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Role of the acquisition of a type 3 secretion system in the emergence of novel pathogenic strains of *Xanthomonas*

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SUMMARY

Cases of emergence of novel plant-pathogenic strains are regularly reported that reduce the yields of crops and trees. However, the molecular mechanisms underlying such emergence are still poorly understood. The acquisition by environmental non-pathogenic strains of novel virulence genes by horizontal gene transfer has been suggested as a driver for the emergence of novel pathogenic strains. In this study, we tested such an hypothesis by transferring a plasmid encoding the type 3 secretion system (T3SS) and four associated type 3 secreted proteins (T3SPs) to the non-pathogenic strains of *Xanthomonas* CFBP 7698 and CFBP 7700, which lack genes encoding T3SS and any previously known T3SPs. The resulting strains were phenotyped on *Nicotiana benthamiana* using chlorophyll fluorescence imaging and image analysis. Wild-type, non-pathogenic strains induced a hypersensitive response (HR)-like necrosis, whereas strains complemented with T3SS and T3SPs suppressed this response. Such suppression depends on a functional T3SS. Amongst the T3SPs encoded on the plasmid, Hpa2, Hpa1 and, to a lesser extent, XopF1 collectively participate in suppression. Monitoring of the population sizes *in planta* showed that the sole acquisition of a functional T3SS by non-pathogenic strains impairs growth inside leaf tissues. These results provide functional evidence that the acquisition via horizontal gene transfer of a T3SS and four T3SPs by environmental non-pathogenic strains is not sufficient to make strains pathogenic. In the absence of a canonical effector, the sole acquisition of a T3SS seems to be counter-selective, and further acquisition of type 3 effectors is probably needed to allow the emergence of novel pathogenic strains.

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INTRODUCTION

Understanding the drivers of the emergence of novel plant diseases should aid in the design of more durable agronomic practices. Disease emergence may result from the introduction of microbial pathogens into novel geographical areas, for example through the commercial exchange of contaminated plant material. Disease emergence may also occur following the introduction of new hosts into an area, resulting in the disruption of geographical isolation and leading to secondary contacts with pathogenic populations (Leroy *et al.*, 2016; Monteil *et al.*, 2013). Alternatively, a microbial strain may acquire new traits that may enable an increase in fitness and/or virulence on its host and, in some cases, can possibly result in a host shift or host jump (Engering *et al.*, 2013). Such an acquisition of new traits is usually explained by recombination and/or horizontal gene transfer of novel genetic material, which may possibly involve either pathogenic or commensal strains. Moreover, global changes may lead to novel ecological conditions more favourable to the development of diseases on plants (Engering *et al.*, 2013).

Strains in the genus *Xanthomonas* collectively cause disease on more than 400 plant species. Strains of *Xanthomonas* have been involved in cases of emerging diseases on plants. In most cases, these emergences can be explained by the introduction of contaminated plant material into novel areas (Boudon *et al.*, 2005; Leduc *et al.*, 2015; reviewed in Jacques *et al.*, 2016). In some cases, however, the emergence of novel diseases may involve host shifts or host jumps (reviewed in Jacques *et al.*, 2016).

Non-pathogenic strains of *Xanthomonas* have been isolated from leaves, where they may live in sympatry with pathogenic

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strains (Boureau *et al.*, 2013; Cesbron *et al.*, 2015; Essakhi *et al.*, 2015; Garita-Cambronero *et al.*, 2016; Merda *et al.*, 2016, 2017). In the species *X. arboricola*, population genomics approaches have shown an epidemic structure with non-pathogenic and unsuccessful pathogenic strains forming a recombinant network, from which epidemic clones have emerged. These epidemic clones are the current successful pathovars *juglandis*, *corylina* and *pruni* (Merda *et al.*, 2016). Recombination and horizontal gene transfer have been shown to be the major forces in the evolution of pathogenic *Xanthomonas* strains (Merda *et al.*, 2016).

Recent genomic comparisons between non-pathogenic strains of *X. arboricola* and their closely related pathogenic strains have highlighted differences in the repertoires of methyl-accepting chemotactic proteins and TonB-dependent transporters (Cesbron *et al.*, 2015; Garita-Cambronero *et al.*, 2016). In addition, differences in resource consumption and chemotactic behaviour have been validated experimentally for some of these strains (Garita-Cambronero *et al.*, 2016).

With the exception of the sugarcane pathogens *X. albilineans* and *X. sacchari*, the virulence of pathogenic strains of *Xanthomonas* depends on a functional type 3 secretion system (T3SS) and its associated effectors (T3Es). The T3SS is a major virulence factor in Gram-negative pathogenic bacteria, which enables bacteria to inject numerous T3Es into the plant cell. Once inside the plant cell, T3Es act collectively to suppress plant defences and divert the metabolism of the host plant to the benefit of the bacterial pathogen. Strikingly, some of the non-pathogenic strains isolated are devoid of a T3SS. Moreover, all non-pathogenic *Xanthomonas* strains described so far harbour a very limited set of T3Es (Cesbron *et al.*, 2015; Essakhi *et al.*, 2015; Garita-Cambronero *et al.*, 2016; Merda *et al.*, 2016, 2017).

In *Xanthomonas* genomes, genes encoding the T3SS form a pathogenicity island. In the genus *Xanthomonas*, at least three independent acquisitions of the T3SS have been hypothesized (Merda *et al.*, 2017). Based on comparative and population genomic studies, the absence of a T3SS in the non-pathogenic strains of *Xanthomonas* described so far has been suggested to be the result of a loss (Essakhi *et al.*, 2015; Merda *et al.*, 2017). Repertoires of T3Es vary greatly between pathogenic *Xanthomonas* strains, and display some degree of correlation with the host specificity of the strains (Hajri *et al.*, 2009, 2012a, 2012b, reviewed in Jacques *et al.*, 2016). The genes encoding T3Es are found scattered in the genome and are often associated with mobile genetic elements (Cesbron *et al.*, 2015; Darrasse *et al.*, 2013). The analysis of the evolutionary history of the species *X. axonopodis* has suggested that the loss or acquisition of T3E genes via horizontal gene transfer may have contributed to the shaping of the host specificity of strains (Mhedbi-Hajri *et al.*, 2013). In the species *X. arboricola*, population genomics approaches have shown that the emergence of pathogenic populations can be associated with the acquisition of a set of core T3Es (Merda *et al.*, 2017).

The acquisition by horizontal transfer of a plasmid carrying a T3SS has been proposed to be the driver of the emergence of pathogenic strains of *Pantoea*. Indeed, in the genus *Pantoea*, numerous strains can be isolated from a wide range of host plants as non-pathogenic, epiphytic or endophytic strains. However, strains of several *Pantoea* species are pathogenic and cause severe losses in crops. In the pathovars *gypsophilae* and *betae* of *P. agglomerans*, the pathogenicity depends on the pPATH plasmid. Amongst other virulence genes, this plasmid contains genes encoding a T3SS and a suite of T3Es, and the inactivation of the T3SS strongly impairs virulence (reviewed in Manulis and Barash, 2003). The pathovars *gypsophilae* and *betae* have been suggested to arise from a commensal strain of *Pantoea* after the acquisition of the pPATH plasmid (De Maayer *et al.*, 2012; Manulis and Barash, 2003; Weinthal *et al.*, 2007). The pPATH plasmids have a broad host range and can replicate in *Xanthomonas* (Weinthal *et al.*, 2007).

In this study, we isolated two non-pathogenic strains of *Xanthomonas* and sequenced their genomes. Comparative genomics analyses highlighted the absence of a T3SS and previously known T3Es in both strains. This provided a good opportunity to test whether the acquisition of a sole T3SS could promote the pathogenicity of *Xanthomonas* commensal strains. Thus, a wild-type copy and Tn5 derivatives of the *hrp* gene cluster of strain *Xcc* 8004 (Arlat *et al.*, 1991) were transferred into the two non-pathogenic strains of *Xanthomonas*. In strain *Xcc* 8004, the *hrp* gene cluster encodes the T3SS and four type 3 secreted proteins (T3SPs), i.e. Hpa1, Hpa2, HrpW and XopF1. The resulting strains were phenotyped on *Nicotiana benthamiana* to determine the impact on the plant–bacterium interaction of the acquisition of a T3SS and its associated secreted proteins by non-pathogenic *Xanthomonas* strains.

RESULTS

Isolation of two non-pathogenic strains of *Xanthomonas* from bean seeds

Amongst the strains isolated from bean seed macerate, strains CFBP 7698 and CFBP 7700 were morphologically similar to *Xanthomonas* strains. Their pathogenicity was tested on bean, their host of isolation. Both strains CFBP 7698 and CFBP 7700 are not pathogenic on bean: inoculation by dipping of strains CFBP 7698 and CFBP 7700 on bean leaves did not result in the appearance of any symptoms, unlike the bean pathogenic strain of *Xanthomonas citri* pv. *fuscans* CFBP 4834 which was used as a positive control (data not shown). No amplicons could be obtained using X4c and X4e primers (Audy *et al.*, 1994), or the Am1F/R and Am2F/R duplex (Boureau *et al.*, 2013),

suggesting that they do not belong to *X. citri* pv. *fuscans* or *X. phaseoli* pv. *phaseoli*. Nevertheless, these isolates belong to the Xanthomonadaceae, as they produced a 1500-bp amplicon when tested by polymerase chain reaction (PCR) with *rpfB*-F and *rpfB*-R primers (Simoes *et al.*, 2007), and to the *Xanthomonas* genus, according to the comparison of their *rpoD* and *gyrB* sequences with the Phylosearch database (https://147.99.127.226/pub/cfbp/phylosearch3.0/index.php).

The genome sequences of strains CFBP 7698 and CFBP 7700 were obtained to screen for the presence of the common features shared between most strains in the genus *Xanthomonas* (Table 1). First, these sequences were used to position both strains in a phylogeny of 82 genomes spanning the diversity of the genus *Xanthomonas* (Fig. 1). The strain CFBP 7698 clusters with strains CFBP 7912 and Nyagatare of the species *X. cannabis*. The average nucleotide identity (ANI) values were determined between CFBP 7698 and its neighbour strains in the phylogeny. According to the ANI results, strain CFBP 7698 belongs to the species *X. cannabis* and strain CFBP 7700 to the species *X. campestris* (Fig. S1, see Supporting Information).

The genomes of strains CFBP 7698 and CFBP 7700 were compared with functional repertoires that are widespread in the

Table 1 General features of the genome sequences of the non-pathogenic *Xanthomonas* strains CFBP 7698 and CFBP 7700.

Strain		CFBP 7698	CFBP 7700
Species		*X. cannabis*	*X. campestris*
Coverage		250 X	250 X
Number of contigs		29	15
Genome size (nt)		4883133	5044897
N50 (nt)		1328031	3787407
Features			
rpf cluster		Yes	Yes
pig cluster		Yes	Yes
Flagellar cluster		Yes	Yes
Methyl-accepting chemotaxis proteins		39	44
Two-component system-related genes		112	111
EPS (exopolysaccharides)	gum cluster	Yes	Yes
LPS (lipopolysaccharides)		Closely related to *X. cannabis Nyagatare* (Aritua *et al.*, 2015)	Distinct from previously sequenced *X. campestris* LPS clusters
		wxc genes that are involved in the elongation and branching of the O-antigen rhamnose chains are absent in both strains CFBP 7698 and CFBP 7700. Overall, in both strains, clusters for LPS biosynthesis diverge significantly from clusters found in model strains of *Xanthomonas*	
TonB-dependent transporters		65	69
T1SS		At least 5	At least 4
T2SS	xcs-type	**Yes**	Yes
	xps-type	**Yes**	Yes
T2 secreted enzymes		79	74
T3SS		No	No
T3Es		No	No
T4SS		**Yes.** Cluster identical to the chromosomal cluster of strain *Xac* 306 (Alegria *et al.*, 2005)	VirB6 lacking. T4SS not functional?
T5SS		*fhaB.* Others? *	*shlB*. Others? *
T6SS		No	Group 3 T6SS similar to *Xff*4834R (Darrasse *et al.*, 2013)
T1 pilus		Yes	Yes
T4 pilus		Yes	Yes

*Other adhesins may have been missed during the assembly of the draft genomes.

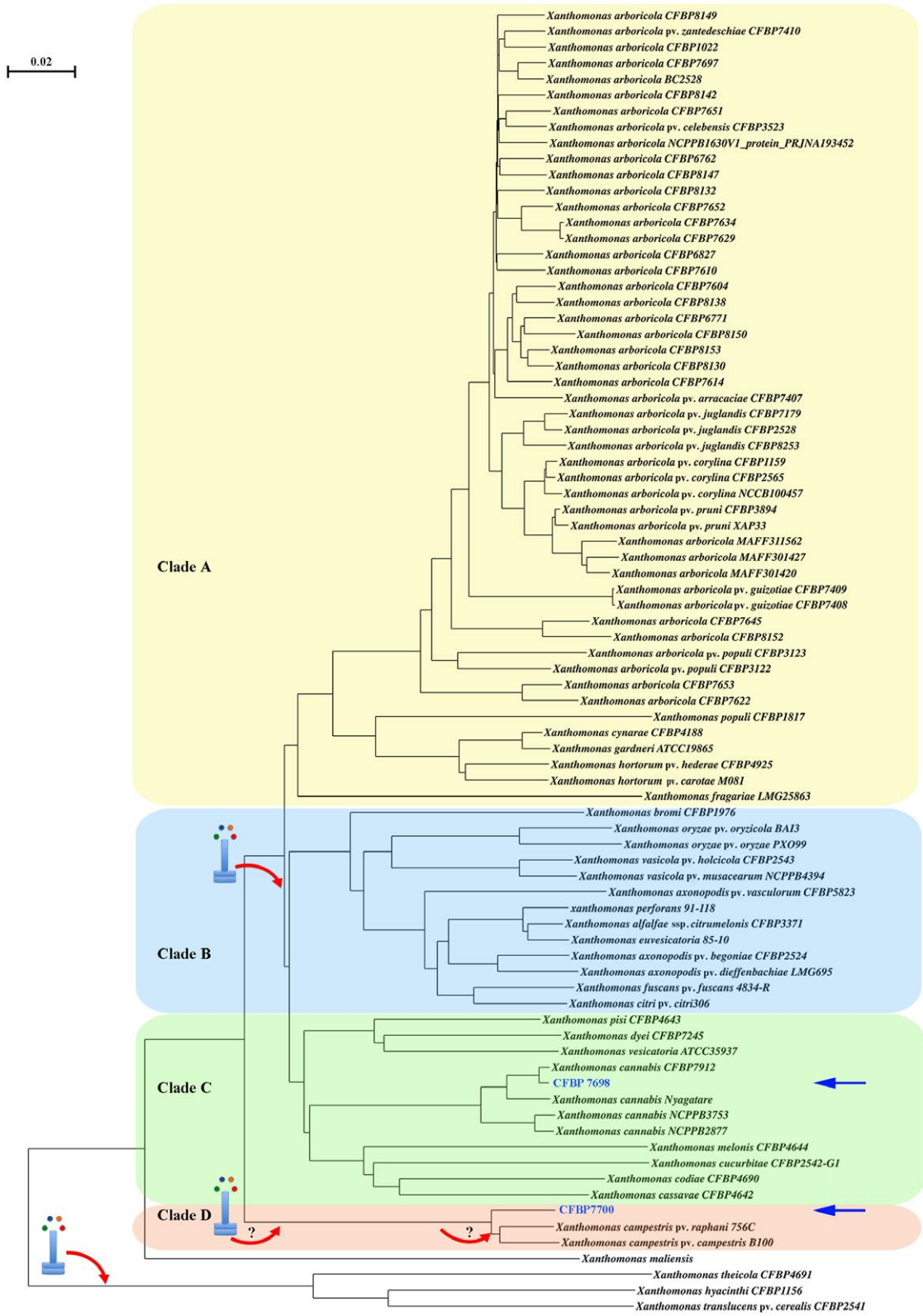

Fig. 1 Phylogenetic positioning of the non-pathogenic strains CFBP 7698 and CFBP 7700. Strains CFBP 7698 and CFBP 7700 were positioned amongst the 82 genomes described by Merda *et al.* (2017). The phylogeny was constructed based on the 968 protein sequences shared by the 84 strains, using CVTree software, with a K-mer size of 6. Bar, 0.02 substitutions per site. The non-pathogenic strain CFBP 7698 clusters with strains belonging to the species *Xanthomonas cannabis*, and CFBP 7700 clusters with strains belonging to the species *Xanthomonas campestris*. The major independent acquisition events of a T3SS (as proposed by Merda *et al.*, 2017) are indicated with a red arrow. Clades A, B, C and D are defined as proposed by Merda *et al.* (2017).

genus *Xanthomonas*. A brief summary of their genomic content is presented in Table 1. Most importantly, the genome sequences of strains CFBP 7698 and CFBP 7700 do not contain any canonical T3SS. When blasting representative sequences of each type of T3SS described in Gram-negative pathogens on the genomes of CFBP 7698 and CFBP 7700, only genes related to the flagellar apparatus produced significant alignments (E-value < 1e-06, data not shown). Moreover, no significant hit was obtained when blasting representative sequences of most putative T3Es previously identified in Gram-negative bacterial pathogens on the genomes of strains CFBP 7698 and CFBP 7700. Hence, strains CFBP 7698 and CFBP 7700 do not feature previously known T3E genes. However, sequences similar to *avrXccA1* are found in the genomes of both strains, and a sequence similar to *avrXccA2* is found in strain CFBP 7698. It is noteworthy that, despite the absence of genes encoding the T3SS and T3Es, the genome sequences of strains CFBP 7698 and CFBP 7700 contain sequences orthologous to genes encoding the master regulators HrpX and HrpG.

We propose that both CFBP 7698 and CFBP 7700 are non-pathogenic strains of *Xanthomonas*, as they are unable to induce symptoms on their host of isolation (bean) and do not feature genes associated with type 3 secretion known to be essential for the pathogenicity of *X. campestris*.

Transfer of the plasmid pIJ3225 into naturally non-pathogenic strains of *Xanthomonas* leads to the formation of a functional T3SS

To test the impact of the acquisition by horizontal gene transfer of a T3SS and four T3SPs by non-pathogenic strains of *Xanthomonas*, the plasmid pIJ3225 was transferred into spontaneous Rif^r derivatives of strains CFBP 7698 (7698R) and CFBP 7700 (7700R) to obtain strains 7698R pIJ3225 and 7700R pIJ3225, respectively. The plasmid pIJ3225 carries the *hrp* cluster of *Xcc* 8004 encoding a T3SS, as well as four genes encoding known T3SPs: *hrpW*, *hpa1*, *hpa2* and *xopF1* (Fig. 2) (Arlat *et al.*, 1991; He *et al.*, 2007).

Moreover, to confirm that the acquisition of pIJ3225 enables the formation of a functional T3SS in non-pathogenic strains naturally devoid of T3SS, the plasmid was conjugated into a spontaneous Rif^r derivative of strain CFBP 7634 (7634R). Indeed, the non-pathogenic strain CFBP 7634 is devoid of T3SS, and contains *hrpX*, *hrpG* and only three known T3E genes, including *avrBs2*. If pIJ3225 is sufficient to express a functional T3SS in a non-pathogenic *Xanthomonas* background, the strain 7634R pIJ3225 should be able to translocate AvrBs2 into plant cells.

The expression of six of the genes present in the *hrp* cluster of *Xcc* 8004 (*hrcC*, *hrcN*, *hrcV*, *hpa1*, *hpa2* and *xopF1*) was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) in all the transconjugants (data not shown). The effective translocation of AvrBs2 was assessed by the inoculation of strains 7634R and 7634R pIJ3225 on *Capsicum annuum* ECW and ECW20R which carry the *Bs2* resistance gene. The recognition of AvrBs2 was shown by the onset of a hypersensitive response (HR) on ECW20R plants carrying the *Bs2* resistance gene (Fig. 3). To confirm that the transfer of AvrBs2 was T3SS dependent, *C. annuum* ECW and ECW20R were also inoculated with the strain 7634R G9 (Table 2), which carries a pIJ3225 derivative with a Tn5 insertion inactivating *hrcJ*, a gene necessary for secretion through the T3SS. The absence of HR induction after inoculation of strain 7634R G9 on *C. annuum* showed that the translocation of AvrBs2 into plant cells by strain 7634R pIJ3225 depends on a functional T3SS (Fig. 3). Therefore, the T3SS encoded by pIJ3225 was functional in non-pathogenic strains naturally devoid of T3SS.

The acquisition of a functional T3SS leads to the suppression of an HR-like necrosis induced by the wild-type, non-pathogenic strains on *N. benthamiana*

When inoculated on leaves of *N. benthamiana*, the wild-type strains CFBP 7698 and CFBP 7700, as well as their rifampicin-resistant derivatives 7698R and 7700R, respectively, induced a rapid HR-like necrosis within 1 and 2 days post-inoculation (dpi) (Fig. 4). In contrast, the inoculation of strains 7698R pIJ3225 and 7700 pIJ3225 only produced a mild chlorosis after 6 dpi (Fig. 4). Therefore, the acquisition of pIJ3225 enabled strains 7698R and 7700R to suppress the onset of HR-like necrosis induced by the wild-type strains.

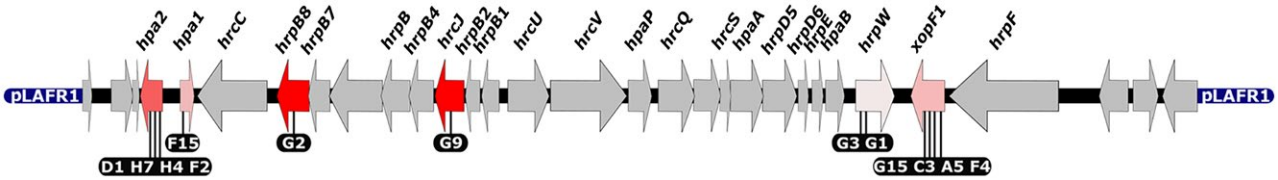

Fig. 2 Map of the Tn5 insertions in the *hrp* cluster of *Xcc* 8004 cloned in pIJ3225. The *hrp* cluster is represented on the basis of the genome sequence of *Xcc* 8004 (He *et al.*, 2007). Approximate positions of Tn5 insertions were determined on the basis of the physical map provided by Arlat *et al.* (1991). The exact positions of Tn5 insertions were determined by sequencing amplicons obtained using primers designed on Tn5 and the putative target genes. Vertical bars show the position of Tn5 insertions in pIJ3225. Genes involved in the suppression of the hypersensitive response (HR)-like necrosis are represented in red. The intensity of the colour is representative of the impact of the Tn5 insertions on the ability of pIJ3225 to suppress the HR-like necrosis induced by the non-pathogenic strains CFBP 7698 and CFBP 7700. [Colour figure can be viewed at wileyonlinelibrary.com]

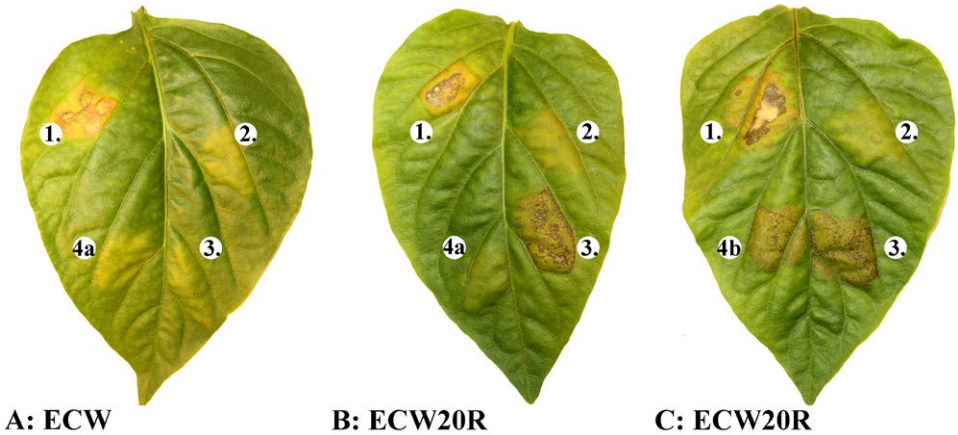

Fig. 3 The type 3 secretion system (T3SS) encoded on pIJ3225 is functional in non-pathogenic strains of *Xanthomonas* naturally devoid of T3SS. *Capsicum annuum* ECW (A) and ECW20R carrying the *Bs2* resistance gene (B, C) were infiltrated with *Xanthomonas* strains to test for a hypersensitive response (HR). 1: Strain *Xcc* 8004 serves as an HR positive control on ECW and ECW20R to ensure that inoculated leaves are fully responsive. 2: Strain 7634R serves as a negative control: it does not induce HR on ECW. Strain 7634R possesses *AvrBs2*, but no T3SS encoding genes, and therefore does not induce HR on ECW20R either. 3: Strain 7634R pIJ3225: HR is observed specifically on ECW20R plants but not on ECW plants, showing that HR is caused by the specific recognition of AvrBs2 by *Bs2*. Therefore, pIJ3225 encodes a T3SS that enables the translocation of AvrBs2 into plant cells in a non-pathogenic *Xanthomonas* background. 4a: Strain 7634R G9: the disruption of *hrcJ* inactivates the secretion through the T3SS. The absence of HR on ECW20R plants shows that the transfer of AvrBs2 is T3SS dependent. 4b: Strain 7634R H7: the disruption of *hpa2* leads to an HR that appears milder than that observed in 3 (strain 7634R pIJ3225). Thus the translocation of AvrBs2 in 4b may be less efficient than that in 3. [Colour figure can be viewed at wileyonlinelibrary.com]

Derivatives of pIJ3225 carrying Tn5 insertions were conjugated into strains 7698R and 7700R (Table 2) in order to: (i) confirm that the lack of HR-like necrosis was dependent on a functional T3SS; and (ii) to identify genes of pIJ3225 involved in this suppression. The development of HR-like necrosis was first assessed by visual phenotyping (Table 3): the pIJ3225 derivatives G9 and G2 did not confer the ability to either strain 7698R or 7700R to suppress the onset of HR-like necrosis. Hence, Tn5 insertions disrupting the genes necessary for secretion via T3SS (*hrpB8* for G2 and *hrcJ* for G9, Fig. 2) abolish the ability of pIJ3225 to suppress HR-like necrosis.

The impact of the other derivatives was more difficult to assess because of intermediate visual phenotypes and heterogeneous leaf responses following inoculation. Therefore, we used chlorophyll fluorescence imaging (CFI) to better calibrate the phenotyping of the plant response to the test strains. To bypass the impact of leaf heterogeneity, each leaf was inoculated with one test strain and three controls: (i) a mock-inoculated control; (ii) the wild-type strain to monitor the reactivity of each leaf (HR-like necrosis control); and (iii) the wild-type strain carrying pIJ3225 to control the suppression of HR-like necrosis (Fig. 5A). Thus, the chlorophyll fluorescence values of the areas infiltrated with the test strains carrying Tn5 derivatives of pIJ3225 were compared with the values of the set of internal leaf controls to determine the occurrence of HR-like necrosis on each leaf. We performed a multiparametric, threshold-based segmentation process based on the minimum fluorescence (F_o) and maximum fluorescence (F_m). Indeed, these two parameters provide complementary contrasts to image the development of symptoms: F_o was mostly impacted by the development of a strong necrosis (Fig. 5B), whereas F_m could reflect different stages between chlorosis and necrosis (Fig. 5C). Within each leaf, pixels of the area inoculated with the test strains were classified into clusters that represent the intensity of the damage. These clusters were built on the basis of the distributions of F_o and F_m fluorescence values in areas inoculated with control strains (Fig. 5D–G). After each pixel of the area inoculated with the test strains had been assigned to one of the four clusters, the clusters were merged to obtain the resulting image of the inoculated area (Fig. 5H).

Using such a multiparametric image analysis, we estimated the ability of the various constructs to suppress HR-like necrosis (Table 3). Indeed, in some cases, visual inspection could hardly discriminate between areas inoculated with the wild-type, non-pathogenic strains and those inoculated with strains carrying pIJ3225 (Table 3). In contrast, the analysis of the distributions of fluorescence values as described above allowed such a discrimination (Table 3). Consequently, phenotyping the impact of the various Tn5 insertions in pIJ3225 using a CFI approach yielded more sensitive results than did visual inspection (Table 3).

Amongst the constructs tested, the Tn5 insertions located in *hpa2* impaired significantly the ability of pIJ3225 to suppress HR-like necrosis induced by the non-pathogenic *Xanthomonas* strains (Table 3). The inactivation of *hpa2* produced a milder phenotype in a 7700R background (Table 3). The disruption of *hpa1* affected the ability of pIJ3225 to suppress HR-like necrosis (Table 3). To a lesser extent, the Tn5 insertions in *xopF1* and *hrpW* also seemed

Table 2 Bacterial strains used in this study.

Strains	Name in the text*	Accession	Genotype or relevant phenotype	Reference
Xanthomonas arboricola	CFBP 7634	CFBP 7634	Non-pathogenic isolate from walnut tree	Essakhi *et al.* (2015)
	7634R	CFBP 13419	Spontaneous Rif^r derivative of strain CFBP 7634	This study
	7634R pIJ3225	CFBP 13429	13419 pIJ3225 Rif^r Tet^r	This study
	7634R A5	CFBP 13437	13419 pIJ3225 *xopF1*::Tn5 Rif^r Tet^r Kan^r	This study
	7634R C3	CFBP 13438	13419 pIJ3225 *xopF1*::Tn5 Rif^r Tet^r Kan^r	This study
	7634R D1	CFBP 13439	13419 pIJ3225 *hpa2*::Tn5 Rif^r Tet^r Kan^r	This study
	7634R F2	CFBP 13441	13419 pIJ3225 *hpa2*::Tn5 Rif^r Tet^r Kan^r	This study
	7634R F4	CFBP 13442	13419 pIJ3225 *xopF1*::Tn5 Rif^r Tet^r Kan^r	This study
	7634R F15	CFBP 13440	13419 pIJ3225 *hpa1*::Tn5 Rif^r Tet^r Kan^r	This study
	7634R G2	CFBP 13444	13419 pIJ3225 *hrpB8*::Tn5 Rif^r Tet^r Kan^r	This study
	7634R G3	CFBP 13445	13419 pIJ3225 *hprW*::Tn5 Rif^r Tet^r Kan^r	This study
	7634R G9	CFBP 13446	13419 pIJ3225 *hrcJ*::Tn5 Rif^r Tet^r Kan^r	This study
	7634R G15	CFBP 13443	13419 pIJ3225 *xopF1*::Tn5 Rif^r Tet^r Kan^r	This study
	7634R H4	CFBP 13447	13419 pIJ3225 *hpa2*::Tn5 Rif^r Tet^r Kan^r	This study
	7634R H7	CFBP 13448	13419 pIJ3225 *hpa2*::Tn5 Rif^r Tet^r Kan^r	This study
Xanthomonas campestris	CFBP 7698	CFBP 7698	Non-pathogenic isolate from bean seeds	This study
	7698R	CFBP 13420	Spontaneous Rif^r derivative of strain CFBP 7698	This study
	7698R pIJ3225	CFBP 13431	13431 pIJ3225	This study
	7698R A5	CFBP 13464	13431 pIJ3225 *xopF1::Tn5* Rif^r Tet^r Kan^r	This study
	7698R C3	CFBP 13465	13431 pIJ3225 *xopF1::Tn5* Rif^r Tet^r Kan^r	This study
	7698R D1	CFBP 13466	13431 pIJ3225 *hpa2::Tn5* Rif^r Tet^r Kan^r	This study
	7698R F2	CFBP 13468	13431 pIJ3225 *hpa2::Tn5* Rif^r Tet^r Kan^r	This study
	7698R F4	CFBP 13469	13431 pIJ3225 *xopF1::Tn5* Rif^r Tet^r Kan^r	This study
	7698R F15	CFBP 13467	13431 pIJ3225 *hpa1::Tn5* Rif^r Tet^r Kan^r	This study
	7698R G1	CFBP 13470	13431 pIJ3225 *hrpW::Tn5* Rif^r Tet^r Kan^r	This study
	7698R G2	CFBP 13472	13431 pIJ3225 *hrpB8::Tn5* Rif^r Tet^r Kan^r	This study
	7698R G3	CFBP 13473	13431 pIJ3225 *hprW::Tn5* Rif^r Tet^r Kan^r	This study
	7698R G9	CFBP 13474	13431 pIJ3225 *hrcJ::Tn5* Rif^r Tet^r Kan^r	This study
	7698R G15	CFBP 13471	13431 pIJ3225 *xopF1::Tn5* Rif^r Tet^r Kan^r	This study
	7698R H4	CFBP 13475	13431 pIJ3225 *hpa2::Tn5* Rif^r Tet^r Kan^r	This study
	7698R H7	CFBP 13476	13431 pIJ3225 *hpa2::Tn5* Rif^r Tet^r Kan^r	This study
	CFBP 7700	CFBP 7700	Non-pathogenic isolate from bean seeds	
	7700R	CFBP 13427	Spontaneous Rif^r derivative of strain CFBP7700	This study
	7700R pIJ3225	CFBP 13436	13427 pIJ3225	This study
	7700R A5	CFBP 13450	13427 pIJ3225 *xopF1::Tn5* Rif^r Tet^r Kan^r	This study
	7700R C3	CFBP 13451	13427 pIJ3225 *xopF1::Tn5* Rif^r Tet^r Kan^r	This study
	7700R D1	CFBP 13452	13427 pIJ3225 *hpa2::Tn5* Rif^r Tet^r Kan^r	This study
	7700R F2	CFBP 13454	13427 pIJ3225 *hpa2::Tn5* Rif^r Tet^r Kan^r	This study
	7700R F4	CFBP 13455	13427 pIJ3225 *xopF1::Tn5* Rif^r Tet^r Kan^r	This study
	7700R F15	CFBP 13453	13427 pIJ3225 *hpa1::Tn5* Rif^r Tet^r Kan^r	This study
	7700R G1	CFBP 13456	13427 pIJ3225 *hrpW::Tn5* Rif^r Tet^r Kan^r	This study
	7700R G2	CFBP 13458	13427 pIJ3225 *hrpB8::Tn5* Rif^r Tet^r Kan^r	This study

(Continued)

Table 2 Continued.

Strains	Name in the text*	Accession	Genotype or relevant phenotype	Reference
	7700R G3	CFBP 13459	13427 pIJ3225 *hprW::Tn5* Rif^r Tet^r Kan^r	This study
	7700R G9	CFBP 13460	13427 pIJ3225 *hrcJ::Tn5* Rif^r Tet^r Kan^r	This study
	7700R G15	CFBP 13457	13427 pIJ3225 *xopF1::Tn5* Rif^r Tet^r Kan^r	This study
	7700R H4	CFBP 13461	13427 pIJ3225 *hpa2::Tn5* Rif^r Tet^r Kan^r	This study
	7700R H7	CFBP 13462	13427 pIJ3225 *hpa2::Tn5* Rif^r Tet^r Kan^r	This study
Xanthomonas campestris pv. campestris	*Xcc* 8004	CFBP 6650	Pathogenic strain isolate from *Brassica oleracea* var. *botrytis*	Turner *et al.* (1984)
Xanthomonas citri pv. fuscans	CFBP 4834	CFBP 4834	Pathogenic strain isolate from *Phaseolus vulgaris* cv. *Michelet*	Darrasse *et al.* (2013)
Escherichia coli	*E. coli K12*	CFBP 5947	Wild-type strain of *E. coli*. syn. CIP 54, CFBP 5947, IFO 3301, NCDO 1984, NCIB 10083	https://catalogue-cfbp.inra.fr/recherche_e.php
Plasmids				
		pIJ3225	*hrp* gene cluster of strain *Xcc* 8004 cloned in pLAFR1. IncP1, Tet^r, *cos*$^+$, Tra$^-$, Mob$^+$	Arlat *et al.* (1991)
		pIJ3225 derivatives	Carries random Tn5-B20 insertions in the *hrp* cluster of strain *Xcc 8004*	Arlat *et al.* (1991)
		pRK600	Cm^r, *tra*RK2, *ori*ColE1	Finan *et al.* (1986)

CFBP, French Collection of Plant-Associated Bacteria, Institut National de la Recherche Agronomique (INRA), Angers, France; 12842, 12895, our own local collection.

CIP, Collection of Institut Pasteur, Institut Pasteur, Paris, France.

IFO, Institute for Fermentation, Yodogawa-kuaka, Osaka, Japan.

NCDO, National Collection of Dairy Organisms, National Institute for Research in Dairying, Reading, UK.

NCIB, National Collection of Industrial Bacteria, Edinburgh, UK.

*In accordance with the names given to the Tn5 insertions in the hrp cluster of Xcc 8004 (CFBP 8340), as described in Arlat et al. (1991).

to affect the ability to suppress HR-like necrosis. As in the case of the disruption of *hpa2*, the impact of Tn5 insertions in *hpa1*, *xopF1* and *hrpW* was weaker in a 7700R background than in a 7698R background (Table 3).

We confirmed that the lack of suppression observed was not caused by a secretion defect by testing all the Tn5 derivatives of pIJ3225 in a 7634R background. Except for the G2 and G9 constructs, all other constructs retained the ability to translocate AvrBs2 into *C. annuum* cells. Therefore, except for G9 and G2, all constructs encoded a functional T3SS. Hence, Hpa2 and Hpa1, and, to a lesser extent, XopF1 and HrpW, participate in the suppression of HR-like necrosis.

The acquisition of a T3SS and four T3SPs by non-pathogenic strains 7698R and 7700R impacts the bacterial population sizes in *N. benthamiana*

During the first 3 days following inoculation on *N. benthamiana*, bacterial populations increased significantly (*c.* 2 logs) for both wild-type strains 7698R and 7700R. However, such an increase was transient as bacterial populations decreased between days 3 and 6 after inoculation (Fig. 6). In both 7698R and 7700R backgrounds, the population sizes of strains complemented with pIJ3225 were significantly decreased by almost 1 log compared with the wild-type strains. Such a decrease was dependent on a functional T3SS, as insertions disrupting the T3SS (*hrcJ* and *hrpB8*) restored levels of population growth comparable with that of the wild-type strains (Fig. 6). The population sizes of strains complemented with construct H4, carrying a Tn5 insertion in *hpa2*, were intermediate between the wild-type strains and strains complemented with pIJ3225, suggesting that Hpa2 contributes to the population size decrease on *N. benthamiana* (Fig. 6).

DISCUSSION

To date, most plant–*Xanthomonas* interactions have been described as pathogenic, and T3SS and its associated effectors (T3Es) are major pathogenicity determinants for pathogenic *Xanthomonas* strains. Although the occurrence of non-pathogenic strains of *Xanthomonas* was initially reported in the mid-1980s (Maas *et al.*, 1985), this topic has received increasing attention only more recently (Boureau *et al.*, 2013; Cesbron *et al.*, 2015; Essakhi *et al.*, 2015; Garita-Cambronero *et al.*, 2016,

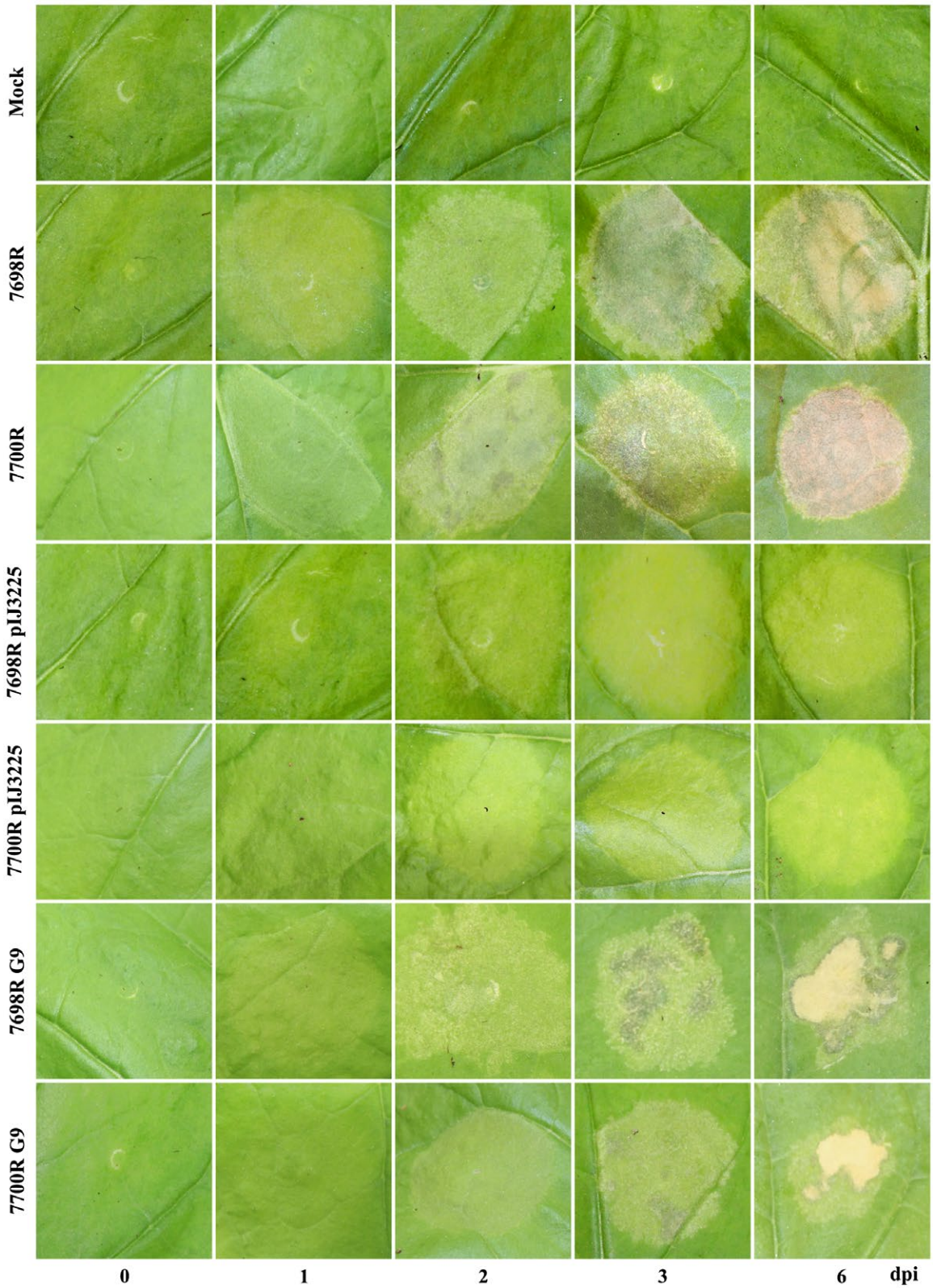

Fig. 4 The acquisition of pIJ3225 enables the suppression of the hypersensitive response (HR)-like necrosis on *Nicotiana benthamiana.* Leaves were inoculated with mock, wild-type strains 7698R and 7700R, as well as their transconjugants carrying pIJ3225 or the pIJ3225::G9 (*hrcJ*) construct. The development of HR-like necrosis was followed over 6 days post-inoculation (dpi). The acquisition of a functional T3SS on pIJ3225 enables the suppression of the HR-like necrosis on *N. benthamiana.* The acquisition of a non-functional T3SS on plasmid pIJ3225::G9 does not enable the suppression of the HR-like necrosis on *N. benthamiana.* [Colour figure can be viewed at wileyonlinelibrary.com]

Table 3 Occurrence of hypersensitive response (HR)-like necrosis with the various strains and constructs tested.

Construct (Arlat *et al.*, 1991)	Localization of the Tn5 insertion	7698R background		7700R background		7634R background
		Visual inspection	Chlorophyll fluorescence imaging	Visual inspection	Chlorophyll fluorescence imaging	Ability to translocate *avrBs2*
G2	*hrpB8*	11/18	13/18	16/18	16/18	–
G9	*hrcJ*	16/18	16/18	16/18	16/18	–
H4	*hpa2*	4/18	9/18	1/18	3/18	+
H7	*hpa2*	7/18	10/18	2/18	4/18	+
D1	*hpa2*	6/18	9/18	0/18	4/18	+
F2	*hpa2*	6/18	8/18	0/18	4/18	+
A5	*xopF1*	0/18	2/18	0/18	0/18	+
C3	*xopF1*	2/18	3/18	3/18	7/18	+
F4	*xopF1*	0/18	2/18	0/18	0/18	+
G15	*xopF1*	1/18	2/18	0/18	2/18	+
G1	*hrpW*	0/18	0/18	0/18	0/18	+
G3	*hrpW*	0/18	3/18	0/18	1/18	+
F15	*hpa1*	5/18	5/18	0/18	2/18	+
pIJ3225 (HR-like necrosis suppression control)	None	8/252	0/252	4/252	0/252	+
Wild-type strain (HR-like necrosis control)	None	250/252	252/252	250/252	252/252	–

Nicotiana benthamiana plants were inoculated with strains 7698R and 7700R complemented with pIJ3225 or Tn5 derivatives. Each leaf tested was inoculated with three control conditions [mock-inoculated control, HR-like necrosis control (7698R or 7700R); HR-like suppression control (7698R pIJ3225 or 7700R pIJ3225)] and a strain carrying one of the Tn5 constructs tested. Each Tn5 construct was tested on 18 independent leaves. In total, each control condition was tested on 252 independent leaves. For each leaf, in each inoculated area, the occurrence of HR-like necrosis was evaluated by visual inspection and by chlorophyll fluorescence imaging at 6 days post-inoculation (dpi). Results are reported as the number of infiltrated areas in which HR-like necrosis was observed. Phenotyping based on chlorophyll fluorescence imaging provided more sensitive results than visual inspection in cases of partial restoration of HR-like necrosis.

2017; Jacques *et al.*, 2016; Merda *et al.*, 2016, 2017; Triplett *et al.*, 2015). In this study, two non-pathogenic strains of *Xanthomonas* were isolated and their genomes were sequenced. Strain CFBP 7698 belongs to the species *X. cannabis*, and strain CFBP 7700 belongs to the species *X. campestris*. The analysis of their genome sequences revealed that these strains are devoid of T3SS and of any previously described T3Es. Other strains of *X. cannabis* devoid of canonical T3SS and T3Es have been reported recently (Jacobs *et al.*, 2015). These strains remained pathogenic as they were able to induce water-soaking on pepper plants, presumably because of the action of cell wall-degrading enzymes (CWDEs). In these X. *cannabis* strains, the HrpG and HrpX key regulators have been suggested to control the induction of the CWDE genes *pehA* and *pehD* (Jacobs *et al.*, 2015). Although their genomic sequences contain orthologues to *hrpG*, *hrpX*, *pehA* and *pehD*, strains CFBP 7698 and CFBP 7700 did not induce symptoms on bean (their host of isolation) or water-soaking on pepper plants. Therefore, we consider that strains CFBP 7698 and CFBP 7700 are non-pathogenic.

Strains CFBP 7698 and CFBP 7700 induce necrosis on *N. benthamiana* in the absence of T3SS, as also observed in the case of strains of *X. cannabis* (Jacobs *et al.*, 2015). Such a necrosis resembles an HR as it appears very rapidly (Fig. 4). Such an HR-like necrosis is rather unusual in the absence of T3SS and T3Es for Gram-negative bacteria. Nevertheless, in response to Gram-positive bacteria, such as *Clavibacter michiganensis,* the onset of HR-like necrosis in the absence of T3SS and T3Es has been reported. In such a case, the HR observed in *Nicotiana* species is triggered by Chp-7 or ChpG serine proteases, which are secreted in the apoplast, but not translocated into plant cells (Lu *et al.*, 2015).

In the case of strains CFBP 7698 and CFBP 7700, the nature of such an HR-like response on *N. benthamiana* remains to be determined. As proposed in the case of strains of *X. cannabis*, such an HR-like necrosis may be a result of the activity of CWDEs (Jacobs *et al.*, 2015). Indeed, the pectate lyase XagP has been shown to trigger an HR on tobacco and pepper (Kaewnum *et al.*, 2006). An orthologue of XagP is found in the genomes of CFBP 7698 and CFBP 7700, and constitutes an excellent candidate inducer of the HR-like necrosis observed on *N. benthamiana*. Another example has been reported in rice, in which the damage resulting from the action of the lipase/esterase LipA produced by *X. oryzae* induced

cell death in roots. To cause disease, *X. oryzae* suppressed such a LipA-induced cell death and other innate immunity responses of rice through the combined additive action of the four T3Es, XopN, XopQ, XopX and XopZ (Sinha *et al.*, 2013).

Surprisingly, despite the rapid necrosis observed in the inoculated area, the non-pathogenic strains 7698R and 7700R multiplied inside leaf tissues in *N. benthamiana* until 3 dpi. Population sizes of strains 7698R and 7700R eventually decreased at 6 dpi, showing that such a multiplication is only transient. Whether the population size decrease observed at 6 dpi is the result of the observed HR-like necrosis or is caused by the onset of another set of plant defences remains unknown. It still remains to be determined whether populations of strains 7698R and 7700R eventually disappear or stabilize in leaf tissues after 6 dpi.

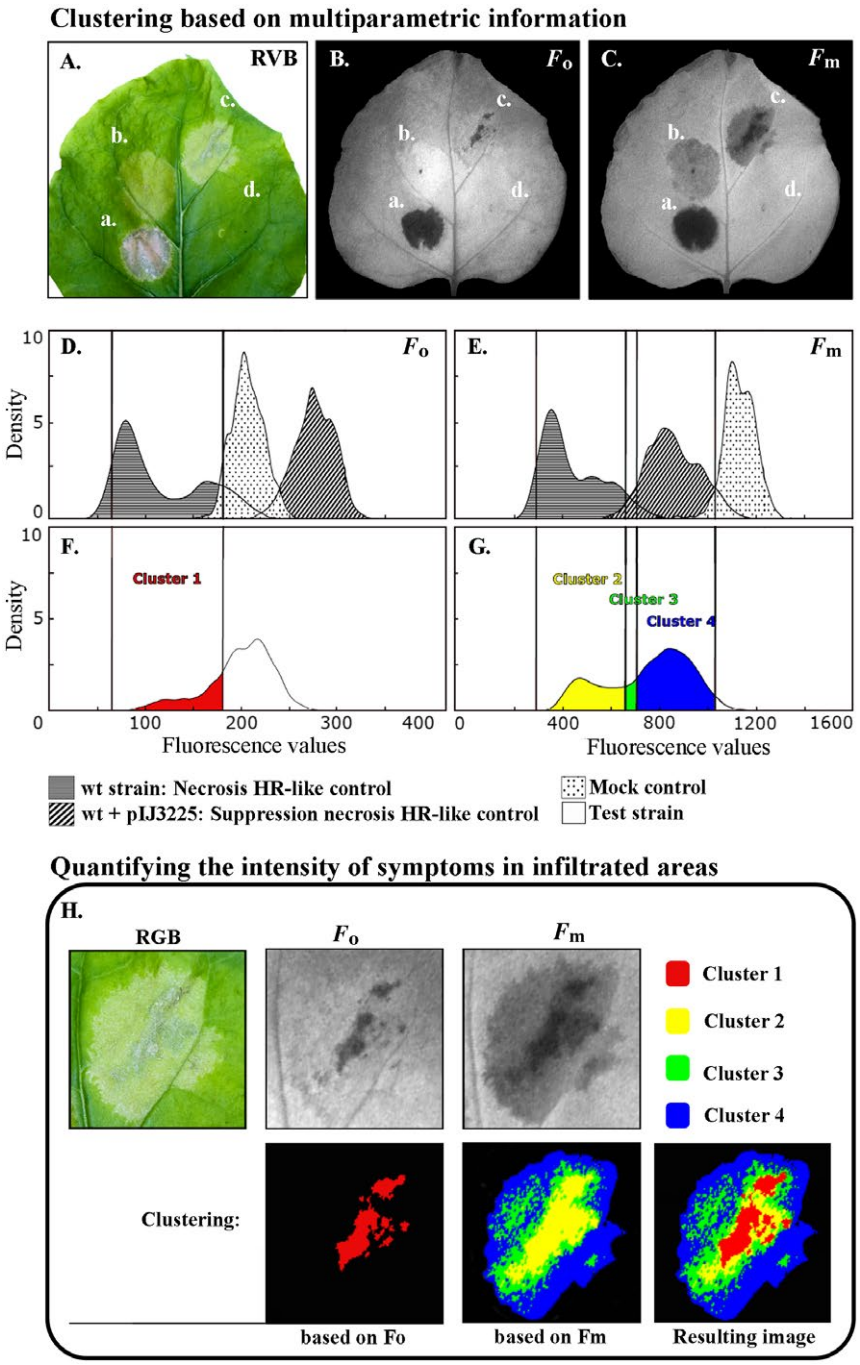

Fig. 5 (A–G) Clustering based on multiparametric information. Thresholds for the segmentation of chlorophyll fluorescence images were determined within each leaf based on the phenotypes obtained in areas infiltrated with control strains. (A) Each leaf features four infiltrated areas: (a) infiltrated with either strain 7698R or 7700R, and used as a positive control for the onset of hypersensitive response (HR)-like necrosis on each leaf; (b) infiltrated with strain 7698R pIJ3225 or 7700R pIJ3225, and used as a control for the ability of pIJ3225 to suppress HR-like necrosis on each leaf; (c) infiltrated with strain 7698R or 7700R carrying Tn5 derivatives of pIJ3225 (Table 2, Arlat *et al.*, 1991), and used to test whether Tn5 insertions alter the ability of pIJ3225 to suppress HR-like necrosis; (d) water-inoculated control. (B, C) Chlorophyll fluorescence imaging of the inoculated leaves: (B) minimum fluorescence (F_o); (C) maximum fluorescence (F_m). (D, E) Distribution of chlorophyll fluorescence values amongst pixels in the areas inoculated with control strains. Histograms associated with F_o and F_m images (D, E) show that distributions of fluorescence values differ amongst control areas. (F, G) Clustering the pixels of the area inoculated with the test strains. Thresholds for clustering are determined on the basis of the distributions of chlorophyll fluorescence values obtained with control strains. For each control area (D, E), the upper and lower thresholds were fixed to correspond to the 20th quantile, i.e. splitting 1/20 of the F_o and F_m pixel distribution values. They were determined for each leaf to take into consideration the variability between leaves. These thresholds were then applied to histograms associated with F_o and F_m images of inoculated area (c) to cluster pixels that display fluorescence values similar to each control area (F, G). (H) Four clusters were determined. Clusters 1 and 2 correspond to tissues found on the HR-like necrosis control (cluster 1, most impacted tissues; cluster 2, less impacted tissues). Cluster 4 corresponds to tissues found in the areas inoculated with pIJ3225 control strains (suppression control). Cluster 3 corresponds to fluorescence values intermediate between HR-like necrosis and the suppression controls. We conclude the presence of HR-like necrosis in the area inoculated with the test strain when the number of cumulated pixels belonging to clusters 1–3 is superior to the 20th quantile. [Colour figure can be viewed at wileyonlinelibrary.com]

The conjugation of the plasmid pIJ3225 encoding the T3SS and four T3SPs into strains CFBP 7698 and CFBP 7700 conferred the ability to suppress HR-like necrosis in *N. benthamiana*. Such suppression is dependent on the ability of the T3SS encoded in pIJ3225 to translocate effectors into the plant cells, as Tn5 insertions inactivating *hrcJ* (G9) or *hrpB8* (G2) abolish the ability of pIJ3225 to suppress HR. However, the acquisition of a functional T3SS reduced the multiplication of strains. Therefore, the acquisition of pIJ3225 encoding a T3SS and four T3SPs is not sufficient, and further acquisition of repertoires of T3Es is needed to make strains CFBP 7698 and CFBP 7700 pathogenic. Similar results have been observed recently with *Pseudomonas syringae* DC3000 derivatives: derivatives lacking T3SS (regardless of effector repertoire) grew better than the polymutant strains of DC3000 deleted of all its T3Es (Chakravarthy *et al.*, 2018). This suggests that the T3SS itself triggers pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) inside plant tissues. The effective suppression of plant defences involves the translocation inside the plant cell of various T3Es that impact cell death in plant tissues and/or population growth of $T3SS^+$ strains *in planta* (Wei *et al.*, 2018).

Nonetheless, the four T3SPs encoded on pIJ3225 interfere with the reaction of *N. benthamiana*. The Tn5 insertions in *hpa2* strongly affect the ability of pIJ3225 to suppress HR-like necrosis and significantly impact microbial growth *in planta*. The protein Hpa2 is a putative lytic transglycosylase which has been reported to possess lytic activity against the bacterial cell walls, presumably to facilitate the assembly of the *hrp*-encoded T3SS in *X. oryzae* (Zhang *et al.*, 2008). The protein Hpa2 has also been shown to be targeted to the plasma membrane of the host plant cell, and to interact with the HrpF translocon protein. Therefore, Hpa2 presumably contributes to the translocation of T3Es inside the plant cells in *X. oryzae* pv. *oryzicola* (Li *et al.*, 2011). Consistently, we observed a mild decrease in the intensity of HR on *C. annuum* ECW20R. This suggests that the Tn5 insertions in *hpa2* (D1, F2, H4, H7) may affect either the efficiency of assembly of the T3SS or the translocation of AvrBs2 into plant cells. However, the translocation of AvrBs2 into *C. annuum* cells was not abolished. Hence, these results suggest that Hpa2 is also injected inside plant cells in *N. benthamiana*, and could play a role in the suppression of HR-like necrosis.

In addition, the Tn5 insertions in *hpa1* and *xopF1* affected, to a lesser extent, the ability of pIJ3225 to suppress HR-like necrosis in *N. benthamiana*, but did not seem to impact significantly on bacterial growth *in planta*. In the case of Hpa1, the results obtained differ from those found in *X. euvesicatoria*, showing that mutation in *hpa1* (syn. *xop*A*)* resulted in reduced bacterial growth *in planta* (Noel *et al.*, 2002). This suggests that Hpa1 may act in combination with other T3Es, and its impact on bacterial growth may only be revealed in strains featuring these other T3Es.

In the case of *xopF1*, hardly any function can be attributed to this effector, which is surprising given that XopF1 belongs to an ancestral core effectome in *Xanthomonas* (Merda *et al.*, 2017; Roux *et al.*, 2015). Indeed, to our knowledge, XopF1 has only been shown to participate in a mild reduction in the induction of *FRK1* in the early response of *Arabidopsis thaliana* protoplasts to flg22 (Popov *et al.*, 2016). In this study, the phenotype associated with the Tn5 inactivation of *xopF1* was, however, subtle and difficult to assess using visual observation. The phenotyping approach using CFI, developed in this study, increased the sensitivity of phenotyping compared with visual inspection. Hence, such an approach may help to reveal weak phenotypes associated with T3Es with subtle impacts on plant tissues. The results obtained in this study argue in favour of a subtle contribution of XopF1 to suppression of HR-like necrosis. In this study, only two chlorophyll fluorescence parameters were used (F_o and F_m) for the phenotyping of HR-like necrosis on *N. benthamiana*.

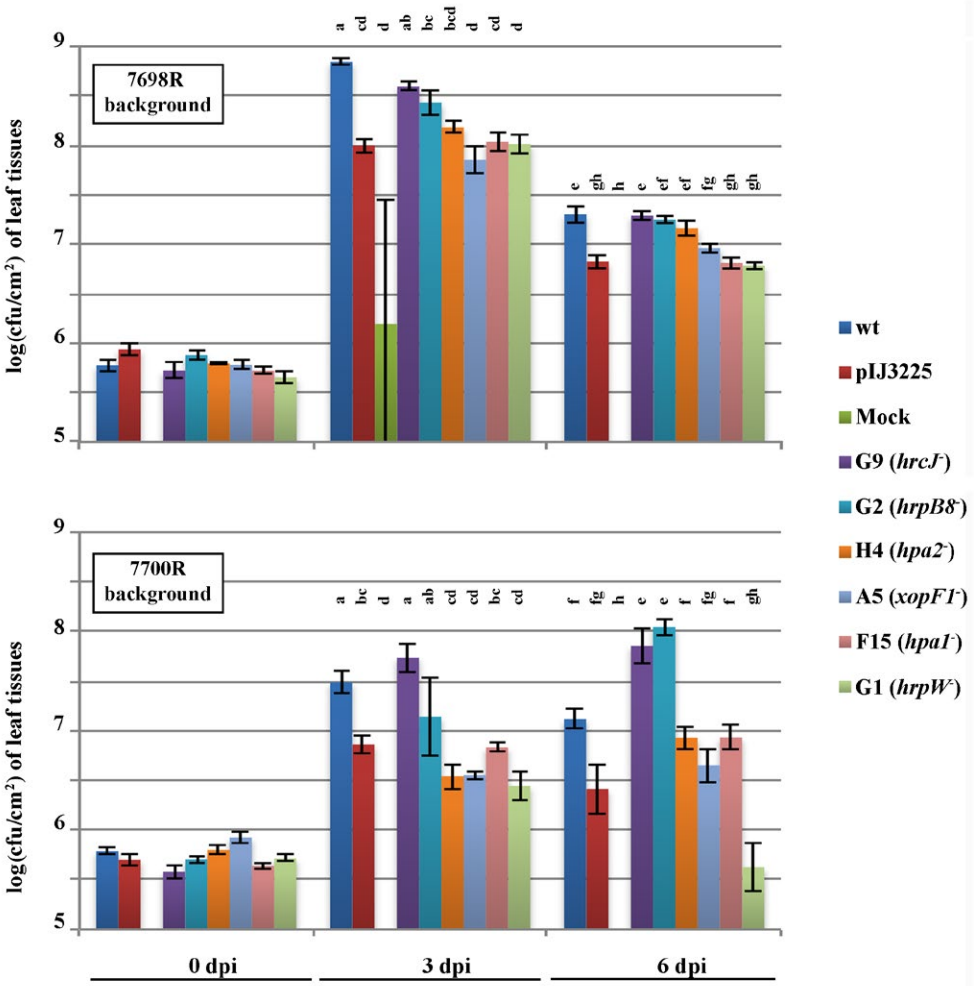

Fig. 6 The acquisition of a functional type 3 secretion system (T3SS) impairs the multiplication of non-pathogenic strains of *Xanthomonas* after inoculation on *Nicotiana benthamiana*. For each strain, inocula calibrated at 10^8 colony-forming units (cfu)/mL were infiltrated into the leaves of *N. benthamiana*. Bacterial population sizes were determined by dilution plating. The charts show the combined results representative of two independent experiments, each experiment involving six independent leaves per strain for each time point. The top panel shows the results obtained with strain 7698R and its derivatives, and the bottom panel shows the results obtained with strain 7700R and its derivatives. Means marked with the same letter were not significantly different using a Kruskall–Wallis non-parametric test. dpi, days post-inoculation; wt, wild-type. [Colour figure can be viewed at wileyonlinelibrary.com]

However, many other chlorophyll fluorescence parameters may be measured or calculated, which carry more information on the status of plant tissues (Berger *et al.*, 2007; Rousseau *et al.*, 2015). Comprehensive compilation of the chlorophyll fluorescence data should increase the sensitivity for phenotyping, and help us to understand the role of T3Es with subtle effects, such as XopF1.

None of the Tn5 insertions located in putative T3Es or T3SPs studied here totally abolished the ability to suppress HR-like necrosis conferred by pIJ3225. Two hypotheses can be proposed. The first is that such a suppression could be the result of the combined action of all four T3SPs. Indeed, in *P. syringae*, complex interplays between T3Es of the repertoire have been reported to impact the outcome of the interaction with *N. benthamiana* (Chakravarthy *et al.*, 2018; Cunnac *et al.*, 2011; Wei *et al.*, 2015, 2018). Alternatively, the possibility that pIJ3225 could allow the translocation of as yet unidentified T3Es encoded in the genomes of CFBP 7698 or CFBP 7700 cannot be ruled out. The mining in the genome of strains CFBP 7698 and CFBP 7700 identified the presence of two *avr* genes, *avrXccA1* and *avrXccA2*, which are related to *avrXca* (Parker *et al.*, 1993). As a result of their *avr* activity on *A. thaliana*, it has been speculated that *avrXccA1* and *avrXccA2* might be T3Es. However, the occurrence of an N-terminal signal peptide for type 2 secretion suggests that they are not T3Es (Bogdanove *et al.*, 2011; Studholme *et al.*, 2011).

The results obtained in this study provide functional evidence to support recent models proposed to explain the evolution of

virulence in the genus *Xanthomonas*. Comparative genomics approaches have suggested that the emergence of pathogenic strains from non-pathogenic ancestral strains would first have involved the acquisition of *hrpG* and *hrpX* regulators and sets of CWDEs (Jacobs *et al.*, 2015). These strains would have formed a recombinant network from which epidemic clones emerged and constituted successful pathovars, and such an emergence has been suggested to be associated with the acquisition of a T3SS and a set of core T3Es (Merda *et al.*, 2016). Three independent acquisitions of a T3SS by ancestral strains in the genus *Xanthomonas* have been suggested by population genomics studies (Merda *et al.*, 2017). Multiple events of horizontal transfer of T3Es have probably occurred in the genus *Xanthomonas* and have been suggested to have shaped, at least in part, the host specificity of strains (Hajri *et al.*, 2009; Merda *et al.*, 2016; Mhedbi-Hajri *et al.*, 2013). Eventually, T3SS and T3E loss events have also been reported, which is probably the case for most non-pathogenic strains isolated recently, including strain CFBP 7698 (Cesbron *et al.*, 2015; Essakhi *et al.*, 2015; Garita-Cambronero *et al.*, 2016, 2017; Merda *et al.*, 2016, 2017). Genomic comparison between strains CFBP 7912 and CFBP 7698 reveals that the absence of T3SS in strain CFBP 7698 is probably a result of a loss event, as the T3SS found in strain CFBP 7912 was probably acquired prior to the split between *X. cannabis* and other *Xanthomonas* species (Merda *et al.*, 2017). In the case of strain CFBP 7700, the lack of T3SS may be a result of either a loss, if the divergence between CFBP 7700 and *X. campestris* occurred after the acquisition of the *hrp* cluster. Conversely, if the divergence between CFBP 7700 and *X. campestris* occurred before the acquisition of the *hrp* cluster, such a strain may also have never possessed a T3SS. In the latter hypothesis, the case of strain CFBP 7700 would resemble that of strains of *X. maliensis* which have possibly derived from ancestral strains that never acquired a T3SS (Merda *et al.*, 2017).

In this study, we have shown that the acquisition of a T3SS and an ancestral repertoire of core secreted proteins allows commensal strains to suppress HR-like necrosis. This suppression is not associated with a better ability of strains to colonize the phyllosphere. On the contrary, the mere acquisition of the T3SS and the core set of effectors reduced the fitness of strains *in planta*. Such an event thus seems counter-selective, thereby explaining the loss events hypothesized by Merda *et al.* (2017). A genomic comparison revealed only four missing regions in strain CFBP 7698 compared with the non-pathogenic strain CFBP 7912, i.e. the T3SS gene cluster and three prophages found in strain CFBP 7912. Alternatively, comparisons with *X. cannabis* Nyagatare revealed more differential features, including T3Es. Strain *X. cannabis* Nyagatare is mildly pathogenic and induces atypical symptoms on bean. Overall, the data obtained here fit the model in which the evolution of pre-emerging strains into real pathogens specialized on specific crops not only involves the acquisition of a T3SS, but also necessitates the acquisition of a repertoire of T3Es (Jacobs *et al.*, 2015; Merda *et al.*, 2017). Indeed, in the absence of a core set of T3Es, genes encoding the T3SS will not be maintained inside the bacterial genome.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and growth conditions

Strains were cultured on classical trypticase soy agar (TSA) medium (tryptone, 17 g/L; peptone soya, 3 g/L; glucose, 2.5 g/L; NaCl, 5 g/L; KH_2PO_4, 5 g/L; agar, 15 g/L; pH 7.2) or 10% TSA, Moka (yeast extract, 4 g/L; casmino acids, 8 g/L; KH_2PO_4, 2 g/L; $MgSO_4$, 0.2 g/L; agar, 15 g/L) and Luria–Bertani (bacto-tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 10 g/L; pH 7.5), supplemented with the appropriate antibiotics. All *Xanthomonas* cultures were incubated at 28 °C. The cultures of *Escherichia coli* were incubated at 37 °C. All strains and plasmids used in this study are listed in Table 2.

Strains CFBP 7698 and CFBP 7700 were isolated from symptomless bean seeds (commercial seed lots) on TSA and deposited at the French Collection of Plant-associated Bacteria (CIRM-CFBP) for storage.

Primers *rpfB* and X4c/X4e, as well as the duplex AM1F/R and Am2F/R, were used to test whether these strains belong to the genus *Xanthomonas* or to groups of *Xanthomonas* strains pathogenic on bean, respectively (Audy *et al.*, 1994; Boureau *et al.*, 2013; Simoes *et al.*, 2007).

Rifamycin-resistant spontaneous mutants of CFBP 7698 and CFBP 7700 were selected on 10% TSA supplemented with 200 μg/mL rifamycin. The absence of growth defects of these mutants was controlled *in vitro* by spectrophotometry (Labsystems Bioscreen C, Helsinki, Finland), and the absence of revertants was checked when culturing on culture medium devoid of antibiotics, as described in Darsonval *et al.* (2009).

pIJ3225 and its derivatives carrying a Tn5 insertion were transferred from *E. coli* to strains CFBP 7698 and CFBP 7700 by triparental mating using the helper strain *E. coli* K12 pRK600. Parental strains were cultured in liquid medium supplemented with the appropriate antibiotics until an optical density at 600 nm (OD_{600}) of 0.4 was reached. Bacterial cells were pelleted by centrifugation at 3950 g, resuspended in Moka. Liquid cultures of each parental strain, as well as the helper strain, were mixed and cultured on Moka plates for 3 days at 27 °C. The transconjugants were selected on Moka plates supplemented with rifamycin for the selection of the *Xanthomonas* background, kanamycin for the selection of pIJ3225 and tetracycline for the selection of the Tn5 insertions. The transconjugants were stored at −80 °C in 40% glycerol (final concentration) after four successive rounds of purification. After purification, the transconjugants were: (i) confirmed to belong to the genus *Xanthomonas*; (ii) checked for the presence of pIJ3225 by PCR using primers designed on *hrpF* (Table S1, see Supporting Information); (iii) when

relevant, checked for the presence of the Tn5 insertion using the primer WDTn5 combined with a reverse primer designed towards pIJ3225. PCR products were sequenced to identify the location of each Tn5 insertion used in this study. Transconjugants carrying Tn5 insertions were controlled for the absence of growth defect *in vitro* by spectrophometry (Labsystems Bioscreen C) compared with strains featuring the native form of pIJ3225. The replication of extra fragments of DNA has been known to increase the cost of genome replication during prokaryotic multiplication (Lane and Martin, 2010). Therefore, the growth *in vitro* of the transconjugants was not compared with that of the plasmidless strains 7698R and 7700R, as we considered that the conjugation of the 46-kb pIJ3225 probably significantly impacted the fitness of the transconjugants compared with their parental strains. After their full characterization, all transconjugants were deposited at CIRM-CFBP.

Genome sequencing and analysis of the genome content of the *Xanthomonas* strains CFBP 7698 and CFBP 7700

Genomic DNAs from the strains CFBP 7698 and CFBP 7700 were extracted, purified and sequenced using Illumina HiSeq 2000 Genoscreen, Lille, France, assembled and annotated as described in Merda *et al.* (2017). This Whole Genome Shotgun projects have been deposited at DDBJ/ENA/GenBank under the accessions PRDN00000000 and PRDO00000000 for strains CFBP 7698 and CFBP 7700 respectively. The versions described in this paper are versions PRDN01000000 and PRDO01000000 for strains CFBP 7698 and CFBP 7700 respectively.

Genome sequences were used to construct a phylogeny of 82 strains representative of the genus *Xanthomonas*, as described by Merda *et al.* (2017). The Family-Companion tool (Cottret *et al.*, 2018) was used to determine the core proteome shared by all strains in the study. The phylogeny was then constructed on the 968 protein sequences of the core proteome, using CV Tree version 4.2.1. (Xu and Hao, 2009), with a K-mer size of 6.

The genomes of strains CFBP 7698 and CFBP 7700 were screened for sequences displaying high similarity (80% in length and 80% in identity) with genes encoding any type of T3SS previously described. In addition, we searched for any sequence with high similarity with genes encoding known T3Es in various pathogenic bacteria (*Xanthomonas*, *Pseudomonas*, *Ralstonia*, *Erwinia*, *Escherichia*, *Salmonella*), as described in Cesbron *et al.*, (2015). A rapid genomic comparison was performed between phylogenetically close relatives using TblastN and BlastP: the predicted protein sequences were blasted, respectively, on genomes and predicted protein sequences of strains *X. cannabis* CFBP 7912, *X. cannabis* pv. *phaseoli* Nyagatare and *X. campestris* pv. *campestris* B100 (Aritua *et al.*, 2015; Merda *et al.*, 2017; Vorhölter *et al.*, 2008). Conversely, protein sequences predicted from genomes of strains CFBP 7912, Nyagatare, B100, *X. citri* pv. *fuscans* Xff4834R, *X. citri* pv. *citri* Xac 306, *X. albilineans* XaPC73 and *X. campestris* pv. *campestris ATCC* 33913 were blasted on genomes and predicted sequences of strains CFBP 7698 and CFBP 7700 using TblastN and BlastP, respectively.

Plant material

Bean plants cv. Flavert were grown in a growth chamber at 23 °C/20 °C (day/night) with a photoperiod of 16 h. They were watered three times a week and supplemented with N : P : K (18 : 14 : 18) at 0.3 g/L once a week. Two-week-old plants harbouring a fully developed first trifoliate leaf were used for inoculations.

Capsicum annuum (pepper), cultivars ECW and ECW20R (carrying the *Bs2 R* gene), and *Nicotiana benthamiana* plants were grown and inoculated in an environmentally controlled growth room under a 16-h photoperiod and an 8-h dark period at 22 °C and 80% relative humidity. *Nicotiana benthamiana* and *C. annuum* plants were maintained under a light intensity of 100 and 280 $\mu E/m^2/s$, respectively, throughout the whole experiment. Seedlings were transplanted 15 days after sowing. Plants were irrigated once a week with nutrient solution (balanced N : P : K of 15 : 10 : 30 with a dosage rate of 1.5 g/L). Auxiliary branches were removed to better manage the plants. Six-week-old plants with at least five fully developed true leaves were used for infiltration.

Preparation of inocula, inoculation and pathogenicity assays on *N. benthamiana*, pepper and bean

For the inoculation of *N. benthamiana* and *C. annuum (*pepper), bacterial suspensions were calibrated at 10^8 and 10^9 colony-forming units (cfu)/mL, respectively. Three fully expanded leaves per plant were inoculated by pressing the blunt end of a 1-mL needleless syringe to the lower side of the leaf whilst supporting the leaf with a gloved finger.

For the pathogenicity assays on *P. vulgaris* (bean), bacterial suspensions were calibrated at 10^7 cfu/mL for subsequent inoculation on plants. The first trifoliate leaves were inoculated by dipping and the plants were incubated at 95% relative humidity at 28 °C for 14 days until symptoms appeared, as described in Darsonval *et al.* (2008).

Phenotyping on *N. benthamiana*

The impact on *N. benthamiana* leaf tissues of strains CFBP 7698, CFBP 7700 and the transconjugants was phenotyped by both visual observations and CFI. Visual observations and CFI were carried out in three independent experiments for each strain tested. Each experiment involved six leaves per strain. Observations were performed each day after inoculation for 6 days.

Prior to CFI, *N. benthamiana* plants were placed in the dark for 20 min. Inoculated leaves were then detached and imaged using a PSI Open FluorCam FC 800-O (PSI, Brno, Czech Republic) to capture F_o and F_m, as described by Rousseau *et al.* (2013). Images were saved as an archive using the software FluorCam 7 (PSI, Drasov, Czech Republic) as recommended by the manufacturer. Images were then exported as TIFF files for subsequent analysis. The image analysis was performed using R software and the EBImage package (R Development Core Team, 2008; Pau *et al.*, 2010).

Assessment of bacterial population sizes on *N. benthamiana*

The bacterial population sizes were measured at 0, 3 and 6 dpi for each strain tested. Each bacterial inoculum was infiltrated on 18 independent leaves. At each sampling date, six 0-78-cm^2 leaf discs were harvested with a cork borer inside the inoculated area. Each leaf disc was homogenized in 3 mL of distilled water using a Mixwel Lab-Blender (Alliance BioExpertise, GUIPRY-MESSAC, FRANCE). Samples were then diluted and plated on 10% TSA plates supplemented with rifamycin, and on 10% TSA plates supplemented with the appropriate antibiotics (rifamycin–kanamycin or rifamycin–kanamycin–tetracycline). For each sample, dilution plating was repeated twice. Plates were incubated at 28 °C and colonies were counted after 3 days. Results are expressed as log(cfu/cm^2). Comparisons were performed using the non-parametric Kruskall–Wallis test with a Bonferroni correction.

Assessment of the expression of genes encoding the T3SS encoded by pIJ3225 in CFBP 7698 and CFBP 7700 backgrounds

Strains were grown *in vitro* under *hrp*-inducing conditions (XVM2 medium). Total RNAs were extracted as described by Darsonval *et al.* (2009). Total RNA (5 μg) was reverse transcribed into cDNA as described by Darsonval *et al.* (2009). The RNA–cDNA mix was ethanol precipitated, dissolved in double-distilled H_2O and further used for PCRs. The presence of cDNAs corresponding to genes *hpa1*, *hpa2*, *xopF1*, *hrcC*, *hrcN* and *hrcV* was assessed by PCR using the appropriate primers (Table S1). Strain *Xcc* 8004 was used as a positive control, and strains CFBP 7698 and CFBP 7700 were used as negative controls.

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SUPPORTING INFORMATION

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Fig. S1 AN1b results for the comparison of strains CFBP 7698 and CFBP 7700 with their neighbor strains in the phylogeny. The ANIb values between CFBP 7698 and *X. cannabis* strains exceeds 95% (100% when comparing CFBP 7698 vs CFBP 7912, and 98% when comparing CFBP 7698 and Nyagatare). When the average nucleotide identity exceeds 95% strains usually belong to a single species. Thus we concluded that strain CFBP 7698 belongs to the species *X. cannabis*. Strain CFBP 7700 clusters with strains belonging to the species *X. campestris*. The ANIb values between CFBP 7700 and *Xanthomonas campestris pv. campestris* strains *Xcc* 8004 or *Xcc* B100 reaches 96%. Thus, strain CFBP 7700 belongs to the species *X. campestris*.

Table S1 PCR primers used in this study.