Identification of new type III effectors and analysis of the plant response by competitive index

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SUMMARY

In recent years, many efforts have been directed towards the identification of new type III-secreted effectors, and the completion of the secretomes of several *Pseudomonas syringae* pathovars. Several functional and bioinformatic screenings have been used to search for candidates, which have been tested for translocation into the plant cell, an essential criterion for the identification of new type III effector proteins. The most common translocation assay is based on the use of Δ AvrRpt2 as a reporter. When fused to a type III effector protein, Δ AvrRpt2 is translocated and elicits a hypersensitive response in leaves of *Arabidopsis thaliana* expressing the RPS2 resistance protein. This approach has been used widely and has allowed the identification of a considerable number of new effectors in a fast and convenient manner. However, as the hypersensitive response is a semi-quantitative assay, and the conditions do not resemble those occurring in nature, effectors with low expression or translocation efficiency could fail to translocate sufficient Δ AvrRpt2 to trigger a clear hypersensitive response. In keeping with these limitations, this test has failed to detect some true effectors that have been confirmed as such by other means. In order to increase the sensitivity of this method, we have developed a modification of the Δ AvrRpt2-based translocation assay using a competitive index in mixed infection to monitor the limitation of growth associated with the induction of the hypersensitive response. We have tested several effector candidates from *P. syringae* pv. *phaseolicola* and other *P. syringae* pathovars, and have compared the results obtained by our competitive index translocation assay with those obtained by standard hypersensitive response assays. We have identified six type III secretion system-translocated proteins using this approach, five of which failed to be identified by hypersensitive response assays. In addition, we have analysed the defence response triggered by one of these effectors using competitive index assays.

INTRODUCTION

Pseudomonas syringae is a Gram-negative, host-specific pathogen that depends on the hypersensitive response and pathogenicity (Hrp) type III secretion system (TTSS) to cause disease in compatible hosts, and to trigger a hypersensitive response (HR) in incompatible hosts (Alfano and Collmer, 1997). TTSS is responsible for the secretion and translocation of effector proteins into the plant cell, where they modulate different cellular processes. The specificity of the TTSS-mediated interaction is based on the set of delivered effectors translocated by each strain, called the secretome (Alfano and Collmer, 2004). In recent years, many efforts have been directed towards the identification of new effectors and the completion of the secretomes of different *P. syringae* pathovars (Lindeberg *et al.*, 2006). These studies have screened for effector candidates by searching for the presence of putative secretion signals, HrpL-dependent promoters, or both. Candidates were then tested for their ability to be translocated in a TTSS-dependent manner. Most common translocation assays are based on the generation of translational fusions between the putative effector gene and the genes encoding either Δ AvrRpt2 (Mudgett *et al.*, 2000) or the adenylate cyclase (hereafter Cya) from *Bordetella pertussis* (Schechter *et al.*, 2004) as translocation reporters. More than 60 *P. syringae* effector candidates have been validated using the Δ AvrRpt2 reporter, expressing the protein fusions either from a constitutive (Vinatzer *et al.*, 2006) or a native (Chang *et al.*, 2005) promoter. In this assay, *P. syringae* strains expressing the fusion proteins are infiltrated into *Arabidopsis thaliana* which carries a functional copy of the RPS2 resistance gene. Thus, the development of a visible RPS2-AvrRpt2-dependent HR indicates that the effector candidate is translocated inside the plant cell. Although this assay has allowed the identification of a considerable number of new effectors, it has certain limitations (Lindeberg *et al.*, 2006). Effectors with low levels of expression or low translocation efficiencies could fail to produce sufficient translocated Δ AvrRpt2 to trigger a clear HR. As the macroscopic HR is a semi-quantitative assay, some translocated effectors may thus be either below or on the limit of detection of these assays, giving a false-negative

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result or a weak or inconsistent HR (Chang *et al.*, 2005; Vinatzer *et al.*, 2005). In keeping with these limitations, there are examples of effectors that, failing to be validated by this assay, have been shown by other methods to be translocated (Lindeberg *et al.*, 2006; Vencato *et al.*, 2006).

In order to increase the sensitivity of the Δ AvrRpt2-based translocation assay, we have designed a modification of this method. In our assay, we monitor the limitation of bacterial growth associated with a resistance–avirulence (R–avr) interaction, rather than the appearance of visible HR symptoms. Furthermore, we measure bacterial growth by the competitive index of bacteria expressing a Δ AvrRpt2 translational fusion to an effector candidate in mixed infection with the wild-type strain. We have established previously that an adequate inoculation dose eliminates the risk of interference between co-inoculated strains (Macho *et al.*, 2007). In this manner, we have shown that the competitive index assay is a more accurate and sensitive measure of bacterial growth within the plant than are traditional growth assays, as a direct comparison between the growth of two strains is carried out in each plant, drastically reducing the experimental variation and increasing the sensitivity (Macho *et al.*, 2007).

In this work, we have applied competitive index-based translocation assays to the analysis of effector candidates from the fully sequenced *P. syringae* pv. *phaseolicola* 1448a strain. Candidates were selected from genes previously reported to be either differentially expressed *in planta*, or HrpL regulated, or those showing similarities to *bona fide* effectors from other pathovars. Some of these candidates had previously given negative or inconsistent results using traditional HR assays. We compare the results obtained by competitive index translocation assays with those obtained by standard HR. We demonstrate translocation for six proteins, five of which failed to give a positive result by HR assay. In addition, we analyse the plant response to one of the effectors using competitive index assays, and find that it is partially independent of the response triggered by AvrRpt2.

RESULTS

Sensitivity of competitive index vs. standard HR as translocation assay

This work was started by testing whether the level of gene expression was important for the detection of translocation of Δ AvrRpt2 fusions. First, we assayed the effectiveness of the full AvrRpt2 protein in triggering HR when expressed from a plasmid under either the medium- to low-level expression lactose promoter *lacZ* or the high-level expression promoter neomycin phosphotransferase type II (*nptII*). *Arabidopsis* wild-type plants (ecotype Col-0; *RPS2/RPS2*) were inoculated with *P. syringae* pv. *tomato* (Pto) DC3000 strain (hereafter PtoDC3000) carrying either of

these two plasmids. AvrRpt2 expressed from the *lacZ* promoter did not elicit any HR symptoms (Fig. 1a), nor did it cause any growth attenuation, as the strain expressing the effector grew as much as the co-inoculated wild-type strain (competitive index not statistically different from unity; $P < 0.05$) (Fig. 1b). Actual competitive indices for all the assays carried out in this work are included as 'Supporting Information' (Table S1). As the competitive index of a given strain in mixed infection with the wild-type is calculated as the output ratio between the two strains divided by their input ratio, a competitive index significantly lower than one reflects a growth defect of the strain being tested. Thus, the level of expression provided by the *lacZ* promoter is not sufficient to trigger an AvrRpt2-dependent HR detectable by either standard or competitive index assays. However, when AvrRpt2 was expressed from the *nptII* promoter, it elicited a clear macroscopic HR (Fig. 1a), and caused a 50-fold attenuation in competitive index assays (Fig. 1b). These results prompted us to test the translocation of the fusion proteins when expressed from the *nptII* promoter. The reason for this is that the level of expression and translocation of AvrRpt2 required for the effective triggering of HR is considerably high, but does not necessarily reflect the level of expression and translocation required for a given effector to carry out its biological function. We also assayed an AvrRpt2 protein generated by fusing its amino (amino acids 1–80) and carboxy (amino acids 81–255) terminal domains (referred to as N + C AvrRpt2 in the corresponding figures and tables), following the same cloning strategy as used to generate translational fusions to the effector candidates. Thus, this AvrRpt2 version has an insertion of two amino acids encoded by the restriction site used to generate the fusion. The results obtained on expression of this modified AvrRpt2 protein from either the *lacZ* or *nptII* promoter were not significantly different from those obtained on expression of the full AvrRpt2 ($P < 0.05$), ruling out any loss of activity caused by the strategy followed to generate the fusions (Fig. 1).

To test the sensitivity of competitive index vs. standard HR as translocation assay, we selected some previously tested effector candidates from *P. syringae* pv. *phaseolicola* 1448a (hereafter Pph1448a). We chose only those effector candidates that, although expressed in an HrpL-dependent manner, gave negative translocation results in standard HR assays, discarding those showing a truncation or frameshift when compared with similar effectors from other pathovars. A detailed list of the selected candidates and their relevant information is included in Table 1. We performed standard HR and competitive index assays to test HopJ1_{Pph1448a}, HopV1_{Pph1448a} (expressed with its putative chaperone, ShcV), HopAK1_{Pph1448a} and HopAJ1_{Pph1448a}. In all cases, the full length of the candidate gene was used to generate the translational fusion to the reporter. However, strains expressing a fusion to the full-length *hopAJ1* gene showed retarded growth and abnormal morphology in rich medium (data not shown). Therefore, we used the first 14 amino acids of HopAJ1 instead, as carried out previously by

Fig. 1 Sensitivity of competitive index vs. standard hypersensitive response (HR) as translocation assay. (a) Standard Δ AvrRpt2-dependent HR translocation assays. PtoDC3000 strains carrying control plasmids (left) or plasmid-encoded candidate fusions to Δ AvrRpt2 (right) were inoculated into *Arabidopsis* Col-0 (*RPS2/RPS2*) wild-type at an optical density at 600 nm (OD_{600}) of 0.07, and leaves were scored for HR at 24 h post-inoculation (hpi). Control plasmids include the full sequence of AvrRpt2, and an AvrRpt2 protein generated by fusing its amino (amino acids 1–80) and carboxy (amino acids 81–255) terminal domains (referred to as N + C AvrRpt2), following the same cloning strategy as used to generate translational fusions to the effector candidates. The photographs illustrate typical symptoms of a minimum of 80 inoculated leaves per experiment. Three independent experiments were performed with similar results. (b) Graphical representation of Δ AvrRpt2-dependent competitive index-based translocation assays. PtoDC3000 wild-type was co-inoculated into wild-type plants with PtoDC3000 strains carrying control plasmids (left) or plasmid-encoded candidate fusions to Δ AvrRpt2 (right). Competitive indices are the mean of three samples, and the error bars represent the standard error. Three independent experiments were performed with similar results. Asterisks indicate results significantly different from one as established by Student's *t*-test ($P < 0.05$), indicating significant attenuation of the strain tested.

Vinatzer *et al.* (2005) to analyse the translocation of the homologous effector HopAJ1_{PmaE54326}. In agreement with previous reports (Chang *et al.*, 2005), neither of these candidate fusions triggered a consistent HR (Fig. 1a). Only the fusion of the first 14 amino acids from HopAJ1_{Pph1448a} induced a weak and inconsistent HR, with mild symptoms present in just one-third of the infiltrated leaves. However, when these candidates were tested by competitive index translocation assays, strains carrying the HopAK1_{Pph1448a} and HopAJ1_{Pph1448a} fusions gave competitive indices significantly different from one ($P < 0.05$). (Fig. 1b). In other words, the growth of these strains is significantly lower than the growth of the wild-type (by twofold and fivefold, respectively), and such growth attenuation is likely to be caused by these fusion proteins inducing resistance in the infiltrated plants. Therefore, the fusion proteins are translocated inside the cell, thus showing that these candidates have the necessary secretion and translocation signals to be considered as TTSS substrates.

Competitive index translocation assays of untested candidates

Once we confirmed that the competitive index translocation assay was an effective and sensitive method to demonstrate the translocation of candidate effectors, we applied the method to other Pph1448a candidates to contribute to the completion of its secretome. Since the publication of the Pph1448a genome sequence (Joardar *et al.*, 2005), many candidates have been proposed on the basis of their similarity to effectors from other pathovars. We selected those candidates that, having been proposed as putative TTSS effectors, had not yet been tested for translocation (Table 1). HopAH2_{Pph1448a}, HopAJ2_{Pph1448a}, HopAN1_{Pph1448a} and PSPPH3757 were chosen for testing. We also selected A0129_{Pph1448a}, a putative HrPL-regulated gene not previously tested for translocation. Standard HR and competitive index assays of the candidate fusions were carried out. None of the Pph1448a candidate fusions elicited a visible HR (Fig. 2a).

Table 1 *Pseudomonas syringae* effector candidates tested for translocation.

Name or locus	Strain	Relevant information	Reference
HopJ1 _{Pph1448a}	Pph1448a	Negative in previous $\Delta 79\text{AvrRpt2}$ translocation assay (Chang <i>et al.</i> , 2005) Translocated homolog in PmaES4326 (Guttman <i>et al.</i> , 2002)	(Lindeberg <i>et al.</i> , 2005)
HopV1 _{Pph1448a}	Pph1448a	Negative in previous $\Delta 79\text{AvrRpt2}$ translocation assay (Chang <i>et al.</i> , 2005) Translocated homolog in PtoDC3000 (Schechter <i>et al.</i> , 2004)	(Lindeberg <i>et al.</i> , 2005)
HopAJ1 _{Pph1448a}	Pph1448a	Negative in previous $\Delta 79\text{AvrRpt2}$ translocation assay (Chang <i>et al.</i> , 2005) Translocated homologue in PmaES4326 (Guttman <i>et al.</i> , 2002), first 14 amino acids also shown as translocated by Vinatzer <i>et al.</i> (2005) Homologue in PtoDC3000 negative in previous $\Delta 79\text{AvrRpt2}$ (Chang <i>et al.</i> , 2005) and Cya (Oh <i>et al.</i> , 2007) translocation assays	(Lindeberg <i>et al.</i> , 2005)
HopAK1 _{Pph1448a}	Pph1448a	Negative in previous $\Delta 79\text{AvrRpt2}$ translocation assay (Chang <i>et al.</i> , 2005) Translocated homologue in PmaES4326 (Guttman <i>et al.</i> , 2002) Homologue in PtoDC3000 translocated at low levels in previous Cya translocation assay (Kvitko <i>et al.</i> , 2007)	(Lindeberg <i>et al.</i> , 2005)
HopAH2 _{Pph1448a}	Pph1448a	Translocated homologues in PtoDC3000 (HopAH2-1, HopAH2-2) (Schechter <i>et al.</i> , 2004; Vinatzer <i>et al.</i> , 2005)	N/A
HopAJ2 _{Pph1448a}	Pph1448a	Similar to translocated effector HopAJ1 _{PmaES4326}	(Lindeberg <i>et al.</i> , 2005)
HopAN1 _{Pph1448a}	Pph1448a	Untested homologue in PtoDC3000 (Boch <i>et al.</i> , 2002)	(Lindeberg <i>et al.</i> , 2005)
PSPPH3757	Pph1448a	Homologue in PtoDC3000, expressed <i>in planta</i> and untested for translocation, known as ORF24 (Petnicki-Ocwieja <i>et al.</i> , 2002) or lpx 39–40 (Boch <i>et al.</i> , 2002)	N/A
A0129 _{Pph1448a}	Pph1448a	Putative HrpL-activated gene	(Vencato <i>et al.</i> , 2006)
HopZ1 _{PsyA2}	PsyA2	Translocated homologue in PmaES4326 (Guttman <i>et al.</i> , 2002) Avirulence phenotype in <i>Arabidopsis</i> plants (Ma <i>et al.</i> , 2006)	(Sundin <i>et al.</i> , 2004)
HopZ2 _{Ppi895A}	Ppi895A	Avirulence phenotype in bean plants	(Arnold <i>et al.</i> , 2001)

However, using competitive index translocation assays, HopAH2_{Pph1448a} and A0129_{Pph1448a} fusions showed significant and reproducible attenuation (twofold and fourfold, respectively) ($P < 0.05$), thus demonstrating that these proteins are translocated (Fig. 2b).

Competitive index assays in *rps2* mutant plants

A positive result in any AvrRpt2-based translocation assay relies on the recognition of the translocated reporter, $\Delta\text{AvrRpt2}$, by a functional RPS2 resistance protein (Mudgett *et al.*, 2000). In keeping with this notion, the attenuation of growth detected by competitive index translocation assays for PtoDC3000 expressing HopAJ1_{Pph1448a}, HopAK1_{Pph1448a}, HopAH2_{Pph1448a} and A0129_{Pph1448a} fusions, as well as the AvrRpt2 controls, should not be detected when the strains are tested in plants lacking a functional copy of *RPS2* (*rps2/rps2* plants). However, if any of the effector candidates were capable of triggering an *RPS2*-independent HR, PtoDC3000 expressing a fusion to that effector would also induce HR in *rps2* plants. Figure 3a shows that, when assayed in *rps2* plants, the competitive index results of the control strains, as well as the translocated fusions, were not significantly different from one ($P < 0.05$), indicating that, in all cases, growth attenuation registered in wild-type plants was only the result of an *RPS2*-mediated response. These results also show that the attenuation of growth detected in wild-type plants is specifically caused by

the activation of a defence response, and not by a lack of fitness or any growth delay caused by the expression of these proteins. No HR was detectable for either the control strains or HopAJ1_{Pph1448a} (Fig. 3b). As expected, no HR was detectable for any of the other translocated fusions (data not shown).

Competitive index analysis of an HR-triggering effector

Like AvrRpt2, some effectors are known to trigger an HR in *Arabidopsis* plants. One is the cysteine protease HopZ1a from *P. syringae* pv. *syringae* A2 (hereafter PsyA2) (Ma *et al.*, 2006). We wished to determine how one of these effectors would perform in competitive index translocation assays. Thus, we carried out fusions to AvrRpt2 of HopZ1a_{PsyA2} and another effector of the same family, HopZ2_{Ppi895A}. Direct translocation of HopZ2_{Ppi895A} has not been tested previously; however, this effector has been confirmed as such on the basis of its ability to induce HR in bean when expressed from Pph1448a (Arnold *et al.*, 2001).

When competitive index translocation assays were performed, bacteria expressing the HopZ1a_{PsyA2} fusion showed 100-fold growth attenuation in wild-type plants, and a reproducible fivefold growth attenuation in *rps2* plants (Fig. 4a). Although resistance in *rps2* plants is clearly weaker, it is consistently detected, thus indicating that an AvrRpt2-independent defence response, triggered by HopZ1a_{PsyA2}, is being detected. To confirm this result,

Fig. 2 Translocation assays of previously untested candidates.

(a) Δ AvrRpt2-dependent hypersensitive response (HR) translocation assays. PtoDC3000 strains expressing candidate fusions to Δ AvrRpt2 were inoculated into wild-type plants at an optical density at 600 nm (OD_{600}) of 0.07, and leaves were scored for HR at 24 h post-inoculation (hpi). The photographs illustrate typical symptoms of a minimum of 80 inoculated leaves per experiment. Three independent experiments were performed with similar results. (b) Graphical representation of Δ AvrRpt2-dependent competitive index-based translocation assays. PtoDC3000 wild-type was co-inoculated into wild-type plants with PtoDC3000 strains expressing candidate fusions to Δ AvrRpt2. Competitive indices correspond to the mean of three samples, and the error bars represent the standard error. The experiments were repeated three times with similar results. Asterisks indicate results significantly different from one as established by Student's *t*-test ($P < 0.05$), indicating significant attenuation of the strain tested.

we determined the competitive index of PtoDC3000 expressing the gene encoding the complete HopZ1a_{PsyA2}, without AvrRpt2, in wild-type and *rps2* plants. This strain displayed a 12-fold growth attenuation in both wild-type and *rps2* plants (Fig. 4a), confirming that HopZ1a_{PsyA2} activates plant defences independent of RPS2. We also determined the competitive index of PtoDC3000 expressing HopZ1a_{PsyA2} and AvrRpt2 simultaneously, but as separate proteins. This strain displayed a 150-fold growth attenuation in RPS2 plants and a 20-fold growth attenuation in *rps2* plants (Fig. 4a). These results confirm that AvrRpt2 and HopZ1a_{PsyA2} trigger different defence responses, and also show that the effectors are less efficient in triggering HR within a fusion than they are when expressed separately. When HR assays were performed, a clear HR was visible when both wild-type and *rps2* plants were infiltrated with PtoDC3000 expressing the

Fig. 3 Translocation assays in *rps2* plants. (a) Graphical representation of Δ AvrRpt2-dependent competitive index-based translocation assays. PtoDC3000 wild-type was co-inoculated into *rps2* plants with PtoDC3000 strains carrying control plasmids (left) or plasmid-encoded candidate fusions to Δ AvrRpt2 (right). Control plasmids include the full sequence of AvrRpt2, and an AvrRpt2 protein generated by fusing its amino (amino acids 1–80) and carboxy (amino acids 81–255) terminal domains (referred to as N + C AvrRpt2), following the same cloning strategy as used to generate translational fusions to the effector candidates. Competitive indices correspond to the mean of three samples, and the error bars represent the standard error. The experiments were repeated three times with similar results. (b) Δ AvrRpt2-dependent hypersensitive response (HR) translocation assays. PtoDC3000 strains carrying control plasmids (left) or plasmid-encoded HopAJ1_{Pph1448a} N-terminal fusion to Δ AvrRpt2 (right) were inoculated into Arabidopsis *rps2* plants at an optical density at 600 nm (OD_{600}) of 0.07, and leaves were scored for HR at 24 h post-inoculation (hpi). The photographs illustrate typical symptoms of a minimum of 80 inoculated leaves per experiment. Three independent experiments were performed with similar results.

HopZ1a_{PsyA2} fusion (Fig. 4b), and a weaker HR (only 75% of the leaves showed HR symptoms) when either plant background was infiltrated with the strain expressing HopZ1a_{PsyA2} without AvrRpt2 (Fig. 4b).

When competitive index translocation assays were applied to the analysis of HopZ2_{Ppi895A}, we that PtoDC3000 expressing the HopZ2_{Ppi895A} fusion displayed a reproducible fourfold growth attenuation in wild-type plants (Fig. 4a), thus corroborating translocation for this effector. In this case, no resistance was detected in *rps2* mutant plants (Fig. 4a), indicating that this effector, unlike HopZ1a_{PsyA2}, is not capable of inducing a defence

Fig. 4 Translocation assays of HopZ1a_{psya2} and HopZ2_{ppi895A}. (a) Graphical representation of Δ AvrRpt2-dependent competitive index-based translocation assays. PtoDC3000 wild-type was co-inoculated into wild-type (left) and *rps2* (right) plants, with PtoDC3000 strains expressing HopZ1a_{psya2}, HopZ1a_{psya2} together with AvrRpt2 as a polycistron, or HopZ1a_{psya2}::AvrRpt2 or HopZ2a_{ppi895A}::AvrRpt2 protein fusions. Competitive indices correspond to the mean of three samples, and the error bars represent the standard error. Experiments were repeated with similar results and pooled for statistical analysis. Results from the control plasmids from Fig. 1 are included in grey for comparison. Control plasmids include the full sequence of AvrRpt2, and an AvrRpt2 protein generated by fusing its amino (amino acids 1–80) and carboxy (amino acids 81–255) terminal domains (referred to as N + C AvrRpt2), following the same cloning strategy as used to generate translational fusions to the effector candidates. Mean values marked with the same letter were not significantly different as established by Student's *t*-test ($P < 0.05$). (b) Δ AvrRpt2-dependent hypersensitive response (HR) translocation assays. PtoDC3000 strains expressing fusions to Δ AvrRpt2 or HopZ1a_{psya2} were inoculated into wild-type (left) and *rps2* mutant (right) plants at an optical density at 600 nm (OD_{600}) of 0.07, and leaves were scored for HR at 24 h post-inoculation (hpi). Results from the control plasmids from Fig. 1 are included in grey for comparison. The photographs illustrate typical symptoms of a minimum of 80 inoculated leaves per experiment. Three independent experiments were performed with similar results.

response in *Arabidopsis*. Standard assays failed to demonstrate HopZ2_{ppi895A} translocation, as no HR symptoms could be recorded in plants infiltrated with PtoDC3000 expressing its fusion (Fig. 4b).

DISCUSSION

The HR has been used widely in the analysis of bacterium–plant interactions to determine the microbial effectors that trigger resistance (e.g. Kunkel *et al.*, 1993), as well as the plant proteins that are required for that process (e.g. Century *et al.*, 1997; Mindrinos *et al.*, 1994). It is well suited for medium to high throughput because of the speed of the outcome. However, in order to obtain a fast response, a high level of bacteria must be used. Although the concentrations found in natural inoculations are in the range 10^5 – 10^7 colony-forming units (cfu)/mL (Goto,

1992), closer to those used in competitive index assays, HR tests require concentrations of up to 10^8 cfu/mL. Using these experimental settings, it is possible to employ Δ AvrRpt2 as a reporter for the translocation of type III effectors. However, our results show that, even when inoculating a high concentration of bacteria, a high level of expression of the protein fusions is also required for the assay to work. Thus, whether or not an effector fusion is capable of triggering an HR depends on several factors, such as the level of expression from the selected promoter, the stability of the fusion protein, the efficiency of each translocation signal and the competence of the fusion to be correctly processed. These issues are particularly relevant, as we had found that fusion proteins can be less efficient than the effectors expressed as independent proteins in the triggering of plant defence responses. As standard HR assays provide semi-quantitative results, and require a considerable level of Δ AvrRpt2 translocation inside the

cell, variations in any of the above-mentioned elements may result in a given effector fusion inducing only a mild defence response, not conducive to visible HR symptoms. Our results show that the use of competitive index analysis of strains expressing AvrRpt2 fusions to candidate effectors is an efficient way of establishing effector translocation into the plant cell. Furthermore, we have demonstrated that the Δ AvrRpt2-based competitive index assay detects translocation in a more sensitive manner than does the standard HR assay, thus allowing the detection of TTSS-translocated substrates failing to be revealed by standard HR assays. It should be noted that this increase in accuracy when measuring growth differences is possible as a result of the sensitivity and range of measurement provided by competitive index assays, which traditional growth assays do not provide (Macho *et al.*, 2007). Moreover, competitive index translocation assays are carried out using bacterial concentrations closer to physiological levels (Goto, 1992), are quick and easy, and are cheaper than Cya-based quantitative translocation assays. We also tested all of our protein fusions in a conductivity assay, as this type of analysis also provides a quantitative outcome. However, the results from the conductivity assay (Fig. S1, see 'Supporting Information') matched those obtained using visible HR assays, not allowing the identification of any new translocated protein.

In this study, we have demonstrated translocation for four previously unconfirmed TTSS substrates from Pph1448a: HopAJ1_{Pph1448a}, HopAK1_{Pph1448a}, HopAH2_{Pph1448a} and A0129_{Pph1448a}. There is a debate within the community about the significance of positive translocation results obtained using constitutive promoters. Our results show that very high expression of AvrRpt2 is required for any of the assays to be able to detect an HR, as only expression from the *nptII* promoter, but not the *lacZ* promoter, provides positive results. In addition, as mentioned above, we have also shown that fusion proteins can be less efficient in triggering plant defence responses than the effectors expressed independently. Therefore, our position is that, although expression *in planta* is a key requisite for a *bona fide* effector, the level of expression provided by the native promoter may be sufficient for effector biological function, but insufficient for the effective triggering of an AvrRpt2-mediated HR, thus validating the use of constitutive promoters to show translocation.

In keeping with our results indicating that standard HR assays are not sufficiently sensitive to detect the translocation of these effectors, Chang *et al.* (2005) classified HopAJ1_{Pph1448a} and HopAK1_{Pph1448a} as not translocated. Interestingly, they showed that these two proteins are expressed in an HrpL-dependent manner. Therefore, we believe that HopAK1_{Pph1448a} fulfils the two criteria (i.e. HrpL-dependent expression and translocation into the plant cell) necessary to be added to the 1448a active secretome. The homologous proteins HopAK1_{PmaES4326} and HopAK1_{PsyB728A} from *P. syringae* pv. *maculicola* ES4326 (hereafter PmaES4326)

and *P. syringae* pv. *syringae* B728A have both been shown to be translocated by standard HR assays (Guttman *et al.*, 2002; Vinatzer *et al.*, 2006). The PtoDC3000 homologue HopAK1_{PtoDC3000} has also been shown to translocate using the Cya reporter, albeit at a low level (Kvitko *et al.*, 2007). Based on this low-level translocation, these authors proposed a harpin role for HopAK1_{PtoDC3000} and, indeed, showed that high concentrations of partially purified protein induced HR when infiltrated into tobacco leaves. However, as the harpin concentration needed to trigger HR is non-physiological (Tampakaki and Panopoulos, 2000), the biological significance of this activity during the infection process remains unclear. Interestingly, these authors also found that HopAK1_{PtoDC3000} is functionally similar to HrpK1 in promoting the translocation of effectors in PtoDC3000. The PtoDC3000 protein shares 70% identity with the Pph1448a homologue HopAK1_{Pph1448a}, thus opening up the possibility that this homologue may also have harpin characteristics or a similar role in promoting translocation. If this were the case for the Pph1448a homologue, we would have a harpin, participating in the translocation of effectors, that is translocated itself. Such a protein may have a dual translocator/effector role. Interestingly, HopP1 and HrpH are both translocated in PtoDC3000, and have been shown to contribute to the translocation of effectors (Oh *et al.*, 2007).

The case of HopAJ1_{Pph1448a} is more complex as we were unable to test the translocation of the full-length protein because of its deleterious effect on bacterial growth. We therefore used the first 14 amino acids of HopAJ1_{Pph1448a} to analyse the presence of a translocation signal within this protein. In keeping with our results, Vinatzer *et al.* (2005) found that the PmaES4326 homologue of HopAJ1 gave uncertain results when the full-length protein was analysed for translocation, but clear positive results when only the first 14 amino acids were tested. Guttman *et al.* (2002) also showed a positive translocation result for HopAJ1_{PmaES4326}, although it is not possible to establish the length of the protein tested from this report. However, using a Cya-based assay and the full-length protein, Oh *et al.* (2007) found no evidence of translocation for the PtoDC3000 homologue HopAJ1_{PtoDC3000}. As these three homologues, HopAJ1_{PmaES4326}, HopAJ1_{PtoDC3000} and HopAJ1_{Pph1448a}, share an overall 95%–98% identity, and the first 102 amino acids from HopAJ1_{PmaES4326} and HopAJ1_{PtoDC3000} are identical, the different results observed are unlikely to be caused by differences between the proteins. Translocation of a truncated version of a protein could give misleading results if there were additional signals within the full-length protein that could interfere with its translocation. Another explanation for the difference in the results could be that expression of the full protein fusion from PmaES4326 or PtoDC3000 may have a pleiotropic effect on bacterial growth, in a similar manner to that seen for the Pph1448a protein. Oh *et al.* (2007) have proposed that the PtoDC3000 homologue could be secreted into the periplasm through a putative *sec* domain. In such a location, it could contribute to TTSS

secretion, allowing the nascent secretion system to penetrate the peptidoglycan layer by the action of its putative lytic transglycosylase domain. As our work and that of others have shown that at least some of the HopAJ1 homologues contain a functional TTSS translocation signal, further research will be necessary to fully elucidate how these proteins contribute to virulence.

Using competitive index assays, we have also demonstrated translocation of the previously untested HopAH2_{Pph1448a}, although expression of this protein *in planta* would need to be determined in order for it to be considered as part of the Pph1448a active secretome. Translocation of its PtoDC3000 homologue, HopAH2_{PtoDC3000}, has also been established using both Δ AvrRpt2-based HR and the Cya assays (Schechter *et al.*, 2006; Vinatzer *et al.*, 2005). However, no expression of the PtoDC3000 *hopAH2* gene has been detected *in planta* (Schechter *et al.*, 2006).

Finally, we have shown translocation for the previously untested A0129_{Pph1448a}. As the gene encoding this protein has an *hrp* box in the promoter region (Vencato *et al.*, 2006), and preliminary results from our laboratory indicate that it is indeed expressed, this protein is highly likely to be part of the Pph1448a active secretome.

Using competitive index assays, we have detected no evidence of translocation for HopJ1_{Pph1448a}, HopV1_{Pph1448a} or HopAJ2_{Pph1448a}. The HopJ1 and HopV1 homologues in PmaES4326 and PtoDC3000 have been shown to translocate; however their N-terminal sequences show sufficient changes when compared with the Pph1448a proteins to justify the different results. Although it is formally possible that translocation of the Pph1448a proteins may take place at such a low level as to require new tests for their detection, it seems more probable that these proteins have lost the ability to translocate in Pph1448a as a result of patho-adaptation. Similarly, sufficient differences can be found between the protein sequences of HopAJ2_{Pph1448a} and HopAJ1_{Pph1448a} to explain why one is translocated, whereas the other is not.

The analysis of HopZ1a_{PsyA2} by competitive index translocation assays is particularly interesting as both the tested effector and the reporter protein are capable of inducing HR in *Arabidopsis*. We found an increase in the level of attenuation detected by the competitive index of PtoDC3000 expressing HopZ1a_{PsyA2} fused to Δ AvrRpt2 (approximately 100-fold), in comparison with that of PtoDC3000 expressing the full AvrRpt2 (20–50-fold) (Figs 1b, 4a; Table S1). This increase in attenuation is consistent with an additive effect of two independent defence responses, each triggered by one of the effectors. When we determined the competitive index of PtoDC3000 expressing HopZ1a_{PsyA2} on its own, we observed an attenuation level close to 12-fold. If these two defence responses were completely independent, we would have expected the competitive index of PtoDC3000 expressing HopZ1a_{PsyA2}:: Δ AvrRpt2 to show a stronger attenuation (250–600-fold), corresponding to the added growth attenuation caused by the two responses. As effectors can undergo diverse modifica-

tions inside the plant cell leading to their activity (Coaker *et al.*, 2005; Mudgett and Staskawicz, 1999), it is possible that the fusion of two effectors may not be as efficient in triggering defence responses as the effectors would be on their own. However, competitive index analysis of PtoDC3000 simultaneously expressing AvrRpt2 and HopZ1a_{PsyA2} as separate proteins showed only a 150-fold growth attenuation in wild-type plants. This attenuation, albeit being significantly stronger than that of the fusion protein ($P < 0.05$), and thus confirming a reduced efficiency of the latter in triggering defence responses, is still significantly weaker than the expected 250–600-fold attenuation of a complete additive effect. One could argue a limitation of the assay in discriminating between strong attenuations as the reason for the difference between the observed and expected attenuation. However, we have previously detected, using competitive indices, attenuations as low as 10^{-4} , without any loss of accuracy or sensitivity (Macho *et al.*, 2007). Therefore, we can conclude that the defence responses induced by HopZ1a_{PsyA2} and AvrRpt2 are not fully additive, either because they are not completely independent, or because they can interfere with each other. In addition, the competitive index analysis of PtoDC3000 expressing HopZ1a_{PsyA2} in *rps2* plants showed a level of attenuation not significantly different from that observed in wild-type plants, indicating that HopZ1a_{PsyA2}-mediated HR is completely independent of RPS2. Whilst this work was in preparation, a study on the HopZ family requirement of myristoylation for virulence and avirulence functions also showed that HR triggered by HopZ1a_{PsyA2} is independent of RPS2 (Lewis *et al.*, 2008). Correspondingly, competitive index analysis in *rps2* plants of PtoDC3000 expressing HopZ1a_{PsyA2}:: Δ AvrRpt2, or the two effectors separately, is expected to reflect only the attenuation caused by the activation of the HopZ1a_{PsyA2}-mediated defence response. Indeed, the competitive index in *rps2* plants of PtoDC3000 expressing AvrRpt2 and HopZ1a_{PsyA2} separately showed a level of attenuation not significantly different from that determined by the expression of HopZ1a_{PsyA2} alone. PtoDC3000 expressing HopZ1a_{PsyA2}:: Δ AvrRpt2 in *rps2* plants displayed a fivefold attenuation, consistent with a reduced efficiency of the fusion protein in triggering the defence responses. It has been reported that AvrRpt2 promotes virulence in *rps2* plants in the ecotype Nossen-0, where the growth of PtoDC3000 is not as efficient as in ecotype Col-0 (Chen *et al.*, 2000). Consistent with this, we did not detect any activity associated with AvrRpt2 in promoting growth in Col-0 *rps2* plants. All of these results are compatible with HopZ1_{PsyA2} being both a bacterial effector that has evolved to exert a bacterial function in the plant cell, and an effector that the plant has evolved to recognize. Based on the level of resistance observed, we can speculate that HopZ1_{PsyA2} and AvrRpt2 are partially independent, unlike other effectors such as AvrRpm1 (Ritter and Dangl, 1996). This is consistent with the virulence target of HopZ1_{PsyA2} in the plant cell not being RIN4, or at least not by the same mechanism as the one

Table 2 Plasmids used in this study.

Name	Parent vector	Promoter	Expressed effector	Reporter/tag	Resistance*	Reference
pBBR1-MCS4	pBBR1-MCS	<i>lacZ</i>	None	None	Amp	(Kovach <i>et al.</i> , 1995)
PAMEX	pBBR1-MCS4	<i>nptII</i>	None	None	Amp, Km	This work
pAME4	pBBR1-MCS4	<i>lacZ</i>	AvrRpt2	None	Amp	This work
pAME5	pBBR1-MCS4	<i>lacZ</i>	None	AvrRpt2 ^{81–255} /FLAG	Amp	This work
pAME8	pAME4	<i>nptII</i>	AvrRpt2	None	Amp, Km	This work
pAME9	pAME5	<i>nptII</i>	None	AvrRpt2 ^{81–255} /FLAG	Amp, Km	This work
pAME5.1	pAME5	<i>lacZ</i>	AvrRpt2 ^{1–80, 81–255}	None	Amp	This work
pAME9.1	pAME9	<i>nptII</i>	AvrRpt2 ^{1–80, 81–255}	None	Amp, Km	This work
pAME9.2	pAME9	<i>nptII</i>	HopAJ1 _{Pph1448a} ^{1–388}	AvrRpt2 ^{81–255} /FLAG	Amp, Km	This work
pAME9.4	pAME9	<i>nptII</i>	HopAN1 _{Pph1448a} ^{1–401}	AvrRpt2 ^{81–255} /FLAG	Amp, Km	This work
pAME9.5	pAME9	<i>nptII</i>	HopJ1 _{Pph1448a} ^{1–157}	AvrRpt2 ^{81–255} /FLAG	Amp, Km	This work
pAME9.6	pAME9	<i>nptII</i>	HopAJ2 _{Pph1448a} ^{1–443}	AvrRpt2 ^{81–255} /FLAG	Amp, Km	This work
pAME9.7	pAME9	<i>nptII</i>	PSPPH3757 ^{1–184}	AvrRpt2 ^{81–255} /FLAG	Amp, Km	This work
pAME9.8	pAME9	<i>nptII</i>	HopAK1 _{Pph1448a} ^{1–548}	AvrRpt2 ^{81–255} /FLAG	Amp, Km	This work
pAME9.9	pAME9	<i>nptII</i>	A0129 _{Pph1448a} ^{1–323}	AvrRpt2 ^{81–255} /FLAG	Amp, Km	This work
pAME9.10	pAME9	<i>nptII</i>	HopZ1 _{PsyA2} ^{1–369}	AvrRpt2 ^{81–255} /FLAG	Amp, Km	This work
pAME9.11	pAME9	<i>nptII</i>	HopZ2 _{Ppi895A} ^{1–362}	AvrRpt2 ^{81–255} /FLAG	Amp, Km	This work
pAME9.12	pAME9	<i>nptII</i>	ShcV + HopV1 _{Pph1448a} ^{1–728}	AvrRpt2 ^{81–255} /FLAG	Amp, Km	This work
pAME9.13	pAME9	<i>nptII</i>	HopAH2 _{Pph1448a} ^{1–416}	AvrRpt2 ^{81–255} /FLAG	Amp, Km	This work
pAME9.14	pAME9	<i>nptII</i>	HopAJ1 _{Pph1448a} ^{1–14}	AvrRpt2 ^{81–255} /FLAG	Amp, Km	This work
pAME21	PAMEX	<i>nptII</i>	HopZ1 _{PsyA2}	None	Amp, Km	This work
pAME23	pAME8	<i>nptII</i>	HopZ1 _{PsyA2} , AvrRpt2	None	Amp, Km	This work

*Amp and Km indicate resistance to ampicillin and kanamycin, respectively.

used by AvrB, AvrRpm1 (Mackey *et al.*, 2002) or AvrRpt2 (Day *et al.*, 2005). However, the partially additive effect indicate that the plant responses to AvrRpt2 and HopZ1a_{PsyA2} share a component of the plant defence system. As the R gene has been discarded, the clearest candidate would be the non-race-specific disease resistance 1 protein (NDR1) (Century *et al.*, 1997).

In summary, we believe that competitive index translocation assays may be useful to determine the translocation of different effector candidates from constitutive or native promoters and to characterize the avirulence responses that may be triggered by these effectors.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Bacteria were grown overnight at 37 °C for *Escherichia coli* DH5α (Hanahan, 1983), or for 48 h at 19 °C or 28 °C for *P. syringae* strains, in Luria–Bertani (LB) medium supplemented with either kanamycin (50 µg/mL for *E. coli* DH5α; 15 µg/mL for *P. syringae* strains) or cycloheximide (50 µg/mL) as appropriate.

Plasmids

The plasmids generated in this work are listed in Table 2. DNA fragments for cloning were polymerase chain reaction (PCR)

amplified using *Pfu* polymerase (Promega, Madison, WI, USA). The genes HopZ1a_{Psy} and HopZ2_{Ppi} were cloned from *P. syringae* pv. *syringae* strain 7B40 (ICMP13516) and *P. syringae* pv. *pisi* strain 870A (ICMP10213), respectively, and confirmed by sequencing to be 100% identical to the corresponding effectors carried by strains PsyA2 and Ppi895A, where they were originally described. Genomic DNA of Pph1448a and Ppi870A was extracted using a Jet Flex Extraction Kit (Genomed, Löhne, Germany), and used as template for PCR amplification. A boiled preparation of Psy7B40 cells was used as template for PCR amplification. Plasmid pVSP61::avrRpt2 (Kunkel *et al.*, 1993) was used as template for PCR amplification of *avrRpt2* fragments. All oligonucleotides used for generation, as well as their restriction sites, are detailed in the 'Supporting Information' (Table S2). Primers included the appropriate restriction sites for cloning of the fragments into the corresponding sites of the cloning vectors (Table S2). The high-expression plasmid pAMEX was generated by cloning the *nptII* gene with its own promoter at the beginning of the pBBR1MCS-4 polylinker. A DNA fragment encoding AvrRpt2 amino acids 81–255 was amplified and tagged with the sequence encoding the FLAG epitope in a single step, and cloned into pBBR1MCS-4 and pAMEX to create low- and high-expression backbone plasmids for candidate fusions, rendering pAME5 and pAME9, respectively. Then, DNA fragments encoding the candidate open reading frames (ORFs) without the stop codon were cloned into pAME9 to create translational fusions to ΔAvrRpt2. Plasmids pAME4,

pAME8 and pAME21 were generated by cloning the PCR-amplified complete ORFs, encoding effector AvrRpt2 (pAME4 and pAME8) or HopZ1a_{PsyA2} (pAME21), in either pBBR1MCS-4 (pAME4) or pAMEX (pAME8 and pAME21). Plasmid pAME23 was generated by subcloning the HopZ1a_{PsyA2} complete ORF from pAME21 using *EcoRV* into pAME8 digested with *HindIII* and blunt-ended. All constructs were checked by sequencing. As indicated in the 'Results' section, PtoDC3000 expressing pAME9.2 showed abnormal growth and morphology (data not shown). None of the other constructs showed any effect on growth or morphology when expressed in PtoDC3000 (data not shown).

HR assays

Seeds of *A. thaliana* accession Col-0 or *rps2* mutants (SALK_087581; European *Arabidopsis* Stock Centre, Nottingham, UK) were germinated and grown in growth chambers with 8-h light/16-h dark cycles at 21 °C light/19 °C dark. Bacterial lawns were grown for 48 h at 19 °C, resuspended in 10 mM MgCl₂ and adjusted to an optical density at 600 nm (OD₆₀₀) of 0.07. Fully expanded leaves of 4–5-week-old plants were inoculated using a 2-mL syringe without needle, and symptoms were scored 24 h post-inoculation (hpi). Three independent experiments were carried out with each strain with similar results. Approximately 80 leaves were infiltrated per experiment and strain.

Competitive index translocation assays

Competitive index assays to measure growth attenuation were performed as described for *Arabidopsis* plants (Macho *et al.*, 2007). Briefly, seeds of *A. thaliana* accession Col-0 or *rps2* mutants were germinated and grown in growth chambers with 8-h light/16-h dark cycles at 21 °C. Four- to five-week-old plants were inoculated with a 5×10^4 cfu/mL mixed bacterial suspension, containing equal cfu of wild-type and effector-expressing strains, using a 2-mL syringe without needle. Serial dilutions of the inoculum were plated onto LB agar and LB agar with kanamycin to confirm the dose and relative proportion between the strains, which should be close to unity. Four days post-inoculation (dpi), bacteria were recovered from the infected leaves by taking three 10-mm-diameter discs with a cork-borer, which were homogenized by mechanical disruption into 1 mL of 10 mM MgCl₂. The bacteria were then enumerated by plating serial dilutions onto LB agar with cycloheximide and LB agar with kanamycin and cycloheximide to differentiate between the strains within the mixed infection. Bacterial enumeration was carried out in the dilution displaying between 50 and 500 colonies per plate. A competitive index in a translocation assay is defined as the effector expressing to weight output ratio divided by the input ratio. Competitive indices are the means of three replicates showing typical results of three independent experiments. Error bars represent the

standard error. Each competitive index was analysed using a homoscedastic and two-tailed Student's *t*-test and the null hypothesis: the mean index is not significantly different from unity, or from another competitive index when otherwise specified (with *P* < 0.05 used).

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SUPPORTING INFORMATION

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

Fig. S1 Conductivity assays for HR detection. Pto DC3000 strains expressing either AvrRpt2 or any of the candidate fusions were inoculated into Arabidopsis Col-0 (*RPS2/RPS2*) wild type, at an OD₆₀₀ of 0.1. Three leaf disks (corresponding to a 1.15 cm² area) were harvested, per each sample, at 16 hours post-inoculation, soaked in water for 45 minutes and transferred to 5 mL of dH₂O. Readings were taken with a Crison 524 conductivity meter (Crison Instruments S.A., Barcelona, Spain). Results show the mean of three replicates, and error bars represent standard error.

An MgCl_2 10 mM solution, and a Pto DC3000 wild type suspension, were used as mock inoculation and negative control, respectively.

Table S1 Competitive indices corresponding to Figs 1–4 of this paper.

Table S2 Oligonucleotides used in this study.

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