflecting collapse of infiltration sites. Data are the average scores from 16 leaves and compare *P. syringae* pv. *phaseolicola* RW60 (pDSK600), marked as 60, and RW60 + *avrPtoB* (60 + B). Note the striking enhancement of lesion formation in Ws-3 and Ws-*eds*. Similar differences were observed in at least three repeated experiments.

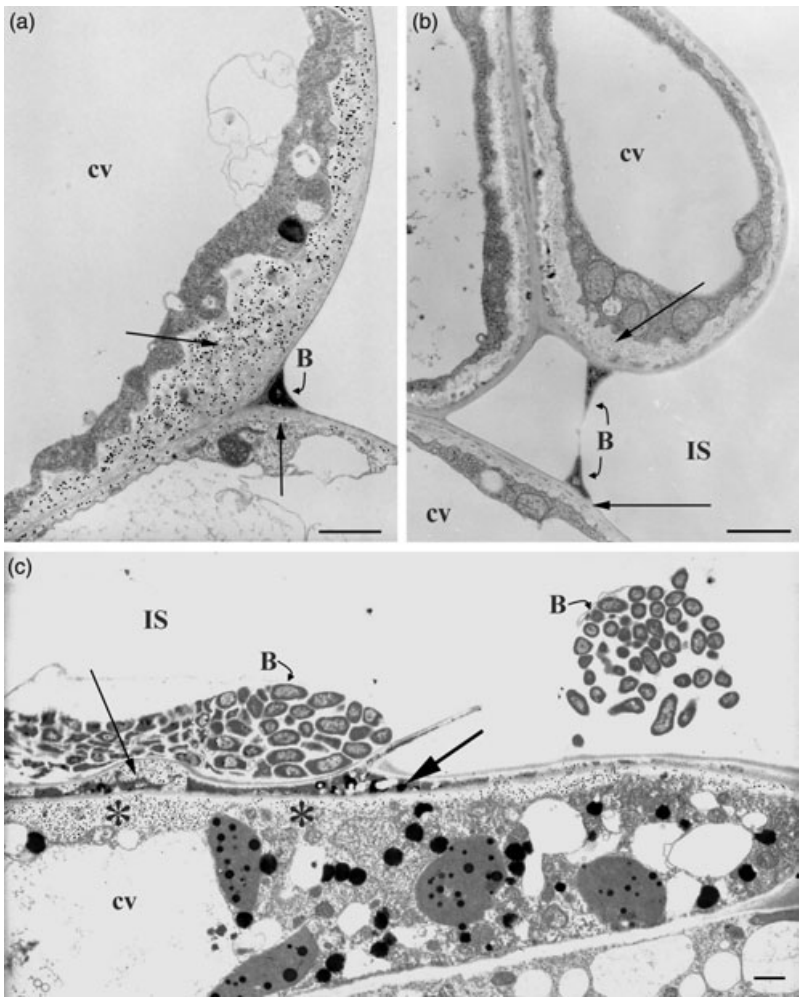


**Figure 3.** Electron microscopy shows that restriction of colonization by *P. syringae* pv. *phaseolicola* RW60 in Arabidopsis is associated with cell wall alterations in mesophyll cells adjacent to bacteria and that AvrPtoB promotes bacterial multiplication in Ws-3.
(a) Restriction of RW60 (pDSK600) in Ws-3; bacteria (B and curved arrow) appear to be agglutinated in the intercellular space and electron dense and translucent deposits containing callose (gold label) have accumulated in the adjacent plant cells (arrows).
(b) RW60 + *avrPtoB*, like RW60 alone, fails to multiply in Nd-1 in which responding mesophyll cells develop extensive papillae (fine arrows) containing electron dense inclusions. In (a) and (b) leaves were sampled 1 day after inoculation.
(c) RW60 + *avrPtoB* proliferates in Ws-3 at a site close to vascular parenchyma cells which have collapsed 3 days after inoculation (broad arrow). Although papillae have formed (fine arrow) they are more dispersed and contain less electron dense material than in cells challenged by RW60 (pDSK600). Cells in the vascular tissue adjacent to the necrotic plant cell have produced a layer of callose (asterisks). Images (a) and (c) are from sections immunogold labelled with anti β1-3 glucan monoclonal antibody. Scale bars = 1 μm; B (with curved arrows), bacterium; cv, cell vacuole; IS, intercellular space.

resistance at certain sites within infiltrated tissues leading to the development of macroscopic symptoms.

### *The AvrPtoB-specific virulence in Ws-3 is due to the absence of functional FLS2*

The differential reactions of Nd-1 and Ws-3 to RW60 + *avrPtoB* were followed in $F_1$ and $F_2$ progeny. In the $F_1$ generation all plants behaved like Nd-1 and in the $F_2$ generation a clear segregation was observed between lesion-forming (susceptible) categories and those producing no lesions (resistant). The initial ratio of resistant:susceptible observed was 284:89, closely matching the 3:1 ratio expected if symptomless resistance was confirmed by a single dominant gene. This gene was mapped using cleaved amplified polymorphic sequences (CAPS) markers to a position on chromosome 5 between *At5g46110* (at 18.715 Mbp) and *At5g48010* (at 19.475 Mbp). The recombinant interval contained the gene encoding the flagellin receptor FLS2 (Felix *et al.*, 1999; Gomez-Gomez and Boller, 2000). Ws-0 is known to lack a functional *FLS2* allele due to a mutation introducing a stop codon before the kinase domain of the protein, which also generates a *Hpy*188III restriction site. We designed CAPS markers to differentiate the *FLS2* alleles of Ws-3 and Nd-1 in our mapping population. Resistance to RW60 + *avrPtoB* was absolutely linked to the presence of the Nd-1 allele of *FLS2*, and both heterozygous and homozygous genotypes were equally resistant in terms of the lack of visible symptoms.

The ability of FLS2 to maintain resistance to RW60 + *avrPtoB* was confirmed using La-er wild type and *fls2-17* mutant lines and through complementation tests using transgenic Ws-0 expressing the Col-0 allele of *FLS2* (Figure 4). As predicted from our mapping, the expression of *FLS2* in Ws-0 led to symptomless resistance to RW60 + *avrPtoB* which, in terms of the failure of bacteria to multiply, was as effective as that observed in wild-type Nd-1 (Figure 4a, b). Conversely, the *fls2-17* mutant of La-er had greatly enhanced susceptibility, allowing detection of the AvrPtoB virulence function (Figure 4c, d). The *fls2-17* mutant was also slightly more susceptible to colonization and lesion formation by RW60 alone or a *hrpA* mutant of DC3000 (with no TTSS), but

 

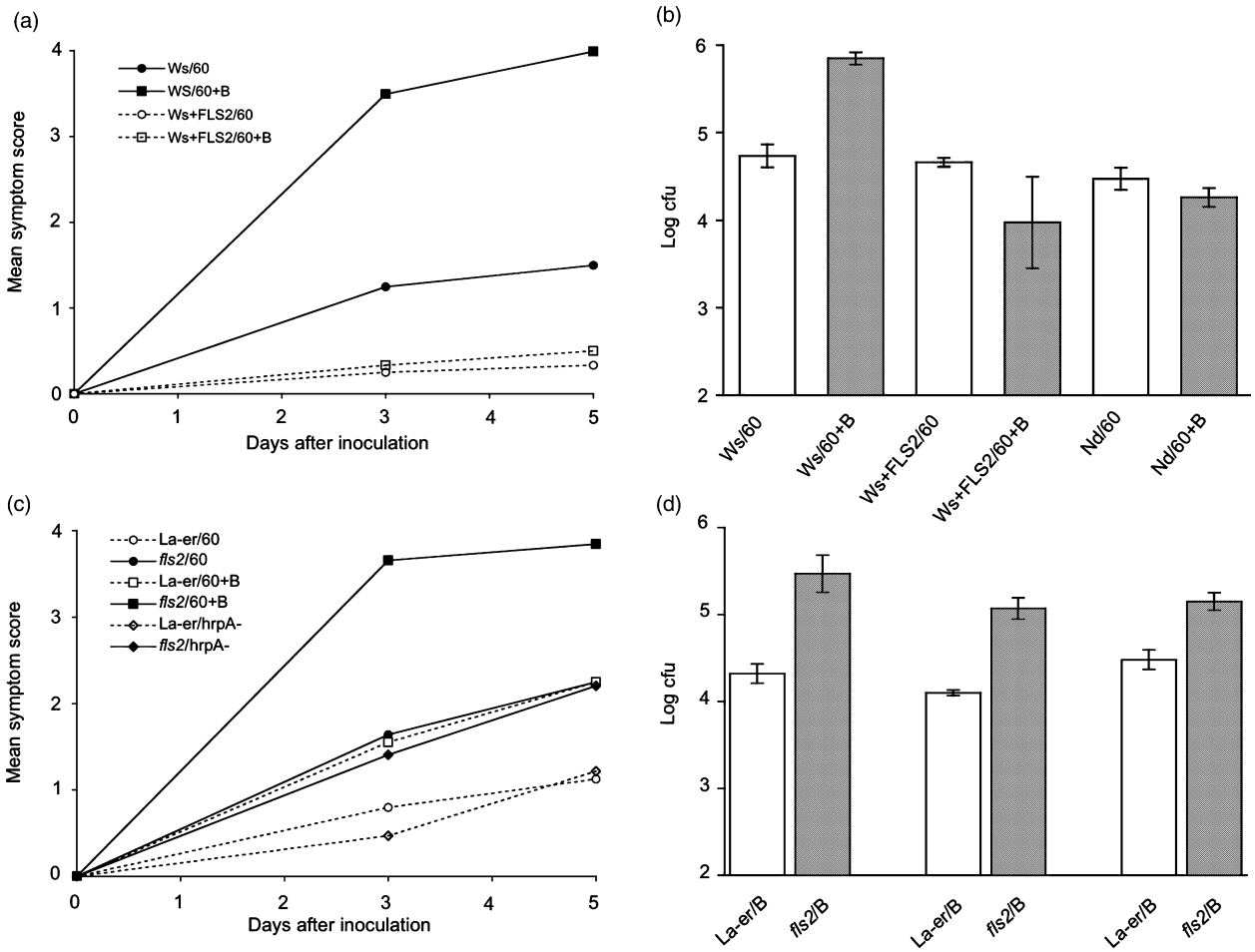


**Figure 4.** The AvrPtoB virulence effect is detectable in the absence of functional FLS2.
(a), (b) Complementation of Ws-0 to resistance to RW60 + *avrPtoB* by the stable expression of *FLS2*.
(a) Symptom development was recorded in 16 leaves of Ws-0 or Ws-0: *FLS2* after inoculation with RW60 (pDSK600) or RW60 + *avrPtoB*.
(b) Bacterial numbers (means ± SD) are reported from infiltration sites sampled 4 days after inoculation and include comparative data with the naturally resistant Nd-1.
(c), (d) The *fls2-17* mutant of La-er (*fls2*) displays increased susceptibility to RW60 + *avrPtoB* both in terms of (c) symptom development and (d) bacterial numbers at infiltration sites. Population data are presented from three separate experiments and record numbers 3 days after inoculation (means ± SD). For symptom development and population counts bacteria were inoculated at $OD_{600\ nm}$ of 0.25 and 0.05, respectively. As indicated for population data (d), similar differences were observed in at least two repeated experiments.

RW60 + *avrPtoB* achieved much more striking increases in symptoms. The same susceptibility phenotypes were demonstrated by both lesion formation and bacterial multiplication. In addition to confirming the role of the flagellin receptor in basal resistance to RW60, our results demonstrated that AvrPtoB is much more able to overcome basal resistance in the absence of FLS2.

### *Expression of AvrPtoB in the plant suppresses resistance to RW60 and* hrp *mutants*

The inducible expression of certain effectors in plants has helped to clarify their role as suppressors of plant defence (Hauck *et al.*, 2003; Kim *et al.*, 2005). We therefore carried out similar experiments using AvrPtoB. We generated homozygous transgenic lines of Nd-1 and Ws-3 expressing *avrPtoB* from a dexamethasone (Dex)-inducible promoter (Aoyama and Chua, 1997). Both accessions responded to *in planta* induction of AvrPtoB, the symptoms observed and response to bacterial challenge being correlated with the level of transgene expression achieved in different plants. Expression of *avrPtoB* in the Nd-1 transgenics was consistently high. In Ws-3, however, some plants from homozygous $T_2$ progeny no longer responded to Dex treatment and did not accumulate *avrPtoB* transcript (data not shown). It was possible to select Ws-3 Dex: *avrPtoB* plants differing in their response by treating detached leaves with 30 μM Dex and following reactions after their incubation at high humidity. Leaves and their plants of origin were classified as low, medium or high responders depending on the recorded

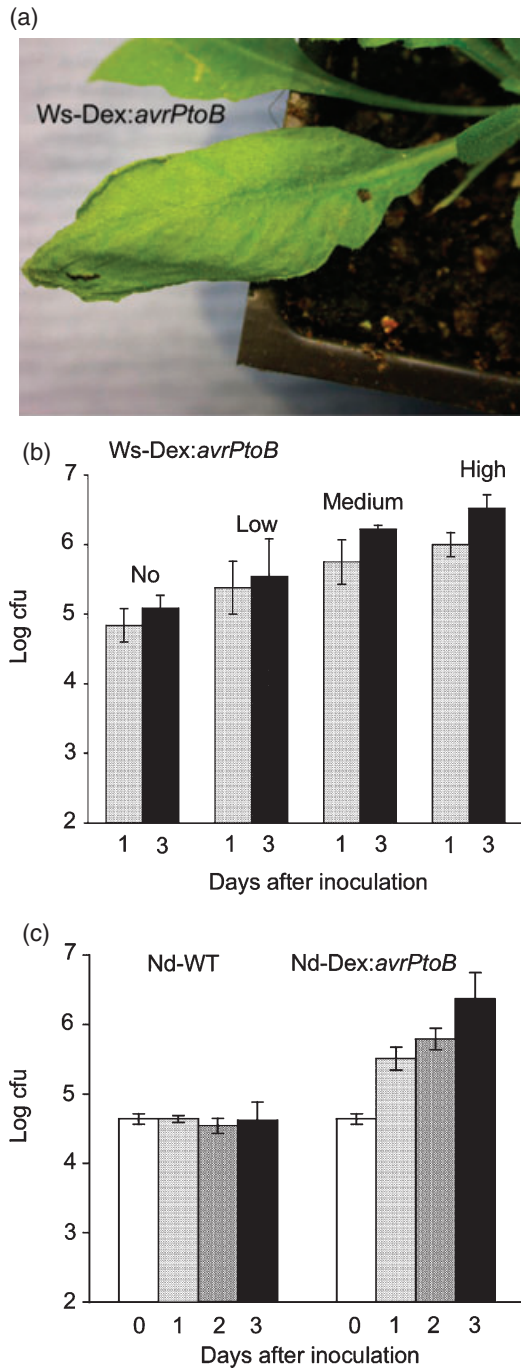


**Figure 5.** Expression of AvrPtoB in Arabidopsis from a dexamethasone (Dex) = inducible promoter causes symptom development and suppresses basal resistance to a *hrpA* mutant of *P. syringae* pv. *tomato* DC3000.
(a) Partial wilting of a leaf of a Ws-3Dex: *avrPtoB* transgenic plant 3 days after painting with a solution of 6 μM Dex in 0.02% silwet.
(b) Promotion of colonization of DC3000 *hrpA*–was correlated with the expression of AvrPtoB in Ws-3 Dex: *avrPtoB* transgenic plants assessed from detached leaf tests as having no, low, medium or high response. Leaves were treated with 6 μM Dex 6 h before inoculation.
(c) Induction of AvrPtoB in leaves of Nd-1 Dex: *avrPtoB* by treatment with 6 μM Dex 6 h before inoculation promoted multiplication of a DC3000 *hrpA* mutant. All inocula were at $OD_{600\ nm}$ of 0.05; means ± SD are given. Similar trends were observed in at least two repeated experiments.

speed of Dex-induced collapse, the most rapid responses being observed after 36 h. Expression of *avrPtoB* in leaves on the plant caused wilting, which developed most rapidly (after 3 days) in high-level responders (Figure 5a). *In planta* expression of *avrPtoB*, induced 6 h before inoculation by treatment with 6 μM Dex allowed greatly increased bacterial multiplication in both Nd-1 and Ws-3 within 1 day of challenge, not only with RW60 but also the TTSS-compromised *hrpA* mutant of DC3000 (Figure 5b, c).

We tested the effect of AvrPtoB expression on the response of Nd-1 to flagellin peptide which was infiltrated as an aqueous solution at 10 μg $ml^{-1}$. The response monitored was the accumulation of callose in paramural deposits which was detected by autofluorescence after aniline blue staining (Figure 6). Effects were found to depend on the time between induction with Dex and elicitation with flg22. Only minor suppression of callose deposition was observed if plants were infiltrated with flg22 2 h before Dex treatment, but much greater reduction was observed with simultaneous inoculation, and with a 3–6 h delay between treatments the plant's response was almost completely suppressed (Figure 6, Table 2). The injection method used involves making a very small cut in the abaxial epidermis to facilitate infiltration. Callose is typically deposited around the wound in Arabidopsis independent of a functional FLS2 receptor system. We noticed that expression of AvrPtoB also reduced wound-induced callose deposition, and again the effect was clear only if Dex induction preceded wounding by 6 h (Figure 6a–d).

Examination of the induced susceptibility of Nd-1 Dex: *avrPtoB* to the DC3000 *hrpA* mutant by electron microscopy (EM) between 6 and 72 h after inoculation revealed a general suppression of cell wall alterations and, finally, collapse of mesophyll cells next to bacterial colonies (Figure 7a–c). No significant deposition of papillae was observed in the AvrPtoB-expressing plants, even when bacterial colonies contained few bacteria 6–12 h after inoculation. As noted in the microscopy of the RW60 + *avrPtoB* challenge, colony expansion was particularly striking next to vascular parenchyma cells.

### In planta *expression of AvrPtoB does not prevent gene-for-gene mediated HR*

Induction of *avrPtoB* in Nd-1 Dex: *avrPtoB* or Ws-3 Dex: *avrPtoB* by treatment with 6 μM Dex 6 h before inoculation did not delay the tissue collapse observed during the HR triggered by DC3000 + *avrB, avrRpm1, avrRps2* or *avrRps4*. The AvrRpm1:RPM1 interaction was studied only in Ws-3 because Nd-1 does not carry the *RPM1* resistance gene. Macroscopic collapse during the HR in transgenic plants with or without Dex induction was associated with the same patterns of emission of biophotons (Bennett *et al.*, 2005 and data not shown).

 

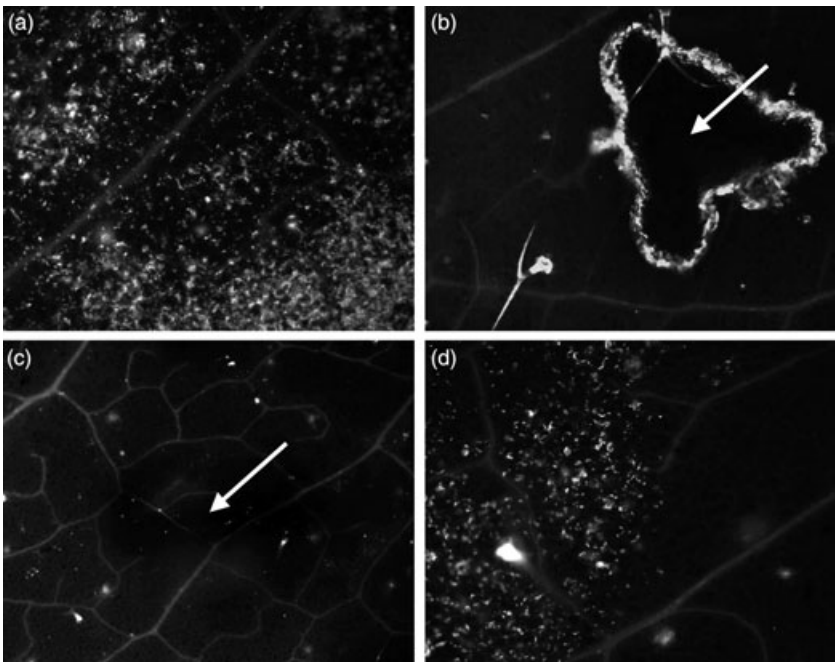


**Figure 6.** Effect of AvrPtoB expression on the response of Arabidopsis accession Nd-1 to the flg22 flagellin peptide (10 μg ml$^{-1}$).
Assessment was at 16 h after infiltration by the formation of scattered deposition of callose which was detected by light blue fluorescence under ultraviolet excitation after staining with aqueous aniline blue. Callose deposition seen at infiltration sites in (a) Nd-1 and (b) Ws-3 after infiltration; note the absence of response in Ws-3 apart from around the wound site used for infiltration (arrowed), a response also observed without flg22 treatment.
(c), (d) Responses in Nd-1 Dex: *avrPtoB* with 6 μM Dex treatment 6 h before and 2 h after flg22 infiltration, respectively; note that the early treatment with Dex suppressed not only the flg22-activated response but also callose deposition normally induced in cells around the wound (arrowed) which is at the centre of image (c). (d) Shows the edge of the zone of infiltration outside of which no callose is induced. The bright fluorescence of trichomes which contain callose is shown in (b) and (d). Similar reactions were observed in all of the three replicate leaves examined for each treatment.

**Table 2** Callose deposition in transgenic Nd-1 Dex: *avrPtoB* plants expressing *avrPtoB* under control of the Dex-inducible promoter, 18 h after injection with a solution of the flagellin peptide (10 μg ml$^{-1}$) into leaves treated with dexamethasone (6 μM) at −6, −3, 0 or +2 h before or after flg22 elicitation

| Time of Dex-induced expression of *avrPtoB* before (−) or after (+) elicitation with flg22 | Callose deposition assessed by autofluorescence after aniline blue staining[a] |
|---|---|
| −6 h | No callose |
| −3 h | + |
| 0 | +++ |
| +2 h | ++++ |
| No Dex control | +++++ |

[a]Callose was assessed on a 1–5 scale with +++++ indicating maximum response of widespread foci of fluorescence visible throughout the area of infiltration in all of the three replicate leaves examined for each treatment (see Figure 6a).

### *AvrPtoB suppresses transcription of genes for basal resistance*

Expression of genes predicted to be involved in basal resistance was examined after challenge of Nd-1 Dex: *avrPtoB* with the DC3000 *hrpA* mutant with or without prior *in planta* induction of AvrPtoB. Using Northern blots, we examined the transcript abundance of pathogen-induced genes (*PIGS*) identified by de Torres *et al.* (2003) and genes reported to be expressed following flagellin treatment (Navarro *et al.*, 2004). The selected genes encoded proteins with diverse functions. Additional transcripts that were not expected to be associated with basal defence were used as control probes to detect any non-specific suppression of the transcription machinery. As reported previously (Navarro *et al.*, 2004; de Torres *et al.*, 2003; Truman *et al.*, 2006; Zipfel *et al.*, 2004), challenge with the *hrp* mutant rapidly induced a subset of *PIGS* including *FRK1*, several members of the *WRKY* transcription factor family, the *FLS2* receptor kinase, *TSA* and *PAL.1*. Transgenic expression of AvrPtoB caused a remarkable suppression of accumulation of the *PIG* transcripts and *NHO1* (encoding a glycerol kinase), but had no effect on other genes such as *HSP70* and *MAP3Kε*. A gene encoding a mitochondrial located protein (*MLP*) previously found to be expressed following challenge with virulent DC3000 (de Torres *et al.*, 2003) was induced in the presence of AvrPtoB with or without bacterial challenge (Figure 8). This experiment demonstrated that AvrPtoB is able to prevent induction of basal defence at the level of gene expression.

## Discussion

The *Pph* RW60 strain was chosen as a delivery vehicle to test the activities of the HopAB family because it has a fully functional type III secretion system but fails to cause an HR in Arabidopsis. It also lacks a 154 kb plasmid that harbours several effectors. We argued that the absence of the plasmid-borne genes such as *avrPphF, avrPphC* and *hopAW1* (Jackson *et al.*, 1999; Lindeberg *et al.*, 2005) might reduce the chance of negative interaction with the delivery and function of introduced, heterologous effectors. Using this approach we clearly demonstrated that AvrPtoB, unlike other HopAB members or indeed the functionally related

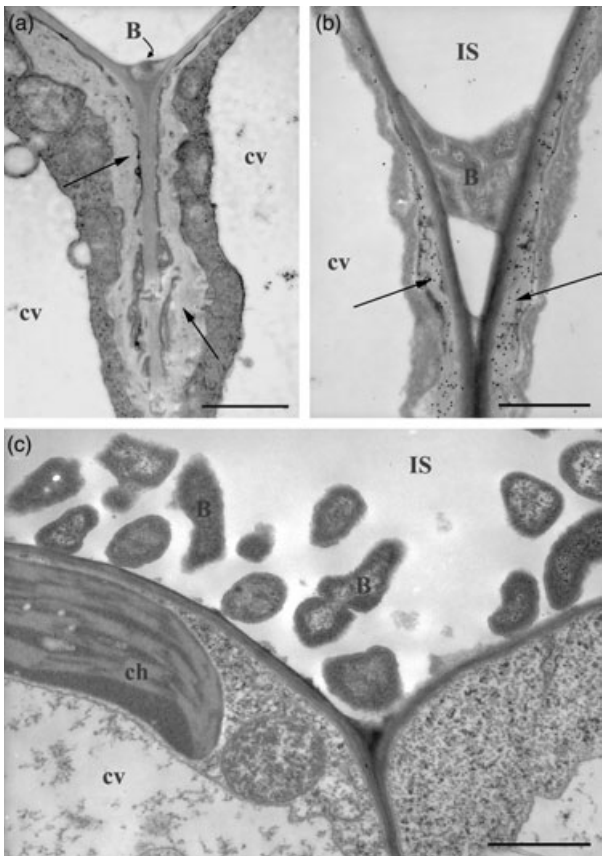


**Figure 7.** Cellular responses to *P. syringae* pv. *tomato* DC3000 *hrpA* mutant are altered 1 day after inoculation in transgenic Nd-1 expressing AvrPtoB.
(a), (b) Deposition of papillae (fine arrows) lining the cell wall at reaction sites in Nd-1 Dex: *avrPtoB* without Dex-induced expression of the effector. In (b) callose has been gold labelled using the monoclonal antibody to β1-3, glucan. (c) Modified reactions and bacterial multiplication observed in Nd-1 Dex: *avrPtoB* treated with 6 μM Dex 6 h before inoculation. Immunolocalization of β1–3 glucan was performed as in (b) but only one gold particle is present. Note the absence of callose deposition or other wall alterations and the proliferation of the DC3000 *hrpA* mutant. All tissues were examined 24 h after inoculation. Scale bars = 1 μm; B, bacterium; ch, chloroplast; cv, cell vacuole; IS, intercellular space.

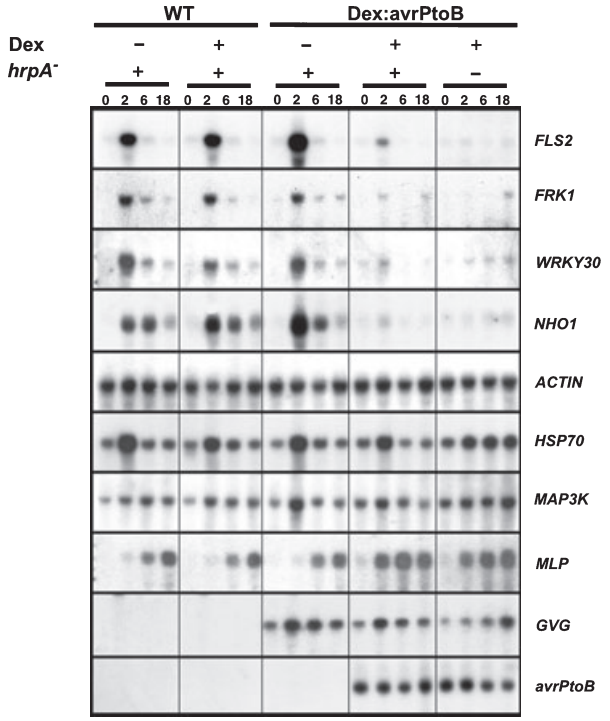


**Figure 8.** Gene expression in Arabidopsis Nd-1 Dex: *avrPtoB*, challenged with a *P. syringae* pv. *tomato* DC3000 *hrpA* mutant, with or without *in planta* expression of AvrPtoB induced by treatment with 6 μM Dex 6 h before inoculation.
Lane headings indicate time after inoculation, i.e. 0 = 6 h after Dex treatment. Northern blots were hybridized with $P^{32}$-labelled probes to *FLS2* (*At5g46330*), *FRK1* (*At2g19190*), *WRKY30* (*At5g24110*), *NHO1* (*At1g80460*), *ACTIN2* (*At3g18780*), *HSP70* (*At5g02500*), *MAP3Kε* (*At3g135302*), *MLP* (*At1g13340*), the chimeric *GVG* transcription factor, and *avrPtoB*. Patterns of transcript suppression by AvrPtoB similar to that observed for *WRKY30* were also found with *PAL1* (*At2g37040*) and *TSA1* (*At3g54640*).

AvrPto, had virulence activity in Arabidopsis by overcoming basal resistance that did not involve the HR. In addition, AvrPtoB and three other HopAB family members promoted virulence by suppressing the HR in bean (Table 1). The EM studies showed that even in Ws-3, RW60 + *avrPtoB* was only able to multiply at certain sites within infiltrated leaves. Delivery of AvrPtoB by RW60 would therefore seem to be unable to achieve full suppression of defence in all challenged cells. The resultant range of phenotypes observed in different accessions of Arabidopsis probably reflects the proportion of plant cells in which basal defence is successfully suppressed by the action of AvrPtoB. In summary, we were able to show that *Pph*, a non-pathogen of Arabidopsis, was able to colonize this plant and elicit disease symptoms after acquiring *avrPtoB*.

Our finding that the C-terminal domain of AvrPtoB is required for virulence functions supports results obtained by Abramovitch *et al.* (2003) using *Nicotiana benthamiana* in their investigation of HR suppression. However, the minimal size requirements for AvrPtoB to function appear to be plant specific. Suppression of the HR in bean is effectively lost with Δ1 (–21 C-terminal amino acids) but failure to overcome basal resistance in Arabidopsis does not become fully apparent until Δ3 (–70 amino acids). These data suggest that different domains within the C-terminus would appear to be regulating the ability to suppress the HR or basal resistance. Janjusevic *et al.* (2006) have recently reported an elegant analysis of the C-terminus of AvrPtoB which has led to the discovery that this effector is an E3 ubiquitin ligase. Furthermore, ligase activity is required for the suppression of plant cell death in tomato by AvrPtoB and is compromised by removal of E2 ubiquitin ligase-binding domains by F479A, F525A and P533A mutations within the C-terminus (Janjusevic *et al.*, 2006). The region encompassing lysines K512 to K529 has also been shown to be essential for

 

AvrPtoB–ubiquitin interactions (Abramovitch *et al.*, 2006). Our data from the simple deletion experiment (Table 1, Figure 1), showed that the virulence function to Arabidopsis was at least partially retained even with removal of the C-terminus to amino acids 508 or 483, including domains essential for ubiquitin interaction and ligase activity. This enzymatic function is, therefore, probably not specifically required for the suppression of basal, cell wall-based defences in Arabidopsis. By contrast, in bean, ligase activity may be essential for HR suppression because this activity was lost by removal of the domain containing the first E2-binding site (around P533), as achieved by Δ1. Virulence activity in both bean and Arabidopsis was clearly separated from induction of the Pto-mediated HR in tomato (Table 1).

Using RW60 to deliver AvrPtoB into Arabidopsis promoted virulence most strongly in accessions lacking functional FLS2, either in wild-type or *fls2* mutant lines. Our demonstration that *FLS2* can be mapped in segregating Nd-1 × Ws-3 populations as if it were a dominant resistance gene operating against RW60 + *avrPtoB* raises important issues about the classification of the flagellin receptor. Rather than representing a first line of basal defence distinct from varietal, gene-for-gene based resistance, *FLS2* might be more accurately considered as a prototype *R* gene which, atypically, does not require the HR for the restriction of microbial colonization. Wild-type Ws-3 or the La-er *fls2-17* mutant were resistant to RW60 or DC3000 *hrpA*– even in the absence of FLS2. The receptor systems mediating the efficient basal defence in these plants remain to be identified. There may be alternative sensors of flagellar components, or receptors targeting other PAMPS such as lipopolysaccharides, EF-Tu or cold shock proteins (Felix and Boller, 2003; Keshavarzi *et al.*, 2004; Kunze *et al.*, 2004). The *in planta* expression experiments show, however, that AvrPtoB is indeed able to act against the FLS2-mediated pathway in Nd-1 provided that sufficient time elapses before cells are challenged with flagellin. The patterns of activity of AvrPtoB observed against Ws-3 and Nd-1 suggest that the effector acts on a signalling pathway common to FLS2 and other PAMP-receptor-mediated defences. However, FLS2 appears to promote a quantitatively more efficient response than other receptors, particularly in the Nd-1 background. The differential susceptibility to RW60 + *avrPtoB* observed between Nd-1 and the Col and La-er accessions, all of which possess functional FLS2, merits more detailed examination. The greater resistance of Nd could be due to the structure or expression of the Nd form of the FLS2 protein or the presence of additional PAMP receptors.

Although RW60 + *avrPtoB* failed to suppress defences sufficiently to promote colonization in Nd-1, expression of AvrPtoB *in planta* in this accession prior to inoculation did allow multiplication of the TTSS-competent RW60 and also a *hrpA* mutant of DC3000. Induction of transgenic *avrPtoB* expression 6 h before elicitation with flg22 also effectively blocked callose deposition. Suppression of other wall alterations was also observed by electron microscopy in tissues challenged with the DC3000 *hrpA* mutant. Timing appears critical in the interaction between AvrPtoB and the flg22-induced response. Following bacterial inoculation, plant cells would be expected to be exposed to the flagellin peptide almost immediately as fragments of flagella diffuse into cells from the bacterial suspension. Effectors such as AvrPtoB have to be synthesized *de novo* and delivered into plant cells through the TTSS which is induced to be constructed in the microenvironment of the plant's intercellular spaces (Alfano and Collmer, 2004; Li *et al.*, 2002; Thwaites *et al.*, 2004). The Hrp pilus has to penetrate the plant cell wall to allow effector delivery which, in *Pph*, has been estimated to take at least 2 h based on analyses of the construction of the TTSS and also the induction time required for viable bacteria to be present within leaves before the HR is induced (Pozidis *et al.*, 2003; Puri *et al.*, 1997). The AvrPtoB protein was detected within 2 h of Dex-mediated induction in Nd-1 Dex: *avrPtoB* transgenics (data not shown). The simultaneous treatment with flg22 and Dex induction (as reported in Table 2) therefore probably mimics the interaction occurring after bacterial challenge. With this time scale the deposition of callose, as an indicator of wall alterations, was not fully suppressed in Nd-1, a result which may help to explain the resistance of this accession to RW60 + *avrPtoB*. In the absence of FLS2, AvrPtoB was demonstrated to be more successful than AvrPto in promoting virulence of *Pph* strain RW60 in Arabidopsis (Table 1). However, Hauck *et al.* (2003) found that *in planta* expression of AvrPto, like AvrPtoB, did suppress wall-based defences. The differences observed between bacterial and *in planta* delivery of AvrPtoB and AvrPto into Arabidopsis highlight the need for the use of several approaches to detect effector activity.

Li *et al.* (2005) have shown, using transient assays with protoplasts, that several effectors from DC3000 are able to suppress the innate immune response activated by flagellin. Our results show that AvrPtoB may now be added to the list of effectors able to suppress the basal defence programme. In view of the ability of a number of effectors from DC3000 to suppress the FLS2-based response, it is surprising that the first infection experiments showing that *FLS2* had a role in the resistance of Arabidopsis to bacterial invasion came from experiments with wild-type DC3000. Using spray inoculation, but not infiltration, an *fls2* mutant was found to be more susceptible than wild-type La-er to colonization (Zipfel *et al.*, 2004). If DC3000 were able to suppress all components of the FLS2-based defence then one might expect no difference in the resistance of wild-type and *fls2* mutant plants, whatever the inoculation procedure. Our experiments show that the timing of exposure of plant cells to flg22 and effectors may provide an explanation for the effect of FLS2 on DC3000. Bacteria on the leaf surface may

 

release flagellin that is able to diffuse into epidermal cells. By contrast, epiphytic bacteria may be unable to penetrate the leaf's cuticle and deliver effectors. In consequence the plant's basal defences might be activated several hours before bacteria penetrate stomata and begin fully to establish parasitism. Colonization by the highly pathogenic DC3000 strain may therefore, under certain circumstances, be partially checked by what might be termed *residual* basal defence. When DC3000 is inoculated directly into mesophyll tissue the time between exposure to flagellin and the delivery of not only AvrPtoB and AvrPto, but also an additional battery of effector proteins is considerably reduced, tipping the balance in favour of defence suppression and the establishment of parasitism.

The results of the expression profile experiment indicate that the effect of AvrPtoB on basal defence is mediated by a specific reprogramming of the transcriptional response to challenge by the *hrp* mutant. Targeting a functionally divergent group of defensive responses in this way helps to explain the complete suppression of the various wall modifications including callose deposition observed after challenge with bacteria or the flg22 peptide, provided there is sufficient time for AvrPtoB to accumulate. Clearly, we need to establish a more complete picture of the families of transcripts suppressed by AvrPtoB through the use of whole genome arrays. It is important to determine which groups of PAMP-induced transcripts are suppressed and to what extent AvrPtoB alone is able to reproduce the pattern of suppression achieved by DC3000. Navarro *et al.* (2004) have presented an appealing model in which PAMP responses are controlled by the removal of negative regulators for proteolysis. Moreover, Truman *et al.* (2006) and also Thilmony *et al.* (2006) provide tantalizing evidence suggesting that effectors act collectively to reimpose this negative regulation. If the primary virulence target of AvrPtoB is a core component of the negative regulation mechanism, then suppression by the effector of a diverse range of transcripts would be expected. Our results indicate that the virulence target of AvrPtoB in Arabidopsis is likely to be a component of the signal transduction cascade leading to activation of a key regulatory factor (either protein or perhaps microRNA) rather than a structural component of the plant's secretion machinery leading to cell wall modification. An intriguing link is that interference with gene regulation may, in Arabidopsis, bean and tomato, also have the observed effect of suppression of the HR (Abramovitch *et al.*, 2003; Jackson *et al.*, 1999; Jamir *et al.*, 2004). Whether or not the ability of AvrPtoB to inhibit Bax-mediated programmed cell death, which was highlighted by Abramovitch *et al.* (2003) and Jamir *et al.* (2004), is mechanistically linked to the suppression of basal resistance described here remains to be determined. Even without inoculation, the expression of AvrPtoB caused some wilting of treated leaves. In addition to regulation of the coordinated defence response, the AvrPtoB target probably also has a role in the control of water loss from healthy plants. Significantly, expression of AvrPtoB in the plant did not prevent the HR triggered by four effectors, AvrB, AvrRpt2, AvrRpm1 and AvrRps4. The AvrPtoB protein does not, therefore, seem to be a broad-spectrum suppressor of eucaryotic cell death as suggested by Abramovitch *et al.* (2006), but may have specific targets. Suppression of basal defences may be the more generally important role for this effector in bacterial pathogenesis.

In conclusion, we have demonstrated that the ability to detect virulence activity of effectors in Arabidopsis depends on the vehicle used for intracellular delivery and the presence or absence of the FLS2-mediated defence response. A clear role for FLS2 in basal resistance against non-pathogenic bacteria has been confirmed. Suppression of basal resistance has been added to the HR-inducing and HR-suppressing multifunctional activities of domains of the AvrPtoB protein. The potent activity of the effector in suppressing flg22-mediated responses following *in planta* expression of the protein indicates that the AvrPtoB–Arabidopsis interaction represents an excellent model system for the identification of what may prove to be a common core regulatory target for effectors secreted through the TTSS. Importantly, our results also highlight the importance of the balance between the speed of delivery of effectors and the onset of activation of plant defences.

## Experimental procedures

### *Plants and bacteria*

*Pseudomonas syringae* strains were grown routinely on King's medium B (KB) agar at 25°C (King *et al.*, 1954). Antibiotics were used at the following concentrations (μg ml$^{-1}$): rifampicin (50) spectinomycin (100) and kanamycin (50). Triparental mating to introduce plasmids was as in Jackson *et al.* (1999). *Arabidopsis thaliana* genotypes were sown in Levingtons $F_2$ compost and vernalized for 2 days at 4°C. Arabidopsis plants were grown under short day conditions in a controlled environment chamber (10 h light, 100–125 μEinstein at 22°C day, 20°C night) for 5–6 weeks before use. French bean (*Phaseolus vulgaris*) and tomato (*Lycopersicon esculentum*) plants were grown in peat-based compost in a greenhouse with natural daylight (about 16 h). Tomato plants were challenged 8 weeks after sowing and young bean pods collected for pathogenicity tests before seeds had expanded.

### *Pathogenicity tests and* in planta *bacterial population counts*

*Pseudomonas syringae* pv. *phaseolicola* RW60 a plasmid-cured strain (Jackson *et al.*, 1999) and *P. syringae* pv. *tomato* DC3000 *hrpA* mutant (Roine *et al.*, 1997) were grown overnight in 10 ml of Luria–Bertani broth supplemented with antibiotics. Overnight cultures were washed once and then resuspended in 10 mM $MgCl_2$. Final cell density was adjusted typically to $OD_{600}$ 0.4 [about $3 \times 10^8$ colony-forming units (CFU) ml$^{-1}$] for pathogenicity tests and 0.05 (about $0.4 \times 10^7$ CFU ml$^{-1}$) for population counts unless otherwise indicated. For Arabidopsis and tomato assays, fully

 

expanded leaves were infiltrated on the abaxial surface with a needleless 1 ml syringe after making a small cut into the epidermis with a razor blade. Bean pods were inoculated with a syringe and needle (Harper *et al.*, 1987). Challenged bean pods and tomato plants were maintained at 23°C with a 16-h photoperiod after inoculation. Symptom development in Arabidopsis was assessed by allocation of infiltration sites to categories as follows: 0, no visible symptoms; 1, patchy pale yellowing; 2, pale yellowing over the infiltrated area; 3, as 2, but with some areas of collapse; 4, collapse of more than half the infection site; 5, collapse of all infiltrated tissue. Mean scores were calculated from at least 12 sites to produce a symptom index. Arabidopsis plants were incubated under the conditions used for growth but maintained at 22°C. Bacterial multiplication was examined by cutting two discs of tissue close to the inoculation site with a 0.4-cm borer, homogenization in 10 mM $MgCl_2$ and serial dilution of the homogenate. Subsequently, five 10- μl aliquots of those dilutions were spotted onto surface-dry KB agar with appropriate antibiotics to allow colony development at 25°C. Two days later, the number of colonies forming per area unit (two discs) of leaf tissue was determined. Each time point determination was done in triplicate from at least three plants. Induction of the HR in Arabidopsis was examined using DC3000-expressing *avrB, avrRpm1, avrRps2, avrRpt2* and *avrRps4* cloned in pVSP1 (Bennett *et al.*, 2005) and an inoculum concentration of about 0.5 or $1 \times 10^8$ CFU $ml^{-1}$. Challenged plants were observed every 4 h after inoculation for 20 h and the onset of tissue collapse recorded.

### *Delivery constructs*

The HopAB family members were first cloned in pBluescript SK + (pBS, Stratagene; La Jolla, CA, USA) either as restriction fragments or PCR amplification products which were verified by sequencing. In order to compare functions without variation imposed through differential expression, open reading frames (ORFs) were transferred to pDSK600 (Murillo *et al.*, 1994) under the control of a triple *lac* UV5 promoter that confers constitutive expression in *Pph*. We first made pDSKHolPmaN by inserting a gel-purified *Bam*HI filled–*Hin*dIII DNA fragment from pBsHolPmaN (carrying the HolPmaN ORF preceded by its Shine–Dalgarno sequence) into *Sma*I–*Hin*dIII doubly digested pDSK600. This plasmid was then used as a backbone to clone other effectors with the same Shine–Dalgarno cassette. This was done by digesting pDSK-HolPmaN with *Nde*I and *Eco*RI or *Hin*dIII and gel purifying the vector fragment, thereby eliminating the *holPmaN* ORF, and inserting the others effector ORFs in this position as corresponding *Nde*I–*Eco*RI or *Nde*I–*Hin*dIII fragments. The pDSKAvrPto construct with high expression of *avrPto* was obtained from Jesus Murillo (University of Navarra, Spain).

### *AvrPtoB deletions*

pBsAvrPtoB was linearized with an *Eco*RV and *Hin*dIII double digest. *Eco*RV and *Hin*dIII enzymes cut approximately 83 bp and 87 bp, respectively, downstream of the *avrPtoB* stop codon. Subsequently, an *Eco*RV–*Hin*dIII adaptor containing stop codons in three different reading frames was inserted, generating the plasmid pBsAvrPtoB-STOP. This plasmid was mutated by oligonucleotide mutagenesis at selected points in the *avrPtoB* ORF, generating in each case a *Pml*I restriction site. The primers used were: (Δ1) 5′-CTCTGGTCAT**CACGTG**CTCAGGC-3′; (Δ2) 5′-GGTCCTTATT**CACGTG**TTGGCTGTAAG-3′; (Δ3) 5′-GGATTACT**CACGTG**CAATTCTCCGC-3′; (Δ4) 5′-GCTGCCCGGA**CACGTG**CCACCACTG-3′; (Δ5) 5′-CGCCTGGCGG**CACGTG**CGGGAGCCAC-3′; (Δ6) 5′-GATTCAACGC**CACGTG**CAAATTCAG-3′; (Δ7) 5′-CTTTCAAGGGC**CACGTG**CAGGTGCTC-3′.

Each independently mutated pBsAvrPtoBSTOP plasmid was doubly digested with *Pml*I and *Eco*RV, gel purified to eliminate the corresponding 3′ *Pml*I-*Eco*RV *avrPtoB* fragment and self-ligated. After verification by sequencing, DNA fragments corresponding to each truncation of *avrPtoB* were excised with *Nde*I (cutting at –1 from the *avrPtoB* start codon) and *Hin*dIII (cutting after the stop codons), gel purified and finally subcloned into *Nde*I–*Hin*dIII-digested pDSK600HolPMaN.

### *Dexamethasone-inducible construct*

*avrPtoB* was cloned in the dexamethasone-inducible vector pTA7002 (Aoyama and Chua, 1997). A 1.7 kb Klenow-filled *Nde*I/*Nhe*I DNA fragment containing the *avrPtoB* ORF was excised from a DC3000 genomic cosmid (Jackson *et al.*, 2002) and subcloned into the *Xho*I-filled site of pTA7002 to create pDEX:avrPtoB. The orientation of the insert was confirmed by restriction digests.

### *Plants transformation*

pDEX:avrPtoB was electroporated into *Agobacterium tumefaciens* GV3101. *Arabidopsis thaliana* plants were transformed using the A *grobacterium*-mediated flower dipping method (Clough and Bent, 1998). $T_1$ transgenic plants were identified on 0.5% Murashige and Skoog (MS) selection plates containing 30 μg $ml^{-1}$ of hygromycin. $T_2$ homozygous transgenic lines were selected by their segregation ratio on selection plates and PCR-mediated amplification using primers to *avrPtoB*.

### *Mapping*

We used a Ws-3 × Nd-1 $F_2$ population kindly donated by Eric Holub (Warwick HRI, UK). Individual $F_2$ plants were screened for susceptibility by hand infiltration with a suspension (about $3 \times 10^8$ CFU $ml^{-1}$) of RW60 + *avrPtoB*. The phenotype was analysed after 5 days. Genomic DNA was subsequently extracted (Edwards *et al.*, 1991) and analysed with various CAPS markers (Konieczny and Ausubel, 1993). A mapping survey of CAPS markers (http://www.mpiz-koeln.mpg.de/masc/) distributed throughout the five Arabidopsis chromosomes placed the gene for resistance to RW60 + *avrPtoB* on chromosome 5. In particular, CAPS markers MASC04588 and MASC04412 allowed us to position our gene on the right arm of chromosome 5 and upstream of the latter. For finer mapping we designed two CAPS markers (P and K) in this region, upstream of MASC04412, based on the Col-0 genomic sequence (TAIR, http://www.arabidopsis.org): The K forward primer (5′-CCATACACCGCGAGATACTGG-3′) and K reverse primer (5′-CTTGAGGAGGTTCGAGTCAATCG-3′) amplified a 1.27 kbp fragment of genomic DNA in both accessions but revealed a polymorphism when digested with *Eco*RI. The P forward primer (5′-CCTGTAGACCCAACTGAAACCAG-3′) and P reverse primer (5′-GATTCGCCTCCTCCAAATGTGAC-3′) amplified a 1.96 kb fragment of genomic DNA in both accessions but revealed a polymorphism when digested with *Hin*dIII. After analysis of DNA from 256 susceptible plants (512 chromosomes), we obtained the following frequency of recombination 37% for P, 5.8% for K and 29.3% for MASC04412 and we were able to locate our gene between marker K (at 18716662) and MASC04412 (at 19474975). In order to differentiate the *FLS2* alleles of Ws-3 and Nd-1 in our mapping population, we amplified genomic

 

DNA with the primers FLS2-F (5′-TTCATCAGAGTCCTCATTACCGG-3′) and FLS2-R (5′-GATCCCGAAGCTGAATACATCGG-3′), which generate a 760 bp DNA fragment. PCR products were subsequently digested with *Hpy*188III.

### *RNA extraction, Northern blots and probes*

Total leaf RNA was isolated by guanidine hydrochloride extraction and 15 μg samples were used for Northern blots as described previously (de Torres *et al*., 2003). Probe templates were amplified by PCR from cDNAs or genomic DNA based on GenBank sequences and labelled with [$P^{32}$]dCTP using the Prime-It$^{R}$ II labelling kit (Stratagene). Information on genes tested is described below with GenBank accession numbers, primers and product sizes: *FLS2* (*At5g46330*), forward primer 5′-GCAGAAGTCGAGCCACTTCTCG-3′; reverse primer 5′-CTCGATCCTCGTTACGATCTTCTC-3′; 1214 bp; *FRK1* (*At2g19190*), forward primer 5′-TCGGATTCGGCGTTTGTT-GATTC-3′; reverse primer 5′-CTCTCGTTTCGCGCTGTTTCTGC-3′; 711 bp;WRKY30 (*At5g24110*), forward 5′-GAACCATAGTAGTG-GAGAGTGGGAG-3′; reverse 5′-GCTCCGAGAATATCCTTCTGGC-3′; 456 bp; *NHO1* (*At1g80460*), forward 5′-GAACTCTCGGGAG-GAAGATCC-3′; reverse 5′-GTTGTTAGCTGTGGCACCACC-3′; 937 bp; *PAL1* (*At2g37040*), forward 5′-CACACAAGAGCAACGGAG-GAGG-3′; reverse 5′-TTTCGTTGCGTAACGGATCACTTCG-3′; 1581 bp; *TSA1* (*At3g54640*), forward 5′-GGCGTCTTCTTCC-TCCAATCC-3′; reverse 5′-GACCAGCTCAATGTCGTTGTTGAGG-3′; 1006 bp; *HSP70* (*At5g02500*), forward 5′-GCTAA-GAGGTTGATCGGTCGTCG-3′; reverse 5′-CCTGAAGAGATCCAT-GTTGAGCTCC-3′; 735 bp; *ACTIN2* (*At3g18780*), forward 5′-GTGCCAATCTACGAGGGTTTCT-3′; reverse 5′-CTTAATCTT-CATGCTGCTTGGTG-3′; 499 bp; *MLP* (*At1g13340*), forward 5′-AT-GGGGAAGAAGCTTGACGCT-3′; reverse 5′-ACCGAATCAGATA-AACCATAACC-3′; 869 bp. The *MAP3Kε* probe (*At3g135302*), was obtained by excising the insert from a full-length cDNA clone (APZL52f07, Kazusa DNA Research Institute, Chiba, Japan). The *avrPtoB* probe was an *Nde*I–*Hin*dIII fragment from pBsavrPtoB containing the complete ORF. For the chimeric *GVG* transcription factor, we amplified a 776 bp fragment from pTA7002 with the following primers: forward 5′-CTGGGAGTGTCGCTACTCTCC-3′ and reverse 5′-GCAGGGTAGAGACATTCTCTGCTC-3′.

### *Microscopy*

Epifluorescence microscopy was used to detect callose after staining with aqueous aniline blue as described in Keshavarzi *et al.* (2004), except that tissue cleared in methanol was incubated directly in aniline blue solution and left for at least 6 h before observation after mounting in the stain. Images were recorded using a Zeiss Axiophot camera. Electron microscopy and immunogold localization of β1-3 glucan (callose) used material embedded in araldite and a mouse monoclonal antibody as described in Soylu *et al.* (2005).

## Acknowledgements

This research was supported by grants from the UK Biotechnology and Biological Sciences Research Council (JWM and MG), the Wain Research Fund (MdeT) and the Deutche Forschungsgemeinschaft (JB).We also wish to thank Eric Holub for the $F_2$ progeny and invaluable discussion on basal defence. Thomas Boller kindly facilitated provision of the La-er *fls2-17* mutant and Ws: *FLS2* transgenic lines. Jonathan Jones offered a generous sample of flg22 and Mark A Bennett helped with microscopy.

## Note added in proof

Following the acceptance of our article, He *et al.* (2006) published research which also highlights the virulence function of AvrPtoB in Arabidopsis. Importantly, their approach was to study responses in protoplasts whereas our work demonstrates that AvrPtoB also functions effectively in whole plants. A second paper of major significance to our understanding of basal defence by Zipfel *et al.* (2006) reported the identification of the EF-Tu peptide receptor.

## References

**Abramovitch, R.B. and Martin, G.B.** (2005) AvrPtoB: a bacterial type III effector that both elicits and suppresses programmed cell death associated with plant immunity. *FEMS Microbiol. Lett.* **245**, 1–8.

**Abramovitch, R.B., Kim, Y.J., Chen, S., Dickman, M.B. and Martin, G.B.** (2003) *Pseudomonas* type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. *EMBO J.* **22**, 60–69.

**Abramovitch, R.B., Janjusevic, R., Stebbins, C.E. and Martin, G.B.** (2006) Type III effector AvrPtoB requires intrinsic E3 ubiquitin ligase activity to suppress plant cell death and immunity. *Proc. Natl Acad. Sci. USA*, **103**, 2851–2856.

**Alfano, J.R. and Collmer, A.** (2004) Type III secretion system effector proteins: double agents in bacterial disease and plant defense. *Annu. Rev. Phytopathol.* **42**, 385–414.

**Aoyama, T. and Chua, N.H.** (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant J.* **11**, 605–612.

**Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M. and Sheen, J.** (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature*, **415**, 977–983.

**Ausubel, F.** (2005) Are innate immune signalling pathways in plants and animals conserved? *Nature Immunol.* **6**, 973–979.

**Bennett, M.A., Mehta, M. and Grant, M.** (2005) Biophoton imaging: a non-destructive method for assaying R gene responses. *Mol. Plant–Microbe Interact.* **18**, 95–102.

**Bestwick, C.S., Brown, I.R., Mansfield, J.W., Boher, B., Nicole, M. and Essenberg, M.** (1998) Host reactions – plants. *Method Microbiol*, **27**, 539–572.

**Beutler, B.** (2005) The Toll-like receptors: analysis by forward genetic methods. *Immunogenetics*, **57**, 385–392.

**Brown, I.R., Mansfield, J.W. and Bonas, U.** (1995) *hrp* genes in *Xanthomonas campestris* pv. *vesicatoria* determine ability to suppress papilla deposition in pepper mesophyll cells. *Mol. Plant–Microbe Interact.* **8**, 825–836.

**Büttner, D. and Bonas, U.** (2003) Common infection strategies of plant and animal pathogenic bacteria. *Curr. Opin. Plant Biol.* **6**, 312–319.

**Chang, J.H., Urbach, J.M., Law, T.F., Arnold, L.W., Hu, A., Gombar, S., Grant, S.R., Ausubel, F.M. and Dangl, J.L.** (2005) A high-throughput, near-saturating screen for type III effector genes from *Pseudomonas syringae*. *Proc. Natl Acad. Sci. USA*, **102**, 2540–2554.

**Clough, S.J. and Bent, A.F.** (1998) Foral dip: a simplified method for Agrobacterium mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

**Edwards, K., Johnstone, C. and Thompson, C.** (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res.* **19**, 1349.

**Espinosa, A. and Alfano, J.R.** (2004) Disabling surveillance: bacterial type III secretion system effectors that suppress innate immunity. *Cell. Microbiol.* **11**, 1027–1040.

 

**Espinosa, A., Guo, M., Tam, V.C., Fu, Z.Q. and Alfano, J.R.** (2003) The *Pseudomonas syringae* type III-secreted protein HopPtoD2 possesses protein tyrosine phosphatase activity and suppresses programmed cell death in plants. *Mol. Microbiol.* **49**, 377–387.

**Felix, G. and Boller, T.** (2003) Molecular sensing of bacteria in plants. The highly conserved RNA-binding motif RNP-1 of bacterial cold shock proteins is recognized as an elicitor signal in tobacco. *J. Biol. Chem.* **278**, 6201–6208.

**Felix, G., Duran, J.D., Volko, S. and Boller, T.** (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* **18**, 265–276.

**Gomez-Gomez, L. and Boller, T.** (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Mol. Cell*, **5**, 1003–1011.

**Gomez-Gomez, L. and Boller, T.** (2002) Flagellin perception: a paradigm for innate immunity. *Trends Plant Sci.* **7**, 251–256.

**Greenberg, J.T. and Vinatzer, B.A.** (2003) Identifying type III effectors of plant pathogens and analyzing their interaction with plant cells. *Curr. Opin. Microbiol.* **6**, 20–28.

**Guttman, D.S., Vinatzer, B.A., Sarkar, S.F., Ranall, M.V., Kettler, G. and Greenberg, J.T.** (2002) A functional screen for the Type III (Hrp) secretome of the plant pathogen *Pseudomonas syringae*. *Science*, **295**, 1722–1726.

**Harper, S., Zewdie, N., Brown, I.R. and Mansfield, J.W.** (1987) Histological, physiological and genetic-studies of the responses of leaves and pods of *Phaseolus vulgaris* to three races of *Pseudomonas syringae* pv. *phaseolicola* and to *Pseudomonas syringae* pv. *coronafaciens*. *Physiol. Mol. Plant Pathol.* **31**, 153–172.

**Hauck, P., Thilmony, R. and He, S.Y.** (2003) A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants. *Proc. Natl Acad. Sci. USA*, **100**, 8577–8582.

**He, P., Shan, L., Lin, N.C., Martin, G.B., Kemmerling, B., Nurnberger, T. and Sheen, J.** (2006) Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in Arabidopsis innate immunity. *Cell*, **125**, 563–575.

**Jackson, R.W., Athanassopoulos, E., Tsiamis, G., Mansfield, J.W. and Sesma, A., Arnold, D.L., Gibbon, M.J., Murillo, J., Taylor, J.D. and Vivian, A.** (1999) Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen *Pseudomonas syringae* pathovar phaseolicola. *Proc. Natl Acad. Sci. USA*, **96**, 10875–10880.

**Jackson, R.W., Mansfield, J.W., Ammouneh, H. *et al.*** (2002) Location and activity of members of a family of *virPphA* homologues in pathovars of *Pseudomonas syringae* and *P. savastanoi*. *Mol. Plant Pathol.* **3**, 205–216.

**Jamir, Y., Guo, M., Oh, H.-S., Petnicki-Ocwieja, T., Chen, S., Tang, X., Dickman, M.B., Collmer, A. and Alfano, J.R.** (2004) Identification of *Pseudomonas syringae* type III secreted effectors that suppress programmed cell death in plants and yeast. *Plant J.* **37**, 554–565.

**Janeway, C.A. and Medzhitov, R.** (2002) Innate immune recognition. *Annu. Rev. Immunol.* **20**, 197–216.

**Janjusevic, R., Abramovitch, R.B., Martin, G.B. and Stebbins, C.E.** (2006) A bacterial inhibitor of host programmed cell death is an E3 ubiquitin ligase. *Science*, **311**, 222–226.

**Kang, L., Li, J., Zhao, T., Xiao, F., Tang, X., Thilmony, R., He, S. and Zhou, J.M.** (2003) Interplay of the Arabidopsis nonhost resistance gene NHO1 with bacterial virulence. *Proc. Natl Acad. Sci. USA*, **100**, 3519–3524.

**Keshavarzi, M., Soylu, S., Brown, I., Bonas, U., Nicole, M., Rossiter, J. and Mansfield, J.** (2004) Basal defenses induced in pepper by lipopolysaccharides are suppressed by *Xanthomonas campestris* pv. *vesicatoria*. *Mol. Plant–Microbe Interact.* **7**, 805–815.

**Kim, M.G., da Cunha, L., McFall, A.J., Belkhadir, Y., DebRoy, S., Dangl, J.L. and Mackey, D.** (2005) Two *Pseudomonas syringae* type III effectors inhibit RIN4-regulated basal defense in Arabidopsis. *Cell*, **121**, 749–759.

**King, E., Ward, M.K. and Raney, D.E.** (1954) Two simple media for the demonstration of pyocyanin and flurescin. *J. Lab. Clin. Med.* **44**, 301–307.

**Konieczny, A. and Ausubel, F.M.** (1993) A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. *Plant J.* **4**, 403–410.

**Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T. and Felix, G.** (2004) The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. *Plant Cell*, **16**, 3496–3507.

**Li, C.M., Brown, I., Mansfield, J., Stevens, C., Boureau, T., Romantschuk, M. and Taira, S.** (2002) The Hrp pilus of *Pseudomonas syringae* elongates from its tip and acts as a conduit for translocation of the effector protein HrpZ. *EMBO J.* **21**, 1909–1915.

**Li, X., Lin, H., Zhang, W., Zou, Y., Zhang, J., Tang, X. and Zhou, J.** (2005) Flagellin induces innate immunity in nonhost interactions that is suppressed by *Pseudomonas syringae* effectors. *Proc. Natl Acad. Sci. USA*, **102**, 12990–12995.

**Lindeberg, M., Stavrinides, J., Chang, J.H., Alfano, J.R., Collmer, A., Dang, J.L., Greenberg, J.T., Mansfield, J.W. and Guttman, D.S.** (2005) Proposed guidelines for a unified nomenclature and phylogenetic analysis of type III Hop effector proteins in the plant pathogen *Pseudomonas syringae*. *Mol. Plant–Microbe Interact.* **18**, 275–282.

**Murillo, J., Shen, M., Gerhold, D., Sharma, A., Cooksey, D.A. and Keen, N.T.** (1994) Characterisation of pPT23B, the plasmid involved in syringolide production by *Pseudomonas syringae* pv. tomato PT23. *Plasmid*, **31**, 257–287.

**Navarro, L., Zipfel, C., Rowland, O., Keller, I., Robatzek, S., Boller, T. and Jones, J.D.** (2004) The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene dependent defense responses and bacterial pathogenesis. *Plant Physiol.* **135**, 1113–1128.

**Nomura, K., Melotto, M. and He, S.Y.** (2005) Suppression of host defense in compatible plant-*Pseudomonas syringae* interactions. *Curr. Opin. Plant Biol.* **8**, 361–368.

**Pozidis, C., Chalkiadaki, A., Gomez-Serrano, A. *et al.*** (2003) Type III protein translocase: HrcN is a peripheral membrane ATPase that is activated by oligomerization. *J. Biol. Chem.* **278**, 25816–25824.

**Puri, N., Jenner, C., Bennett, M.A., Stewart, R., Mansfield, J.W., Lyons, N. and Taylor, J.** (1997) Expression of *avrPphB*, an avirulence gene from *Pseudomonas syringae* pv. *phaseolicola*, and the delivery of signals causing the hypersensitive reaction in bean. *Mol. Plant–Microbe Interact.* **10**, 247–256.

**Roine, E., Wei, W., Yuan, J., Nurmiaho-Lassila, E.L., Kalkkinen, N., Romantschuk, M. and He, S.Y.** (1997) Hrp pilus: an *hrp*-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl Acad. Sci. USA*, **94**, 3459–3464.

**Soylu, S., Brown, I.R. and Mansfield, J.W.** (2005) Cellular reactions in Arabidopsis following challenge by strains of *Pseudomonas syringae*: from basal resistance to compatibility. *Physiol. Mol. Plant Pathol.* **66**, 232–243.

**Thilmony, R., Underwood, W. and He, S.Y.** (2006) Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. *tomato*

 

DC3000 and the human pathogen *Escherichia coli* O157:H7. *Plant J.* **46**, 34–53.

**Thwaites, R., Spanu, P.D., Panopoulos, N.J., Stevens, C. and Mansfield, J.W.** (2004) Transcriptional regulation of components of the type III secretion system and effectors in *Pseudomonas syringae* pv. *phaseolicola*. *Mol. Plant–Microbe Interact.* **17**, 1250–1258.

**de Torres, M., Sanchez, P., Fernandez-Delmond, I. and Grant, M.** (2003) Expression profiling of the host response to bacterial infection: the transition from basal to induced defense responses in RPM1-mediated resistance. *Plant J.* **33**, 665–676.

**Truman, W., de Torres, M. and Grant, M.** (2006) Type III effectors orchestrate a complex interplay between transcriptional networks to modify basal defense responses during pathogenesis and resistance. *Plant J.* **46**, 14–33.

**Tsiamis, G., Mansfield, J.W., Hockenhull, R. *et al.*** (2000) Cultivar-specific avirulence and virulence functions assigned to *avrPphF* in *Pseudomonas syringae* pv. *phaseolicola*, the cause of bean halo-blight disease. *EMBO J.* **19**, 3204–3214.

**Wu, A.J., Andriotis, V.M., Durrant, M.C. and Rathjen, J.P.** (2004) A patch of surface-exposed residues mediates negative regulation of immune signaling by tomato Pto kinase. *Plant Cell*, **16**, 2809–2821.

**Zhu, M., Shao, F., Innes, R.W., Dixon, J.E. and Xu, Z.** (2004) The crystal structure of *Pseudomonas avirulence* protein AvrPphB: a papain-like fold with a distinct substrate-binding site. *Proc. Natl Acad. Sci. USA*, **101**, 302–307.

**Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T. and Felix, G.** (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts agrobacterium-mediated transformation. *Cell*, **125**, 749–760.

**Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D.G., Felix, G. and Boller, T.** (2004) Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature,* **428**, 764–767.

 