Towards the Identification of Type III Effectors Associated with *Ralstonia solanacearum* Virulence on Tomato and Eggplant

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ABSTRACT

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For the development of pathogen-informed breeding strategies, identifying the microbial genes involved in interactions with the plant is a critical step. To identify type III effector (T3E) repertoires associated with virulence of the bacterial wilt pathogen *Ralstonia solanacearum* on Solanaceous crops, we used an original association genetics approach combining DNA microarray data and pathogenicity data on resistant eggplant, pepper, and tomato accessions. From this first screen, 25 T3Es were further full-length polymerase chain reaction-amplified within a

35-strain field collection, to assess their distribution and allelic diversity. Six T3E repertoire groups were identified, within which 11 representative strains were chosen to challenge the bacterial wilt-resistant eggplants 'Dingras multiple Purple' and 'AG91-25', and tomato Hawaii7996. The virulence or avirulence phenotypes could not be explained by specific T3E repertoires, but rather by individual T3E genes. We identified seven highly avirulence-associated genes, among which *ripP2*, primarily referenced as conferring avirulence to *Arabidopsis thaliana*. Interestingly, no T3E was associated with avirulence to both eggplants. Highly virulence-associated genes were also identified: *ripA5_2*, *ripU*, and *ripV2*. This study should be regarded as a first step toward investigating both avirulence and virulence function of the highlighted genes, but also their evolutionary dynamics in natural *R. solanacearum* populations.

Effective and durable control of plant pathogens is a critical challenge for global food security. Once a pathogen has established in an area, the use of genetically resistant plants is one of the most effective control approaches, particularly against bacterial pathogens for which chemical control and agricultural practices remain ineffective or impractical solutions (Strange and Scott 2005). Now the central challenge of this approach is the identification of broad-spectrum and durable resistance genes/quantitative trait loci (QTL). Resistance durability was first measured retrospectively (see for example van den Bosch and Gilligan 2003), but recent studies demonstrated that this trait may be inferred from the resistance level and spectrum of the plant R genes/QTL and their genetic background (Palloix et al. 2009; Quenouille et al. 2014). Evolutionary potential of the pathogen (McDonald and Linde 2002), and specifically evolutionary forces at work on the pathogen genes involved in virulence are also important traits for resistance durability. The seminal paper of Leach et al. (2001) proposed that durability of plant R genes could be inferred from the pathogen fitness penalty induced by adaptation to this gene, including the loss of the cognate avirulence gene (Leach et al. 2001).

In the model currently describing the evolution of plant-pathogen microbes interactions (Jones and Dangl 2006), pathogens secrete effectors that suppress or subvert the resistance responses triggered by microbe-associated molecular patterns (MAMPs). This dichotomy between MAMP-triggered immunity (MTI) and effector-

triggered immunity (ETI) has been recently questioned by Cook et al. (2015), who proposed to consider the plant innate immunity as "a system that evolves to detect invasion." Plant-pathogenic bacteria possess a large repertoire of secreted effectors, where the type III secretion system (T3SS) plays a central role in virulence (Kenny and Valdivia 2009). Numerous functional genetic assays have tried to decipher the respective role of many individual effectors (for a review, see for example Chang et al. 2014; Feng and Zhou 2012), but the association of individual effectors to an avirulence or virulence phenotype remains problematic due to functional redundancy (Cunnac et al. 2011). Genome-wide association mapping may be a promising alternative approach, allowing to associate not only individual genes but gene repertoires to a phenotype (Falush and Bowden 2006). We applied such an approach on the soilborne Beta-Proteobacterium *Ralstonia solanacearum*, a vascular plant pathogen whose host range is particularly huge (more than 54 botanical families). It induces bacterial wilt on the family *Solanaceae* but also more than 50 other families (among which include *Zingiberaceae*, *Fabaceae*, *Myrtaceae*, ...), causing heavy losses throughout the tropical and subtropical belt in Asia, Africa, and America. Breeding efforts for resistance to this disease have been hindered for years by the lack of complete resistance genitors, the strong interactions between resistance and local strains, as well as by the huge genomic and phenotypic plasticity of the pathogen. Among proteobacterial plant pathogens, *R. solanacearum* possesses a very large repertoire of genes contributing to virulence, including genes involved in aero- and chimio-tactism (Yao and Allen 2006, 2007), reactive oxygen species (ROS) detoxification (Flores-Cruz and Allen 2011), multidrug efflux pumps (Brown et al. 2007), and Tat secretome (Gonzalez et al. 2007), but the secreted proteins (effectors) distributed by the T3SS constitute the key virulence factors (Poueymiro

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and Genin 2009). Whereas most proteobacterial plant pathogens possess 20 to 30 type III effectors (T3E), *R. solanacearum* meta-repertoire gathers 94 families (orthologous groups) (Peeters et al. 2013), and individual strains usually carry 60 to 75 effectors (Deslandes and Genin 2014). Genes governing specific plant–*R. solanacearum* interactions have been identified in the model plants *Arabidopsis thaliana* and *Medicago truncatula* (for a review, see Deslandes and Genin [2014]). The most documented example is the acetyltransferase popP2 (Deslandes et al. 2003), recently renamed ripP2 (Peeters et al. 2013), whose interaction with the *A. thaliana* gene *RRS1-R* (Deslandes et al. 1998, 2002) and the cysteine protease RD19 (Bernoux et al. 2008) triggers plant immunity, making it the first avirulence gene described in *R. solanacearum*. It was recently demonstrated that RRS1-R forms a receptor complex with RPS4 (conferring resistance to *Pseudomonas syringae*) that detect the WRKY-targeting effectors, and convert the lysine-acetylase activity of RipP2 to immunity (Le Roux et al. 2015; Sarris et al. 2015). Other effectors have been identified, such as the cysteine protease ripP1 that elicits a hypersensitive response (HR) on some *Petunia* (Poueymiro et al. 2009; Poueymiro and Genin 2009) and tobacco species, and RipG7 which is required for pathogenicity on *Medicago truncatula* (Angot et al. 2006). Whereas, bacterial genetic factors that are critical for virulence and avirulence to cultivated species remain largely unknown. The zinc-dependent protease rip36 (Nahar et al. 2014), renamed ripAX2 (Peeters et al. 2013), induces a HR on *Solanum torvum*, a wild relative of eggplant. RipA2 contributes to pathogenicity to tomato, whereas ripA5 elicits a HR-like on some tobacco species (Sole et al. 2012). The ripG2, ripG3, ripG6, and ripG7 effector proteins collectively contribute to pathogenicity to tomato and *Arabidopsis* (Remigi et al. 2011).

By challenging reference resistance genitors of tomato, eggplant, and pepper (Core-TEP) to a worldwide collection of *R. solanacearum* strains (core-RS2), Lebeau et al. (2011) identified several cases of “incompatible interactions,” phenotyped as “no wilt and no colonization,” that may be indicative of gene-for-gene interactions. One of the accessions involved in such incompatible interactions was eggplant AG91-25, which carries the *ERs1* resistance gene (Lebeau et al. 2013). The core-RS2 strains were hybridized on a DNA microarray (Guidot et al. 2009; Remenant et al. 2010) in order to get access to their gene content. The combined analysis of such genotypic and phenotypic data was carried out in order to identify phenotype-associated genes.

In this study, we thus aimed to identify, by a top-down approach sensu Falush and Bowden (2006), the *R. solanacearum* T3E gene repertoires associated with avirulence or virulence phenotypes on resistant eggplant, pepper, and tomato cultivars. The specific questions we addressed were as follows. (i) What is the T3E distribution variability in a large strain collection? (ii) Is the virulence on *Solanaceae* explained by T3E repertoires, individual T3E presence/absence, or individual T3E allelic differences? (iii) Are the avirulence/virulence effectors involved in interactions with several cultivars, or is there cultivar specificity (Lewis et al. 2014)?

MATERIALS AND METHODS

Bacterial strains. Two *R. solanacearum* strain collections were used for this study. A first set of 12 international strains called core-RS2 was selected from a global reference collection, based on their aggressiveness on tomato, eggplant, and pepper susceptible reference accessions (Lebeau et al. 2011); this was used from the first step of this study. Two additional strains belonging to the highly harmful “emerging ecotype” (Wicker et al. 2007) were added to this set but were studied only from the second step of our study. The second set gathered 35 environmental strains collected on different diseased hosts throughout Reunion Island (Table 1). Bacterial strains were all stored in Cryobank beads at -80°C . They were grown first on nutrient broth overnight at 28°C , and then streaked (50 μl) on Kelman’s triphenyl tetrazolium chloride (TZC) agar medium (Kelman 1954) supplemented with 1% yeast extract and

subcultured 2 days at 28°C . Bacterial DNA was extracted from fresh cultures (~ 1.0 to 2.0×10^9 cells), using the DNeasy Blood and Tissue kit (QIAGEN) following the manufacturer’s instructions for gram-negative bacteria. DNA solutions were then quantified with a NanoDrop ND-8000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), adjusted to $10 \text{ ng } \mu\text{l}^{-1}$, and stored at -20°C until use.

Plant accessions. Tomato, eggplant, and pepper accessions were chosen within the core-TEP according to their bacterial wilt-resistance level and spectrum (Lebeau et al. 2011) (Table 2). For tomato, L390 (coded T10 throughout the article) is highly susceptible (Lebeau et al. 2011; Truong et al. 2008; Wang et al. 1998). The accession Hawaii7996 (encoded T5) displays the highest resistance level, and controls the broadest spectrum of strains (Lebeau et al. 2011); it is also the best known bacterial wilt-resistant tomato accession, with several mapped QTLs (Carmeille et al. 2006; Wang et al. 2013; Wang et al. 2000). For eggplant, the susceptible accession chosen was MM738 (encoded E8 by Lebeau et al. [2011] and in the following), given its high susceptibility (Lebeau et al. 2011) and its status of susceptible parent of the mapping population MM738 $\times$ AG91-25 (Lebeau et al. 2013). The resistant accessions chosen display complete incompatible interactions (i.e., no wilt and no stem colonization) with at least one *R. solanacearum* strain of the core-RS2: (i) Dingras multiple Purple, referenced as MM853 in INRA germplasm collection (E1 in this study), displays both a high resistance level and a broad resistance spectrum; (ii) SM6, referenced as MM643 (E2 in this study), is completely resistant to phylotype IIB “emerging strains” (Deberdt et al. 2014; Lebeau et al. 2011) but also IIA and III strains (detailed in Table 3); (iii) Ceylan, referenced as MM152 (E3 in this study), is completely resistant to phylotype I, IIA, and III strains (Table 3); (iv) Surya, referenced as MM1811 (E4 in this study), is resistant to IIA and III strains; and (v) AG91-25, referenced as MM960 (E6 in this study), is totally resistant to strain CFBP6942 (encoded RUN0145) and is poorly colonized by CFBP7032 (RUN0150) and PSS366 (RUN0155), whereas susceptible to virulent strains (N’Guessan et al. 2012). AG91-25 also carries the dominant gene *Ers1*, the first bacterial wilt-resistance gene identified in a crop (Lebeau et al. 2013). For pepper, resistant accessions were (i) P687 (P2 in this study), resistant to phylotype I strains; (ii) CA8, referenced as PM1580 (P6 in this study), resistant to phylotype IIA strains; and (iii) Perennial, referenced as PM659 (P8 in this study), resistant to phylotype III strains (Table 3).

Comparative genomic hybridization (CGH) data. The DNA microarray used in these experiments, generated by C. Boucher and collaborators (INRA-CNRS, Toulouse, France), consisted of 6,516 65-mer and 70-mer oligonucleotides representative of the genes identified within the *R. solanacearum* genomes GMI1000 (phylotype I), IPO1609 (phylotype IIB/clade 5/sequevar 1), and MOK2 (phylotype IIB/clade 5/sequevar 3), as previously described (Remenant et al. 2010; Wicker et al. 2012). Each gene was represented by a single oligonucleotide, except for 117 T3E or effector-like genes, which were represented by two to six oligonucleotides to distinguish allelic forms of a given gene. Each oligonucleotide was spotted twice on a microarray. DNA extraction and labeling, microarray hybridization, hybridization analyses, and gene detection threshold are detailed in Guidot et al. (2007) and Remenant et al. (2010). Effector data sets were complete for 10 core-RS2 strains; however in the case of PSS4 (RUN0157) and PSS366 (RUN0155), only 65 effectors out of 117 were correctly identified. In downstream analyses, these two strains were thus analyzed apart from the others.

Identification of genes associated with effector probes. The correspondence between original probe names and Rip T3E new nomenclature (Peeters et al. 2013) was established by using the RalstoT3E website hosted by the Laboratory of Plant Microbe Interactions (LIPM), Toulouse (<https://iant.toulouse.inra.fr/bacteria/annotation/site/prj/T3Ev2/>).

Genotype/phenotype association workflow. The whole process, articulated in three main steps, is summarized in Figure 1.

Step 1: CGH screening of gene repertoires for association with virulence. In the literature, genes that were experimentally demonstrated as involved in *R. solanacearum* virulence are genes encoding type III effectors (T3E) and the T3SS (Poueymiro and Genin 2009), genes involved in bacterial motility (Tans-Kersten et al. 2001, 2004), aero- and chemotaxis (Yao and Allen 2006, 2007), transcription regulation, toxin resistance (Brown et al. 2007; Gonzalez et al. 2007), and genes encoding extracellular enzymes secreted through the type II secretion system (Denny 2006). In this work, we focused on the distribution of type III effectors or T3E-like coding sequences within the 12 strains of the first collection, and on their association with virulence phenotypes. Distribution patterns of the other genes were investigated in a preliminary study but no correlation with virulence phenotypes was found.

We considered 117 genes present on the 7K microarray, including 79 T3E sensu Poueymiro and Genin (2009) and 10 putative T3Es,

six genes (*hpaB*, *hrpZ*, *hrpY*, *hrpX*, *hrpW*, and *hrpV*) coding for the T3SS, and 22 coding sequences that shared homologous domains with T3E from different bacterial genera, or that contained homeobox domain. Each gene or coding sequence was represented by two to five probes.

Association of type III effectors with virulence or avirulence on Solanaceae. To identify genes associated with virulence or avirulence, we considered published phenotyping data obtained on the core collections of bacterial wilt-resistant tomato, eggplant, and pepper (core-TEP) (Lebeau 2010; Lebeau et al. 2011). We particularly focused on plant accessions displaying incompatible interactions (defined on this pathosystem as “zero wilting and zero colonization”) with at least one *R. solanacearum* strain, i.e., (i) eggplant lines Dingras Multiple Purple (MM853), SM6 (MM643), Ceylan (MM152), Surya (MM1811), and AG91-25 (MM960), respectively encoded here as E1, E2, E3, E4, and E6; (ii) pepper lines PM687, CA8 (PM1580), and Perennial (PM659), respectively encoded P2, P6, and P8; and (iii) tomato Hawaii7996 (encoded T5) (Lebeau et al. 2011) (Table 3). Thus, we distinguished an avirulent

TABLE 1. Characteristics of *Ralstonia solanacearum* strains belonging to the world collection core-RS2 and to the Reunion Island collection

Strain	RUN number	Host	Origin	Phylotype	Sequevar
Core-RS2 (Lebeau et al. 2011)					
GMI1000	54	<i>Solanum lycopersicum</i>	French Guiana	I	18
PSS366	155	<i>Solanum lycopersicum</i>	Taiwan	I	15
PSS004	157	<i>Solanum lycopersicum</i>	Taiwan	I	15
PSS358	159	<i>Solanum lycopersicum</i>	Taiwan	I	15
CFBP7058	215	<i>Solanum scabrum</i>	Cameroon	I	13
CFBP2957	36	<i>Solanum lycopersicum</i>	Martinique	IIA	36
CFBP7032	150	<i>Solanum lycopersicum</i>	Cameroon	IIA	41
CFBP6783	17	<i>Heliconia rostrata</i>	Martinique	IIB	4NPB
CFBP7029	147	<i>Solanum lycopersicum</i>	Cameroon	IIB	1
CFBP3059	39	<i>Solanum melongena</i>	Burkina Faso	III	23
CFBP6941	133	<i>Solanum lycopersicum</i>	Cameroon	III	29
CFBP6942	145	<i>Solanum scabrum</i>	Cameroon	III	29
Emerging ecotype strains					
CFBP6784	16	<i>Anthurium andreanum</i>	Martinique	IIB	4NPB
ANT80	18	<i>Anthurium andreanum</i>	Martinique	IIB	4NPB
Collection from Reunion Island					
JT519	471	<i>Pelargonium</i>	—	I	31
JT523	608	<i>Solanum tuberosum</i>	—	I	31
JQ1044	930	<i>Pelargonium</i>	Trois Bassins	I	NA
JQ1143	58	<i>Solanum tuberosum</i>	Bois Court	IIA	39
JT510	59	<i>Solanum tuberosum</i>	Notre Dame de la Paix	IIB	1
JT516	160	<i>Solanum tuberosum</i>	—	IIB	1
JS529	476	<i>Solanum tuberosum</i>	—	IIB	1
CFBP4801	623	<i>Solanum lycopersicum</i>	—	IIB	1
LNPV28.23	654	<i>Solanum tuberosum</i>	—	IIB	1
JT511	681	<i>Solanum tuberosum</i>	—	IIB	1
JT514	697	<i>Solanum tuberosum</i>	—	IIB	1
JQ1006	843	<i>Solanum tuberosum</i>	Notre Dame de la Paix	IIB	1
JQ1073	845	<i>Solanum lycopersicum</i>	Saint Pierre	IIB	1
JQ1023	848	<i>Solanum lycopersicum</i>	Mont Vert	IIB	1
JQ1078	880	<i>Solanum lycopersicum</i>	Saint Pierre	IIB	1
JQ1107	889	<i>Solanum tuberosum</i>	Bois Court	IIB	1
JQ1006	919	<i>Solanum tuberosum</i>	Notre Dame de la Paix	IIB	1
JQ1007	921	<i>Solanum tuberosum</i>	Grand Tampon	IIB	1
JQ1009	923	<i>Solanum tuberosum</i>	Piton Maho	IIB	1
JQ1017	924	<i>Solanum tuberosum</i>	Plaine des Cafres	IIB	1
JQ1018	925	<i>Solanum tuberosum</i>	Piton Hyacinthe	IIB	1
JQ1019	926	<i>Solanum tuberosum</i>	Notre Dame de la Paix	IIB	1
JQ1023	928	<i>Solanum lycopersicum</i>	Mont Vert	IIB	1
JQ1051	931	<i>Solanum lycopersicum</i>	Grand Anse	IIB	1
JQ1078	934	<i>Solanum lycopersicum</i>	Saint Pierre	IIB	1
JQ1101	936	<i>Solanum lycopersicum</i>	Grand Tampon	IIB	1
JQ1131	941	<i>Solanum tuberosum</i>	Piton Hyacinthe	IIB	1
CFBP2148	944	<i>Solanum tuberosum</i>	—	IIB	1
JT525	60	<i>Pelargonium</i>	—	III	19
JT528	61	<i>Solanum tuberosum</i>	—	III	19
NCPBP1029	77	<i>Pelargonium</i>	—	III	19
CFBP4963	523	<i>Solanum tuberosum</i>	—	III	19
CFBP2146	657	<i>Pelargonium</i>	—	III	19
CFBP4964	693	<i>Pelargonium</i>	Trois Bassins	III	19
JQ1092	913	<i>Solanum tuberosum</i>	Tan Rouge	III	NA

strains pool and a virulent strains pool for each accession considered, and compared their gene content as estimated from CGH data. All probes present in avirulent pool and absent in virulent pool were assigned to the putative avirulence gene probes, whereas all probes absent in avirulent pool and present in virulent pool were assigned as putative virulence gene probes. We then assessed the association of each T3E gene with avirulence/virulence by considering the frequency of its respective probes within avirulent strains and virulent strains pools.

Step 2: PCR genotyping on selected type III effector genes. The CGH data screening allowed to retain a set of 25 genes,

TABLE 2. Genetic resources in tomato, eggplant, and pepper, selected in the core-TEP collection (Lebeau et al. 2011)

Code	Accession	Species	Inoculated in step 3
Tomato			
T5	Hawaii7996	<i>Solanum lycopersicum</i>	Yes
T10	L390	<i>S. lycopersicum</i> var. <i>cerasiforme</i>	Yes
Eggplant			
E1	Dingras multiple Purple, MM853 ^a	<i>S. melongena</i>	Yes
E2	SM6, MM643	<i>S. melongena</i>	No
E3	Ceylan, MM152	<i>S. melongena</i>	No
E4	Surya, MM1811, EG203	<i>S. melongena</i>	No
E6	AG91-25, MM960	<i>S. melongena</i>	Yes
E8	MM738	<i>S. melongena</i>	Yes
Pepper			
P2	PM687	<i>Capsicum annuum</i>	No
P6	PBC631A, CA8, PM1580	<i>C. annuum</i>	No
P8	Perennial, PM659	<i>C. annuum</i>	No

^a MM and PM numbers correspond to accession codes in the INRA Germplasm Collection.

TABLE 3. Summary of the avirulent/virulent *Ralstonia solanacearum* strain pools, as defined by their virulence phenotypes on eggplant (E code), tomato (T code), and pepper (P code) accessions^a

Accession (code)	Avirulent (phenotype 0)	Virulent (phenotypes 4 to 5)
Eggplant		
Dingras (E1)	CMR32 ■ CFBP2957 ○ GMI1000 □	CFBP6783 ●
SM6 (E2)	CMR32 ■ CFBP2957 ○ CFBP6783 ●	PSS4 □ PSS366 □
Ceylan (E3)	CMR32 ■ CMR15 ■ CFBP2957 ○ CMR39 ○ PSS358 □	CFBP6783 ●
Surya (E4)	CMR32 ■ CFBP2957 ○	CMR15 ■
AG91-25 (E6)	CMR32 ■ CMR39 ○	CFBP3059 ■ CMR15 ■ CFBP6783 ● CMR34 ●
Tomato		
Hawaii7996 (T5)	CMR32 ■ CMR39 ○	CMR15 ■ CMR34 ● CFBP6783 ●
Pepper		
PM687 (P2)	PSS358 □	CFBP3059 ■ CFBP6783 ● CMR39 ○
CA8 (P6)	CFBP2957 ○ CMR39 ○	CFBP6783 ●
Perennial (P8)	CMR15 ■	CFBP6783 ●

^a Avirulence was defined as complete absence of wilt and colonization on the plant accession. Virulent strains were able to colonize and wilt accessions, inducing a phenotype 4 or 5 sensu Lebeau et al. (2011). *R. solanacearum* strains belong to phylotype I (□), IIA (○), IIB (●), or III (■).

associated with virulence or avirulence. The distribution of these 25 genes was PCR-checked within the two strain collections described above. These genes consisted of two harpin genes, *popW* (Li et al. 2010) and *hrpZ* (Lin et al. 2010), and 23 putative or validated type III effectors (Peeters et al. 2013).

PCR primer design. Since effector gene sequences are available for only a few strains belonging to our collections, we aligned orthologous effector sequences of GMI1000 (phylotype I), CMR15 (phylotype III), PSI07 (phylotype IV), IPO1609, Molk2 (both in phylotype IIB), and CFBP2957 (phylotype IIA). Orthologous gene families were defined from the T3E sequences found within the complete genome sequences harbored in the Mage Web interface (<https://www.genoscope.cns.fr/agc/microscope/home/index.php>) of the MicroScope platform. On this platform, search for orthologous genes was performed by applying the following parameters: (i) gene identity above 80%; (ii) ratios of alignment lengths computed for each comparison using the BLAST software (minLrap and maxLrap) above 90%. We also used the RalstoT3E website (<https://iant.toulouse.inra.fr/bacteria/annotation/site/prj/T3Ev2/>) to assign each coding sequence to a *rip* family following the nomenclature proposed in the reference paper of Peeters et al. (2013).

Based on these alignments, we identified conserved zones and designed the primers that would enable to amplify putative T3E in all the strains studied. When possible, the primers were designed to amplify entire genes; for gene sizes exceeding 1 kb, primers were designed to amplify several gene fragments. Primer design was performed using the Primer 3 software (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm>) following the parameters: annealing temperature around 60°C and primer size between 18 and 25 nucleotides.

Gene amplification and visualization. For each strain, putative effectors were PCR amplified on 20 ng of sample DNA template. PCR reactions (total volume of 25 µl) consisted of 1 U of Red Goldstar Taq DNA polymerase, 25 pmol of each primer, 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM of each dNTPs, and 1× Q-solution. The reaction was cycled in Eppendorf Mastercycler Gradient or Applied Biosystems GenAmp PCR System 9700 thermocyclers with a first denaturation step at 96°C for 5 min followed by 30 cycles of 30 s at 95°C, 60 s at 56°C, and 60 s at 72°C, and a final elongation step of 10 min at 72°C. All PCR products were resolved on a 2% agarose gel and visualized with UV light after ethidium bromide staining (5 µg ml⁻¹); fragment sizes were estimated as compared with a 100-bp DNA ladder (New England BioLabs).

Step 3: Pathogenicity tests (phenotyping) with representative strains. Tomato (susceptible T10, and resistant T5) and eggplant (susceptible E8, resistant E1 and E6) seeds were sown in a greenhouse for 4 and 3 weeks, respectively, before inoculation and were transplanted 1 week later into FLORADUR potting mix (9 × 9 cm pots). Once three to four fully expanded leaves was reached, plants were transferred (at least 2 days before inoculation) into a high quarantine security level (NS3) growth chamber (Rotoplan), to cope with inoculation of exotic strains (notably from the “emerging ecotype” [phylotype IIB/clade 4/sequevar 4NPB]). Climatic parameters were set at 85% relative humidity, with a photoperiod of 12 h, and a thermoperiod of 30°C day/24°C night (±2°C). Bacterial suspensions of selected strains were prepared in Tris buffer (10⁸ cells ml⁻¹) and inoculated (5 ml per plant) as previously described (N’Guessan et al. 2012) after root scarification. Each strain was inoculated on 15 plants from each accession. The experiment was repeated once. Bacterial wilt incidence and severity were monitored every 2 to 3 days for 28 days on a 0 to 4 scale that conveys the percentage of leaves wilted (N’Guessan et al. 2012). Each plant that scored 3 and 4 was considered wilted. Plants showing no symptoms at the end of the experiment were harvested and sampled for latent infections as previously described (Deberdt et al. 2014; Lebeau et al. 2011; N’Guessan et al. 2012). The percentage of wilted plants and the colonization index (Prior et al. 1996) were thus accessed.

Data analysis. All statistical analyses were done using R software version 3.1.3 (R Development Core Team 2013).

Typologies of T3E gene repertoires (step 2). For each strain, the expected PCR results were (i) presence or absence of the T3E, as estimated by PCR amplification success or failure, and (ii) the size of the amplified fragment. For each putative gene, class 1 corresponded to a fragment amplification of the expected size, class 2 referred to no amplification, and classes 3 to 6 corresponded to the different alleles (band size) obtained after amplification. Each class, named “allele score” further down in the paper, was considered a qualitative factor within each variable (T3E gene or coding sequence [CDS]). The strains were then clustered on the basis of each allele score for all 25 genes amplified, using an agglomerative hierarchical clustering (Maechler et al. 2015) with the Euclidean distance and considering

the “Ward” method. We identified the variables (genes) best describing each cluster (named type III effector repertoire group [TRG] further down) by using a χ^2 test (Husson et al. 2009, 2015).

Comparison of wilting and colonization rates across strains (step 3). The effect of strain and plant accession on wilting and colonization incidence, and area under the disease progression curve (AUDPC), was assessed using a generalized linear model considering, respectively, binomial data (wilted versus nonwilted) with χ^2 test ($P = 0.05$), and an analysis of variance with Fisher-Snedecor test ($P = 0.05$). Incidence and AUDPC means were compared within each plant accession using a pairwise comparison test based on the Tukey’s methods at 0.05 (Hothorn et al. 2008).

Typologies of virulence phenotypes (step 3). The combination of final wilting incidence and colonization index scores was used

Fig. 1. Genotype-phenotype association workflow. Data priming this study were generated by Lebeau (2010), Lebeau et al. (2011), and Guidot et al. (2009). T3E: type III effector; HAC: hierarchical ascending classification; and TRG: type III effector repertoire group. In step 3, the eggplant accessions were either resistant (R) or susceptible (S).

to calculate reference phenotypes using the “*k-nearest neighbor*” algorithm (Venables and Ripley 2002) as previously described (Lebeau et al. 2011; N’Guessan et al. 2012). Virulence phenotypes ranged from 1 (highly resistant) to 5 (highly susceptible). The 0 class used in this study corresponded to no wilt and no colonization. Each representative strain was thus assigned to a virulence phenotype on the different plant accessions. The virulence phenotypes were considered as ordered factors ranked in ascending order from 0 to 5. A phenotype clustering was constructed by using an agglomerative hierarchical clustering as described above, considering each accession separately.

A principal component analysis based on gene presence/absence patterns and the phenotype on the different resistant accessions (E1, E6, and T5) was performed using the *ade4* package (Dray and Dufour 2007).

RESULTS

T3E distribution within the core-RS2 as estimated by CGH. We first aimed to assess the distribution of 117 genes coding for proteins secreted by, or constitutive of the structure of, the type III secretion system, within the reference 12-strain core-RS2 collection. Among the 117 genes considered, 91 were T3E (78) or putative T3E (13) (Supplementary Table S1). Considering the 10 strains with complete validated hybridization results (i.e., apart from PSS366 [RUN0155] and PSS04 [RUN0157]), the distribution of the 91 T3E genes was compared across the core-RS2 strains. It is important to note that we considered the gene present in a strain if this strain was positive for at least one probe of this gene. We also compared this CGH-based repertoire to the repertoire of 26 genomes available on the RalstoT3E website. Twenty T3E genes, sensu Peeters et al. (2013), were absent or were not detected in our conditions, from this core-RS2 collection: *ripAF2*, *ripAG*, *ripAI* (RSp0838), *ripAZ2*, *ripBA*_fragment2 (RSc0228), *ripBB*, *ripBC*, *ripBD*, *ripBF*, *ripBG*, *ripC2*_Fragment 1 and 2 (RSp0593 and 0592, respectively), *ripF2*, *ripG8*, *ripH4*, *ripK*, *ripO2*, *ripP3*_fragment 1 and 2 (RSc3444 and 3443, respectively), *ripS6*, *ripS7*, and *ripS8* (RSc3447). Except *ripAI* which was found to be a core-effector (Ailloud et al. 2015; Clarke et al. 2015; Peeters et al. 2013), most of these “lacking effectors” were either phylotype IV-specific (as detailed below) or found within phylotype IIB strains.

Type III effector repertoires ranged from 61 (CMR32) to 79 (GMI1000). Although there was no clear relationship between phylotype and repertoire richness, phylotype III strains seemed to contain fewer T3Es (61 to 67) than the other phylotypes: 74 to 78 in phylotype I, 73 to 77 in phylotype IIA, and 74 to 75 in phylotype IIB (Supplementary Table S2). Forty-one T3E genes and three putative T3Es were common to all 10 strains, among which four *ripA* (*ripA2*-5), four *ripG* (*ripG2*-5), three *ripH* (*ripH1*-3), and four *ripS* (*ripS1*-4). Conversely, *ripA5_2* was the least shared T3E (20% strains), as well as *ripT* and *ripG1* (30% strains). To check for the effector prevalence evenness within our collection, and detect eventual phylotype-specific effectors, we compared the distribution of the 91 T3E genes within each phylogenetic group (phylotype I, three strains; phylotype II, four strains; and phylotype III, three strains). Effector distributions within phylotypes were not significantly different from expected (χ^2 test, P value = 0.999 to 1), indicating that phylotype had no detectable influence on effector distribution. Considering the distribution of individual T3E across phylotypes, only *ripA5_2* (PTO1391) and *ripC1* had a distribution different from expected (χ^2 test, P value = 0.038 for both), the first one appearing specific to phylotype II while the latter was found absent from phylotype III strains.

Considering the 12 strains (including PSS366 and PSS4), only 65 T3Es gave unambiguous scorable results. From the comparison of T3E distribution across phylotypes (phylotype I containing five strains this time), the conclusions were highly similar (no apparent phylotype-specific T3E composition), except that (i) *ripG1* looked

significantly overrepresented in phylotype I, and (ii) *ripBH* was overrepresented in phylotype II (χ^2 test, P value = 0.044 and 0.020, respectively).

The effector prevalence evenness was also assessed on the 26 *R. solanacearum* genomes harbored on the RalstoT3E website (Supplementary Table S3). It is important to note that this data set included phylotype I genomes (four, including GMI1000), numerous phylotype II genomes (four IIA including CFBP2957, and 14 IIB including CFBP6783), phylotype IV genomes (three), but contained one single phylotype III genome (CMR15). Type III effector repertoires ranged from 68 to 75 for phylotype I, from 54 to 67 for phylotype IIA, from 54 to 70 for phylotype IIB, 59 for phylotype III, and from 46 to 62 for phylotype IV. Effector distributions within phylotypes were significantly different from expected for phylotype IV and phylotype I (χ^2 test, P value = 0.014 and 0.029, respectively). Considering the distribution of individual T3E across phylotypes, 15 effectors had a distribution different from expected. Most of them were significantly specific to phylotype IV (*ripAF2*, *ripAG*, *ripAK*, *ripAZ2*, *ripBF*, *ripH4*, and *ripO2*; χ^2 test, P values ranging between 5.88×10^{-6} and 0.029), or to both phylotypes IV and I (*ripT*, P = 0.004), or specific to phylotype I (*ripAI*, *ripAH*, *ripBA*, *ripS6*, *ripS8*, and *ripTAL*; P values ranging from 3.25×10^{-4} to 0.025). *ripG8* was specific to phylotype III (P = 1.831×10^{-6}). We confirmed that *ripG1* was significantly overrepresented within phylotype I, whereas absent within phylotype II genomes (P = 0.004); *ripBH* was found in both phylotype II and phylotype IV genomes, and its distribution was thus not different from expected (P = 0.171).

Analysis of effector repertoires of the core-RS2 reveals the association of some T3Es to specific virulence phenotypes. Comparing the presence of each probe within each couple of avirulent and virulent strain panels (Table 3), we chose to rely on the ratio “present probes/total probes per gene” to infer a degree of association to a particular phenotype. As an example, *ripP1* was 100% associated with avirulence to eggplant ‘Surya’ (Table 4) because all three *ripP1* probes were present in all avirulent strains, whereas absent in all virulent strains.

Association with avirulence. Sixty genes were associated with avirulence, mainly to pepper P8 (35 genes), then eggplant E6 (28 genes), E2 (11 genes), E1 (10 genes), E3 (four genes), and E4 (two genes). One single gene, *ripAX2*, was associated with avirulence to tomato T5 (Table 4). Interestingly, 20 genes were associated with avirulence to two accessions (mostly one eggplant and pepper P8), and five genes were associated with avirulence to three cultivars: *ripA4* and *ripN* on E1-E6-P8; *ripAS* on E3-E6-P8; *ripD* on E2-E6-P8; and *ripG5* on E1-P6-P8 (Supplementary Table S4).

We focused on the 31 genes (one harpin, four putative T3Es, 26 T3Es among which *ripAP* was in two fragments) (i) whose at least 50% probes (corresponding to 0.5 in Table 4) were associated with avirulence to at least one accession, and/or (ii) were associated with avirulence to E1 or E6 even with less than 50% probes (Table 4), and/or (iii) were related to fitness on one or several hosts in a previous study (Macho et al. 2010). Highest association scores (from 1 down to 0.67) were observed with avirulence to E6 (*ripAP-ripE1*, *ripP2*, *ripAJ-ripAU-ripAZ1-ripP1*), P8 (RSp0216, RSp0218-*ripAS*, *ripG3*), E4 (*ripP1*, *ripAZ1*), and T5 (*ripAX2*). Some effectors were associated with avirulence to more than one accession. Indeed, avirulence to both E1 and P8 was associated with four effectors (*hrpZ*, *ripG4*, *ripS5*, and RSc1723); avirulence to E3 and P8 with two (RSp0216 and RSp0218), like E4 and E6 (*ripAZ1* and *ripP1*); and avirulence to E6 and P8 was associated with *ripG3* (Table 4). Three genes were even associated with avirulence on three accessions: *ripAS* (E3-E6-P8), *ripN* (E1-E6-P8), and *ripG5* (E1-P6-P8). Effectors associated with avirulence to E1 were unexpectedly few and mildly associated (one probe/gene): *hrpZ*, *ripG4*, *ripG5*, *ripN*, *ripS5*, and RSc1723. Only *ripN* was associated with avirulence to both E1 and E6.

Association with virulence. Seventy-three genes (including 51 T3Es and seven putative T3Es) were associated with virulence (Supplementary Table S5), mainly on pepper P8 (58 genes) and P2 (22 genes), then eggplants E2 (17 genes) and E3 (11 genes), tomato T5 (9 genes), eggplants E1 (7 genes), E4 (6 genes), E6 (5 genes), and pepper P6 (4 genes). Focusing on the 37 genes with at least 50% probes (score of 0.5 in Table 5) were associated with virulence to at least one accession, highest association scores (1 down to 0.67) were observed on P8 (18 genes and one *ripM* fragment) and P2 (8 genes); virulence on eggplants was associated with five (E2), three (E3), two (E1 and E6), or the gene RSp0213 (E4). Some genes were associated with virulence on several accessions: BA02498 on E1-E3-P2-P6-P8; PTO7001 on E1-E3-E6-P2-P8-T5; PTO3558 on E1-E3-E6-P2-P8-T5; *ripA5_2* on E3-E6-P2-P8-T5; and *ripE2* on E1-E3-P2-P6-P8.

Association with both avirulence and virulence. Thirty-seven genes were both associated with avirulence and virulence, but in most cases high association scores (over 50%) were with one unique phenotype, avirulence, or virulence. Some interesting cases were observed on high association scores, several effectors being associated with avirulence to eggplant and virulence to pepper. Hence, *ripP1* was associated with avirulence to both eggplants E4 and E6 and to virulence to pepper P8; and *ripAZ1*, avirulent to E4 and virulent to P8. Conversely, *ripAF1* was associated with

avirulence to P8 and virulence to both E2 and E4; RSp0213 was associated with avirulence to P8 and to virulence to E4 and P2.

From this first screening, the effectors associated with interaction (avirulence or virulence) to eggplants E6 and E1 and tomato T5 were retained for further experiments. Twenty-five T3Es and effector-like genes were thus selected (Table 6), among which were 16 avirulence-associated and nine virulence-associated genes.

The avirulence/virulence-associated effectors repertoire can be described in six distribution patterns, some of which are phylotype-specific. The 25 T3Es associated with interaction to eggplants and tomato were selected based on CGH results, targeting specific short regions of the gene. To assess their actual presence and size within the two *R. solanacearum* strain collections ($n = 48$), all of these genes were PCR-amplified using consensus primers (sequences and conditions detailed in the Supplementary Table S6). From this PCR screening, it appeared that T3Es were (i) successfully amplified giving the expected gene size, (ii) not amplified despite two or three independent replications, or (iii) successfully amplified but giving a gene size different from the expected one. These different states of each effector within each strain were used to build a typology of the T3E repertoires.

Each strain was thus described by a pattern of 25 variables (gene) that may have up to six different levels: 1 = amplified at the expected size; 2 = not amplified, and 3 to 6 = amplified at sizes

TABLE 4. Type III effectors (T3E) and T3E-like genes associated with avirulence on eggplant, pepper, and tomato accessions, as determined by the ratio present probes/total probes per gene in each avirulent strains panel

Gene code	Rip name	Former/other name, description	Number of probes/gene	Eggplant					Pepper	Tomato	Related to fitness on ^a
				Dingras (E1)	SM6 (E2)	Ceylan (E3)	Surya (E4)	AG91-25 (E6)	Perennial (P8)	Hawaii7996 (T5)	
RSc1723	–	Putative T3E	4	0.25					0.5		
RSc2897	–	Putative T3E	4		0.25			0.5			
RSp0216	–	Putative T3E	4			0.5			1		
RSp0218	–	Putative T3E	4			0.5			0.75		
RSp0854	–	hrpZ	2	0.5					0.5		
RSp0099	<i>ripA2</i>	AWR2	4	0.25					0.25		
RSp0847	<i>ripA4</i>	AWR4	4	0.25				0.25	0.25		
RSp0822	<i>ripAF1</i>	HopF1-like	4					0.25	0.5		Eggplant, bean
RSc2101	<i>ripAJ</i>	–	3					0.67			
RSp1218	<i>ripAP</i> -fragment 1	–	1					1			
RSp1215	<i>ripAP</i> -fragment 2	–	3					1			
RSp1384	<i>ripAS</i>	–	4			0.25		0.5	0.75		
RSp1460	<i>ripAU</i>	–	3					0.67	0.33		
RSp0572	<i>ripAX2</i>	HopH1-like	4							0.75	
RSp1022	<i>ripAY</i>	–	3					0.33	0.33		Eggplant
RSp1582	<i>ripAZ1</i>	–	3				0.67	0.67			
RSp0304	<i>ripD</i>	HopD1-like	3		0.33			0.33	0.33		Tomato, eggplant, bean
RSc3369	<i>ripE1</i>	–	4					1			
RSp0028	<i>ripG3</i>	GALA3	5					0.5	0.6		
RSc1800	<i>ripG4</i>	GALA4	4	0.25					0.25		
RSc1801	<i>ripG5</i>	GALA5	4	0.25					0.25		
RSc1357	<i>ripG7</i>	–	4		0.5						
RSp0215	<i>ripH2</i>	HLK2	4						0.5		
RSp0160	<i>ripH3</i>	HLK3	4					0.5	0.25		
RSp1130	<i>ripN</i>	–	3	0.33				0.33	0.33		
RSc0826	<i>ripP1</i>	PopP1	3				1	0.67			
RSc0868	<i>ripP2</i>	PopP2	4					0.75			Tomato, eggplant, bean
RSc1839	<i>ripS4</i>	–	5					0.2			Eggplant
RSp0296	<i>ripS5</i>	SKWP5	4	0.25					0.5		
RSc1815	<i>ripTAL</i>	AvrBs3-like	5						0.2		Eggplant
RSc2775	<i>ripW</i>	–	4	0.25					0.25		
RSp0877	<i>ripX</i>	popA	2					0.5			

^a This selection gathers genes whose (i) at least 50% probes (corresponding to 0.5 score) are associated with avirulence to at least one plant accession, and/or (ii) were associated with avirulence on E1 and E6, and/or (iii) significantly contributed to in planta fitness (Macho et al. 2010). Genes selected for step 2 analysis are written in bold.

different from the expected one. The correspondences between alleles and amplicon sizes are detailed in Supplementary Table S7. The screening of the 25 T3Es in the two *R. solanacearum* strain collections thus led to the identification of six main groups of strains sharing similar T3E repertoire patterns, thus named type III effector repertoire groups (TRG) (Table 7). The *catdes* function (Husson et al. 2009) allowed identifying the variables' levels best describing each TRG (Supplementary Table S8). These TRG seem to be phylotype-specific, except for TRG3 and TRG6. The TRG1 gathered strains of phylotype IIB, and was best characterized by BA02498 presence (allele 1) and the absence of *ripG3*, *ripS5*, and *ripP2* (*P* values ranging from 2.31×10^{-4} to 9.54×10^{-3}). TRG2 gathered phylotype IIA strains and was best characterized by BA07003-allele 3 and the presence of *ripAS* (allele 1) (*P* values ranging from 2.66×10^{-3} to 2.63×10^{-2}). TRG3 was dominated by phylotype IIB strains ($n=25$) and was best characterized by *RipG3*-allele 4 and the presence of PTO1265, *ripV2*, and BA07003 (allele 1) and the absence of *RipN* and *ripAS* (*P* values ranging from 9.86×10^{-13} to 3.31×10^{-8}). TRG4 contained 80% of phylotype III strains and was best characterized by *RipG3*-allele 3, *ripW*-allele 4, and *ripAU*-allele 1 and the absence of *ripU*, PTO3558, and BA07003 (*P* values ranging from 2.38×10^{-8} to 7.96×10^{-6}). TRG5 only contained

phylotype I strains and was best described by the presence of *ripG3* (allele 1), *ripW*-allele 3, *ripG4*, *ripAZI*, and *ripS5* and the absence of BA07003 (*P* values ranging from 3.50×10^{-6} to 2.45×10^{-4}). TRG6, containing only RUN0930 (phylotype I) and RUN0657 (phylotype III), was characterized by *ripG3*-allele 5 and PTO1265-allele 3 (*P* = 0.042 for both).

The TRG representatives display high variability in virulence and aggressiveness on eggplants and tomato. To test the hypothesis that the T3E repertoire may explain the virulence phenotype, we selected 11 strains representative of the six TRG to challenge the resistant tomato and eggplant accessions. Considering both T3E content and phylogenetic position (phylotype/clade/sequence), we thus chose (i) RUN0017 (IIB/clade 4/sequence 4NPB) to represent TRG1, (ii) RUN0058 (IIA/clade 2/sequence 39) for TRG2, (iii) RUN0059, RUN0147, RUN0928, and RUN0941 (all in IIB/clade 5/sequence 1) for TRG3, (iv) RUN0145 and RUN0523 (both in phylotype III/clade 6/sequence 29 and 19, respectively) for TRG4, (v) RUN0054 (the reference GMI1000) and RUN0157 (both in phylotype I/clade 1/sequence 18 and 15, respectively) for TRG5; and (vi) RUN0657 (III/clade 6/sequence 19) for TRG6.

The strains were inoculated in two sets at different dates, with RUN0017 inoculated on both dates as a control. Because the wilting

TABLE 5. Type III effectors (T3E) and T3E-like genes associated with virulence on eggplant, pepper and tomato accessions, as determined by the ratio present probes/total probes per gene in each virulent strains panel^a

Gene or probe	Rip Name	Former/other name, description	Number of probes/gene	Eggplant					Pepper			Tomato
				Dingras (E1)	SMA6 (E2)	Ceylan (E3)	Surya (E4)	AG91-25 (E6)	PM687 (P2)	CA8 (P6)	Perennial (P8)	Hawaii7996 (T5)
BA02498	—	CPUF, AvrPtoB-like domain	3	1		1			1	1	1	
BA07003	—	Pcad2	2	0.5		0.5			0.5	0.5	1	
PT00619	—	Putative glycosyltransferase	2								0.5	
PT01265	—	CPUF	3	0.33	0.33	0.33		0.33	0.67		0.33	0.33
PT03045	—	CPUF (TPR domain)	2								1	
PT03558	—	(RRSL_04659)	3	0.33		0.33		0.33	0.33		0.67	0.33
PT04098	—	Putative T3E (RALIP_4318)	3								1	
PT04281	—	Cpuf	3								1	
PT04284	—	Putative T3E (RALIP_4533)	2								1	
PT07000	—	Putative T3E (RALIP_1709)	3						0.67			
PT07001	—	Putative T3E (ripM fragment)	2	1		1		1	1		1	1
RSc2131	—	Puf	3		0.67				0.33		0.33	
RSc3174	—	Putative T3E	4						1			
RSp0213	—	Putative T3E	2				1		1			
RSc2139	<i>ripA1</i>	Awrl	4		0.75							
PT01391	<i>ripA5_2</i>	Awrl5-2 (RALIP_1563)	3			1		1	1		1	1
RSc0321	<i>ripAE</i>	—	4								0.75	
RSp0822	<i>ripAF1</i>	Hopfl1-like	4		0.5		0.5					
RSc0895	<i>ripAH</i>	—	3		0.67							
RSc2359	<i>ripAK</i>	—	4		0.5							
RSp1582	<i>ripAZI</i>	—	3								0.67	
PT04502	<i>ripBH</i>	RALIP_4767	3								1	
PT04434	<i>ripBI</i>	RALIP_4696	3								1	
RSp1239	<i>ripCI</i>	—	4		0.25				0.25		0.75	
PT04834	<i>ripE2</i>	RALIP_0863	4	0.5		0.5			0.75	0.5	0.5	
RSp0914	<i>ripG1</i>	Gala1	3		0.67				0.33			
RSc1800	<i>ripG4</i>	Gala4	4								0.5	0.25
RSc1356	<i>ripG6</i>	Gala6	4								0.5	
RSc2132	<i>ripJ</i>	—	3								1	
RSc0826	<i>ripP1</i>	Popp1	3								1	
BA00250	<i>ripS4</i>	RCFBP_11536	3								0.67	
RSc1839	<i>ripS4</i>	Rsc1839	5								0.4	
RSc3212	<i>ripT</i>	—	2						0.5			
RSc1815	<i>ripTAL</i>	—	5		0.6						0.2	
PT03560	<i>ripU</i>	RRSL_04660	2								1	0.5
RSp1212	<i>ripU</i>	—	5						0.8			
PT01326	<i>ripV2</i>	RALIP_1493	3								1	0.33

^a This selection gathers genes whose at least 50% probes (score 0.5) are associated with virulence on at least one plant accession. Genes selected for step 2 analysis are represented in bold.

incidence, colonization index, and AUDPC were found not to be significantly different across the two dates ($P = 0.143, 0.082$, and 0.615 , respectively), we pooled the two sets in one. After 28 days of incubation, the control susceptible accessions were significantly more diseased than the resistant accessions (detailed results are shown in Supplementary Fig. S1). However some strains induced few symptoms on the susceptible controls. RUN0523 (TRG4) induced less than 20% wilt and colonization on T10 and E8 and was thus regarded a poorly aggressive strain on eggplant and tomato. RUN0058 (TRG2) induced a low wilting incidence but more than 60% colonization to T10. All the other strains induced more than 70% wilt and 80% colonization. On eggplant E8, strains RUN0017 (TRG1), RUN0059 and RUN0928 (TRG3), RUN0523 (TRG4), and RUN0657 (TRG6) caused less than 20% wilt and colonization; highest aggressiveness (more than 80% wilt and colonization) was observed for RUN0157 (TRG5), RUN0147, and RUN0941 (TRG3). The resistant eggplant E1 showed the highest resistance level since only RUN0147 induced more than 20% wilt on it. The accession E6 was susceptible (30% wilt and 60% colonization or above) to TRG3 strains RUN0147, RUN0928, and RUN0941, and to TGR5 strain RUN0157. The resistant tomato T5 was most highly affected (80% wilt and colonization or above) by TRG3 strains RUN0147, RUN0941, and RUN0928, and by RUN0017 (TRG1). Incompatible interactions (no wilt and no colonization) were observed on the couples (i) E1 $\times$ RUN0523 and E1 $\times$ RUN0058, and (ii) E6 $\times$ RUN0054, E6 $\times$ RUN0523, E6 $\times$ RUN0145, and E6 $\times$ RUN0059.

The T3E repertoire is not globally descriptive of the virulence phenotype. The hierarchical ascending classification of the 11 strains representative of the six TRG built based on their T3E repertoire did not clearly match with their virulence phenotypes on eggplant and tomato (Fig. 2). More precisely, strains RUN0058 (TRG2) and RUN0017 (TRG1), despite having very similar T3E repertoires, highly differed in virulence on the five cultivars. Similarly, RUN0147 and RUN0059 belong to the same TRG3 but the former is highly virulent on E6 and T5, whereas the latter is avirulent on E6 and poorly aggressive on T5. The strains RUN0157 and RUN0054, though gathered in the same TRG5, greatly differ in virulence on the resistant accession E6 (Fig. 2).

From these results, we concluded that the entire T3E repertoire does not determine the phenotype of a strain.

Some T3Es are individually highly associated with avirulence or virulence. We performed a principal component analysis based on the presence or absence of genes, regardless of their allelic state. Projecting the phenotypic classes (0 to 5) and the gene presence on the same factorial plan, we considered that genes projected in the same zone as extreme phenotypes (avirulence, coded as 0; virulence; coded as 3 for E1, or 4 and 5 for E6 and T5) were associated with this phenotype. On eggplant E1, the two axes opposed avirulent strains (bottom left) to virulent strains (top right) (Fig. 3A). *RipA5_2* (PTO1391) was associated with virulence, whereas *ripAS* (RSp1384), and more secondarily *ripN* (RSp1130), were associated with avirulence. On eggplant E6 (Fig. 3B), avirulent strains were in the top left quarter of the plan, whereas virulent strains were in the top right and bottom left quarters. PTO3558 and *ripU* (PTO3560), more secondarily *ripA5_2* (PTO1391), were associated with virulence, while *ripP2* (RSc0868), *ripAU* (RSp1460), and *ripG3* (RSp0028), and more secondarily *ripAX2* (RSp0572) and *ripP1* (RSc0826), were associated with avirulence. On tomato T5 (Fig. 3C), the phenotypes were mainly separated along the first axis, from quasi-avirulent strains on the left to virulent strains on the right. *RipV2* (PTO1326), BA07003, PTO3558, and *ripU* (PTO3560) were associated with virulence, whereas *RipP1* (RSc0826), *RipN* (RSp1130), *ripAX2* (RSp0572), and more secondarily *ripP2* (RSc0868), *ripG3* (RSp0028), *RipAU* (RSp1460), and *ripG5* (RSc1801) were associated with avirulence.

Then, we followed a stringent approach, hypothesizing that the phenotype could be explained by the presence or absence of key T3Es. For each resistant cultivar, we identified T3Es called (i) virulence effectors that were present in virulent strains and absent in avirulent strains, and (ii) avirulent effectors that were absent in virulent strains and present in avirulent strains. We thus identified the following effectors as highly associated with strain phenotypes (Table 8): *ripA5_2* was associated with virulence on both eggplant E1 and tomato T5; *ripE2*, but also PTO1265 and PTO7001 were associated with virulence on eggplant E1; PTO3558 and *ripU* associated

TABLE 6. Type III effectors and effector-like genes selected on basis of their association with phenotype (avirulence or virulence) to eggplants E1 and E6 and tomato T5 following step 1^a

Gene Code	Rip name/other name	Eggplant					Pepper			Tomato
		E1	E2	E3	E4	E6	P2	P6	P8	T5
BA02498	–	V	–	V	–	–	V	V	V	–
BA07003	–	V	–	V	–	–	V	V	V	–
PT01265	–	V	V	V	–	V	V	–	V	V
PT03558	–	V	–	V	–	V	V	–	V	V
PT07001	–	V	–	V	–	V	V	–	V	V
RSc1723	–	A	–	–	–	–	–	–	A	–
RSp0854	<i>hrpZ</i>	A	–	–	–	–	–	–	A	–
RSp0099	<i>ripA2</i>	A	–	–	–	–	–	–	A	–
RSp0847	<i>ripA4</i>	A	–	–	–	A	–	–	A	–
PT01391	<i>ripA5_2</i>	–	–	V	–	V	V	–	V	V
RSp1384	<i>ripAS</i>	A	–	A	–	A	–	–	A	–
RSp1460	<i>ripAU</i>	–	–	–	–	A	–	–	A	–
RSp0572	<i>ripAX2</i>	–	–	–	–	–	–	–	–	A
RSp1582	<i>ripAZ1</i>	–	–	–	A	A	–	–	–	–
PT04834	<i>ripE2</i>	V	–	V	–	–	V	V	V	–
RSp0028	<i>ripG3</i>	–	–	–	–	A	–	–	A	–
RSc1800	<i>ripG4</i>	A	–	–	–	–	–	–	A	V
RSc1801	<i>ripG5</i>	A	–	–	–	–	–	–	A	–
RSp1130	<i>ripN</i>	A	–	–	–	A	–	–	A	–
RSc0826	<i>ripP1</i>	–	–	–	A	A	–	–	V	V
RSc0868	<i>ripP2</i>	–	–	–	–	A	–	–	–	–
RSp0296	<i>ripS5</i>	A	–	–	–	–	–	–	A	–
PT03560	<i>ripU</i>	–	–	–	–	–	–	–	V	V
PT01326	<i>ripV2</i>	–	–	–	–	–	–	–	V	V
RSc2775	<i>ripW</i>	A	–	–	–	–	–	–	A	–

^a Phenotypes: A, avirulence; V, virulence.

with virulence to both E6 and T5; and BA7003 was associated with virulence to tomato T5. *ripAS* was associated with avirulence to E1, *ripP2* and *ripAX2* to avirulence on E6; and *ripP1*, *ripP2*, *ripAX2*, *ripN*, and *ripS5* were associated with avirulence to T5.

DISCUSSION

Although bacterial wilt has been a major biotic stress in solanaceous crops throughout the world, knowledge remains scarce about the plant genes allowing hosts to resist *R. solanacearum* attacks, and about the bacterial genes most contributing to

adaptation to new host or to a resistant accession. To prime the development of durable resistance sources, given the huge genomic and phenotypic plasticity of the pathogen, it is now critical to favor pathogen-informed resistance breeding. Such a strategy requires identifying the bacterial genes involved in virulence to hosts and selected for that function, as well as those counter-selected based on detection in resistant hosts, for further exploring their diversity and evolutionary dynamics in natural populations. Studies on *R. solanacearum*–plant interactions have been focused for a long time on the model species *Arabidopsis thaliana* (Deslandes et al. 1998, 2003; Dignonnet et al. 2012) and *Medicago truncatula* (Ben

TABLE 7. Distribution of the 25 type III effectors (T3E) and T3E-like genes selected among *Ralstonia solanacearum* strains from Core-RS2 and Reunion Island, as determined by polymerase chain reaction (PCR) amplifications^a

RUN	Phylotype	TRG ^b	Gene name, Rip name																								
			–	<i>ripP1</i>	–	<i>ripAX2</i>	<i>ripG4</i>	<i>ripN</i>	<i>ripAZ1</i>	<i>ripAS</i>	<i>ripG5</i>	<i>ripAU</i>	<i>ripE2</i>	<i>ripP2</i>	<i>ripS5</i>	<i>hrpZ</i>	<i>ripG3</i>	–	–	<i>ripU</i>	<i>ripA5_2</i>	–	<i>ripV2</i>	–	<i>ripW</i>	<i>ripA2</i>	<i>ripA4</i>
			BA2498	RSc0826	RSc1723	RSp0572	RSc1800	RSp1130	RSp1582	RSp1384	RSc1801	RSp1460	PTO4834	RSc0868	RSp0296	RSp0854	RSp0028	BA7003	PTO3558	PTO3560	PTO1391	PTO1265	PTO1326	PTO7001	RSc2775	RSp0099	RSp0847
16	IIB	1	1	2	2	2	2	2	2	2	2	1	2	2	3	2	1	1	1	1	2	2	2	1	1	1	
17	IIB		1	2	2	2	2	2	2	2	2	2	1	2	2	3	2	1	1	1	1	2	2	2	1	1	1
18	IIB		1	2	2	2	2	2	2	2	2	2	1	2	2	3	2	1	1	1	1	2	2	2	1	1	1
36	IIA	2	2	2	2	2	2	1	2	1	2	3	3	3	2	1	2	3	1	1	2	2	1	2	1	1	1
58	IIA		2	2	2	2	2	2	2	1	2	2	2	3	3	3	2	1	1	1	2	2	1	2	1	1	1
150	IIA		1	2	2	1	2	2	2	1	2	2	3	3	2	1	3	3	1	1	1	2	1	2	1	1	1
59	IIB	3	2	2	2	2	2	2	2	2	2	2	3	3	3	4	1	1	1	1	1	1	1	1	1	1	
147	IIB		2	2	2	2	2	2	2	2	2	2	3	2	2	3	4	1	1	1	1	1	1	1	1	1	
160	IIB		2	2	2	1	2	2	2	2	2	2	3	3	2	3	4	1	1	1	1	1	1	1	1	1	1
476	IIB	4	2	2	2	2	2	2	2	2	2	3	3	3	3	4	1	1	1	1	1	1	1	1	1	1	
623	IIB		2	2	2	2	2	2	2	2	2	2	2	2	3	3	4	1	1	1	2	1	1	1	1	1	
654	IIB		2	2	2	2	2	2	2	2	2	2	3	2	3	3	4	1	1	1	1	1	1	1	1	1	1
681	IIB	5	2	2	1	1	2	2	2	2	2	2	2	3	3	4	1	1	1	2	1	1	1	1	1	1	
697	IIB		2	2	2	2	2	2	2	2	2	2	3	3	3	3	4	1	1	1	1	1	1	1	1	1	
843	IIB		2	2	2	2	2	2	2	2	2	2	3	3	3	3	4	1	1	1	1	1	1	2	1	1	1
845	IIB	6	2	2	2	2	2	2	2	2	2	3	3	3	3	4	1	1	1	1	1	1	1	1	1	1	
848	IIB		2	2	2	2	2	2	2	2	2	2	3	2	3	3	4	1	1	1	1	1	1	1	1	1	
880	IIB		2	2	2	2	2	2	2	2	2	2	3	2	3	3	4	1	1	1	1	1	1	1	1	1	
889	IIB	7	2	2	1	1	2	2	2	2	2	2	2	2	3	4	1	1	1	2	1	1	1	1	1	1	
913	III		2	2	2	2	2	2	2	2	2	2	3	2	3	3	4	1	1	1	1	1	1	1	1	1	
919	IIB		2	2	2	2	2	2	2	2	2	2	3	1	3	1	4	1	1	1	1	1	1	1	1	1	
921	IIB	8	2	2	2	2	2	2	2	2	2	3	3	3	3	4	1	1	1	1	1	1	1	1	1	1	
923	IIB		2	2	2	2	2	2	2	2	2	2	3	3	3	3	4	1	1	1	1	1	1	1	1	1	
924	IIB		2	2	2	2	2	2	2	2	2	2	3	3	3	3	4	1	1	1	1	1	1	1	1	1	
925	IIB	9	2	2	2	2	2	2	2	2	2	3	3	3	3	4	1	1	1	1	1	1	1	1	1	1	
926	IIB		2	2	2	2	2	2	2	2	2	2	3	3	3	3	4	1	1	1	1	1	1	1	1	1	
928	IIB		2	2	2	2	2	2	2	2	2	2	3	3	2	3	4	1	1	1	1	1	1	1	1	1	
931	IIB	10	2	2	2	2	2	2	2	2	2	3	3	2	3	4	1	1	1	1	1	1	2	1	1	1	
934	IIB		2	2	2	2	2	2	2	2	2	2	3	3	3	3	4	1	1	1	1	1	1	2	1	1	
936	IIB		2	2	2	2	2	2	2	2	2	2	3	3	2	3	4	1	1	1	1	1	1	2	1	1	
941	IIB	11	2	2	1	1	2	2	2	2	2	2	3	3	3	4	1	1	1	2	1	1	1	1	1	1	
944	IIB		2	1	1	1	2	2	2	2	2	2	1	3	3	3	4	1	1	1	2	1	1	1	1	1	
39	III		2	1	1	2	3	1	1	2	2	1	1	3	1	1	3	2	2	2	2	2	2	2	1	1	1
145	III	12	2	1	1	1	2	1	2	2	1	1	1	3	1	1	3	2	2	2	2	2	2	4	1	1	
133	III		2	2	1	1	1	1	2	1	2	1	2	3	1	1	3	2	2	2	2	2	2	4	1	1	
60	III		2	2	1	1	1	1	1	1	1	1	2	1	1	1	3	2	2	2	2	2	2	4	1	1	
61	III	13	2	2	1	1	1	1	1	1	1	1	2	1	1	1	3	2	2	2	2	2	2	4	1	1	
77	III		2	2	1	1	1	1	1	1	1	1	2	1	1	1	3	2	2	2	2	2	2	4	1	1	
523	III		2	2	1	1	2	1	1	1	1	1	2	1	1	1	3	2	2	2	2	2	2	4	1	1	
693	III	14	2	2	2	2	1	1	1	1	1	4	3	1	1	3	2	2	2	2	2	2	2	4	1	1	
54	I		2	1	1	1	1	1	1	1	2	2	2	1	1	1	1	2	2	2	2	2	2	3	1	1	
215	I		2	1	1	1	1	1	1	1	2	2	2	1	1	1	1	2	1	1	2	2	2	3	1	1	
155	I	15	2	1	1	1	1	1	1	2	2	2	1	3	1	1	2	1	1	2	2	2	2	3	1	1	
157	I		2	2	1	2	1	1	1	3	2	2	2	2	1	1	1	2	1	1	2	2	2	3	1	1	
471	I		2	1	1	1	1	1	1	1	1	4	3	3	1	1	1	2	1	1	2	2	2	1	1	1	
608	I	16	2	2	1	1	1	1	1	1	1	1	1	3	1	1	2	2	1	2	2	2	2	1	1	1	
657	III		2	2	2	2	1	1	1	1	1	4	3	1	1	1	4	1	1	1	1	3	2	1	1	1	
930	I		2	2	2	2	1	1	1	1	2	2	3	3	1	1	5	1	1	1	1	2	2	1	1	1	

^a Genes were either amplified at the expected size (allele 1), or not amplified (allele 2). In this latter case, absence of amplification may be interpreted as absence of the gene, or high divergence in the region targeted by the PCR primers. Genes were also amplified at a different size than expected (alleles 3, 4, and 5). Gene typology led to identify six T3E repertoire groups (TRG) within which were chosen representative strains (written in bold) to be inoculated on eggplant and tomato.

^b Type III effector repertoire group, determined using the ascending hierarchical classification (function *agnes*, package *cluster*) with strains as individuals and genes as variables.

^c Pattern, determined using the ascending hierarchical classification (function *agnes*, package *cluster*) with genes as individuals and strains as variables.

et al. 2013; Turner et al. 2009; Vailleau et al. 2007), but transposition of these findings to crops has been problematic. Some critical mechanistic differences having been identified between pathogenesis to model species on one hand and to crops on the other hand (Lin et al. 2008; Remigi et al. 2011). Deciphering plant–microbe interactions on crop species is thus essential.

Our three-step association genetics approach constituted a first published snapshot of the type III effector diversity existing in natural plant-associated *R. solanacearum* populations, and allowed us to identify avirulence and virulence candidate genes. Because population structure can cause bias in the association with phenotype (Falush and Bowden 2006), we checked using χ^2 test that there was no correlation between the phylotype and the effector presence.

A first snapshot of the T3E distribution in a large natural collection. This study gives a different view of the core-effectome within the *R. solanacearum* species complex (RSSC [Genin and Denny 2012]). The core-RS2 core-effectome contains 40 T3Es, which does not completely match the core-effectome identified by Peeters et al. (2013). Hence, the Peeters's core-effectors *ripAO*, *ripE1*, and *ripG7* were present in 90% core-RS2 strains; the core *ripAC* was present in 80% core-RS2 strains, and the core *ripAA* was even more variable, being absent from the phylotype III strains (except CMR15) and from two phylotype I strains, PSS366 and PSS358. The core *ripC1* and *ripU* were also variable in our study, but we observed a discrepancy between our results and Peeters' results on CMR15. This absence in our data set may be explained by technical artifacts. Recent genomics studies focusing on phylotype II genomes identified core-effectomes of different composition, including either 14 T3Es (Ailloud et al. 2015) or 31 T3Es (Clarke et al. 2015). Our own analysis on publicly available genomes identified 11 core-effectors on a quite phylogenetically unbalanced data set. Additional genomic sequences, especially within phylotype III, are needed to approach the actual composition of the RSSC core-effectome.

The T3E repertoires from the Reunion collection should be regarded partial since 25 T3Es only were considered, but they still give interesting insights on the prevalence and diversity of these effectors. Effector distribution could indeed be described in six patterns (TRG) based on effector presence but also alleles. The most polymorphic effector was *ripG3*, whose alleles were major descriptors of the different TRGs: allele 1 for TRG5, allele 3 for TRG4, allele 4 for TRG3, and allele 5 for TRG6 (Table 7). Such diversity may be a signature of the diversifying selection evoked for this gene in the reference paper describing the GALA family evolution (Remigi et al. 2011).

Some effectors were present throughout the collection, like *ripA2*, *ripA4*, and *ripW* (pattern 5 in Table 7). *ripA2* and *ripA4* were monomorphic, but it is important to note that these were partial sequences (3,065 bp over the 3,381 bp of *ripA2*_{GMI1000}, and the 960-bp region of the 3,990-bp *ripA4*_{GMI1000}). Whereas *ripA2* (formerly AWR2) was primarily characterized as contributing to virulence (Sole et al. 2012), it could be successfully amplified in the T5-avirulent strains. This difference could be due to sequence polymorphism or functional redundancy; it also may indicate that *ripA2* plays a dual role within tomato, supporting the Invasion Model (Cook et al. 2015). Whereas, *ripW* (formerly called *popW*) gave three different alleles: one being widespread in the collection (1,202 bp with IIB primers and 1,119 bp with GMI1000 primers), another (allele 4: 1,202 bp with IIB primers only) being specific to TRG 4, composed of phylotype III strains, and a third one (allele 3: 1,119 bp with GMI1000 primers only) specifically found in TRG5 (phylotype I strains). We did not find correlation between these alleles and an avirulence phenotype. *ripW* was previously characterized as a two-domain protein (Li et al. 2010), whose harpin activity (detected on tobacco) resides in its N-terminal domain (first 159 residues), and which doesn't significantly contribute to virulence on tomato. The analysis of a 20-strain collection, covering six countries but whose phylotypes were not determined, allowed the identification of six alleles ranging from 1,131 to 1,155 bp. The range of variation that we observed was thus higher, and it may be interesting to

determine the critical residues involved in the harpin activity on tobacco and solanaceous.

An approach with rich outputs, but with some limitations. Although most of the associations detected in step 3 were consistent with those found in step 1, some distortions should be evoked and discussed. Indeed, *ripAP*, *ripE1*, and *ripAJ*, though associated with avirulence to E6 from step 1 (Table 5), were not retained in the downstream analyses. *RipAP* is present in two fragments in the GMI1000 genome, and experiments established that it is not secreted by the T3SS (A. C. Cazalé and N. Peeters, *personal communication* 2014). Moreover, the associations found in step 1 were not always confirmed in STEP 3 (Table 9). Main discrepancies concerned avirulence to Hawaii7996 (T5): several associations found on full-length gene analysis (for *ripAU*, *ripG3*, *ripN*, *ripP1* and *ripP2*, *ripG5*, and *ripS5*) were not primarily detected from probe data analysis. Avirulence to eggplant detected in step 1 was mostly confirmed in step 3, with some exceptions. The E6-avirulence of *RipAX2* was found only in step 3, whereas this of *ripN* was not confirmed in step 3; *ripAS*, primarily associated with avirulence on E3 and E6, was found avirulent to E1 only from S step 3. Regarding virulence effectors, the virulence of *ripE2* on E1, *ripA5_2* on E6, and *ripV2* on T5, were confirmed, whereas *ripU*, virulent to T5 from step 1, was found virulent to T5 but also E6 in step 3. It is thus strongly recommended to combine both approaches and to favor full-length gene analysis for the choice of candidate genes.

Avirulence and virulence phenotypes are not explained by repertoires, but rather by individual effectors which constitute promising candidates interacting with eggplant and tomato. Strains of identical phylotype and TRG displayed very different virulence phenotypes (see RUN0054 and RUN0157 as an example), whereas other strains displaying identical phenotypes were in different TRG (see RUN0157 [TRG5] and RUN0941 [TRG3]). The “repertoire-for-repertoire” hypothesis, stated to

TABLE 8. Type III effectors (T3E) highly associated with strain phenotypes on the three resistant accessions

Plant accession	Strain (RUN)	Associated T3E ^a
Dingras multiple Purple (E1)	Virulent strains	147
		941
		17
		PTO1265
		PTO7001
		ripE2 (PTO4834)
	Avirulent strains	523
		58
AG91-25 (E6)	Virulent strains	941
		157
		147
		PTO3558
		ripU (PTO3560)
		147
	Avirulent strains	523
		145
		59
		54
Hawaii7996 (T5)	Virulent strains	941
		147
		17
		PTO3558 ^c
		ripU (PTO3560)
		BA7003
		ripA5_2 (PTO1391)
		ripP1 (RSc0826)
		ripAX2 (RSp0572)
		ripP2 (RSc0868)
	Quasi-avirulent strains ^d	145
		54
		ripN (RSp1130)
		ripS5 (RSp0296)

^a The T3Es associated with virulence were absent in all avirulent strains and present in all but one virulent strains, whereas those associated with avirulence were absent in all virulent strains and present in all but one avirulent strains.

^b The avirulence T3Es marked in bold were absent in all virulent strains and present in all avirulent strains.

^c The virulence T3Es marked in bold were present in all virulent strains and absent in all avirulent strains.

^d The quasi-avirulent strains induced no wilt, but were able to colonize Hawaii7996, corresponding a virulence phenotype of 1 instead of 0.

explain the *Xanthomonas* host specificity (Hajri et al. 2009), does not match the *R. solanacearum* situation.

Collectively, the final principal component analysis and the stringent analysis have helped to define a short list of avirulence- and virulence-associated effectors to be further investigated.

Among avirulence effectors, *ripP2* is one of the best candidates, associated with both eggplant E6 and tomato T5. Then, follow *ripAU* and *ripG3*, as well as *ripP1* and *ripAX2* (also for avirulence to E6 and T5). Then we identify *ripN* (avirulence to E1 and T5) and *ripAS* (avirulence to E1 only). Since *ripG5* is only associated with

Fig. 2. Type III effector (T3E) genotypes do not match virulence phenotypes. Hierarchical ascending classification of *Ralstonia solanacearum* strains (named by their RUN number and phylotype) based on their T3E repertoire, and correspondence with phenotypes observed on resistant (R) and susceptible (S) eggplants and tomatoes. TRG, type III effector repertoire group. The phenotype score was defined by the combination of final wilting incidence and colonization index, and calculated following Lebeau et al. (2011), where 0 = complete resistance (no wilt and no colonization), 1 = highly resistant, 2 = moderately resistant, 3 = partially resistant, 4 = moderately susceptible, and 5 = highly susceptible.

Fig. 3. Distribution of the type III effector (T3E) genes on the factorial plan of the two mostly informative axes of the principal component analysis performed on gene presence-absence data and phenotypic data collected from pathogenicity tests on **A**, Dingras multiple Purple (E1), **B**, AG91-25 (E6), and **C**, Hawaii7996 (T5). T3E genes associated with avirulence are shaded in blue, while those associated with virulence are shaded in red.

avirulence to T5, and because it is expressed in operon with *ripG4*, we chose not to keep it on our short list.

Among virulence effectors, most promising candidates are PTO3558 and *ripU* (virulence to both E6 and T5), *ripA5_2* (virulence to both E6, E1, and T5), and then *ripV2* and BA07003 (virulence to T5). It will be critical first to check the expression and secretion of PTO3558 and BA07003 by the T3SS. This short list of effectors is now nominated for further experiments that may validate their avirulence or virulence function.

ripP2 (former *popP2*) has been the first described *R. solanacearum* avirulence gene to interact with the RRS1-R gene, carried by the *Arabidopsis thaliana* accession Nd1 (Deslandes et al. 2003), whose functions have been most thoroughly investigated (see the review of Deslandes and Genin [2014] as well as Le Roux et al. [2015] and Sarris et al. [2015]). Amplifying the 8-to-989-bp region (over 1,464 bp in total), we identified two alleles: 982 bp (allele 1) found within almost all phylotype III strains and two phylotype I strains (RUN0054 and RUN0215), and 950 bp (allele 3) found in most phylotype IIA and IIB strains, three phylotype III strains (RUN0039, RUN0145, and RUN0133), and two phylotype I strains (RUN0155, RUN0471). To our knowledge, this is the first published paper describing *ripP2* diversity. Whether these *ripP2* alleles keep their enzymatic functions and their ability to be detected within plant (their “invasion pattern” sensu Cook et al. [2015]) remains to be tested (Tasset et al. 2010). It is now tempting to speculate that the interaction eggplant AG91-25/*ripP2* may follow the *Arabidopsis* RRS1-R/*ripP2* model (Bernoux et al. 2008; Deslandes et al. 1998, 2002, 2003; Le Roux et al. 2015; Sarris et al. 2015). According to this model, the AG91-25 major resistance gene *ERs1* (Lebeau et al. 2013) could be homologous of RRS1-R, or coupled to an RPS4 homolog. However, preliminary BLAST search of RRS1-R on the eggplant sequence genome (Hirakawa et al. 2014) returned no hit (S. Salgon and C. Sauvage, *personal communication* 2015). Recently, *ripP2* was demonstrated to specifically interact with a new eggplant resistance protein, RE-BW (Xiao et al. 2014); it remains to be determined whether this gene colocalizes with *ERs1*.

ripAX2, also called *rip36* (Peeters et al. 2013; Poueymiro and Genin 2009), codes for the other demonstrated avirulence effector, eliciting HR on *Solanum torvum* (Nahar et al. 2014), a wild relative

of eggplant. This paper gives first insights on the diversity on this poorly known effector, whose prevalence looks highest in phyloypes III and I strains.

ripP1 (former *popP1*), whose protein was demonstrated as an avirulence factor on Petunia (Lavie et al. 2002) and HR-elicitor on *Nicotiana glutinosa* (Poueymiro et al. 2009), was only detected in phylotypes I and III, giving a single allele of 1,088 bp (over the 1,104 bp total gene), and its presence was quite variable, even within phylotype I strains (absent in two over six strains). This variability was also reported in a Japanese phylotype I 22 strain-collection, but *popP1* presence was not correlated with HR on tobacco (Liu et al. 2009). Its homolog in *Xanthomonas perforans*, XopJ4, was also reported as avirulence protein recognized by the *Solanum penellii* *RXopJ4* gene (Sharlach et al. 2013).

ripA5_2 (former AWR5-2) on the other hand, was not reported as a virulence effector in the literature. AWR5 was indeed recognized by *Arabidopsis* and induced an HR on *Nicotiana tabacum* (Sole et al. 2012). It remains to assess the actual expression and the respective roles of the two *ripA5* paralogs in plant–pathogen interactions.

Potential candidate effectors in interaction with other species. Because the resistance levels observed were highest on eggplant, we focused our quest on this species. But scientists should also use these results to further investigate the pepper–*R. solanacearum* molecular interactions. One should focus on the role, distribution, diversity, and evolution of the two harpins, *hrpZ* and *ripW*, and the 10 effectors associated with avirulence to both eggplant and the pepper ‘Perennial’ (P8). Among these are two members of the *ripA* family (former AWR family), *ripA2* and *ripA4* (Sole et al. 2012), and three members for the *ripG* family (former GALA family), *ripG3*, *ripG4*, *ripG5*, whose functions remain unknown on pepper.

Furthermore, some cases of eggplant-pepper differential phenotypes may deserve further investigation. *ripP1* and *ripAZ1* were associated with avirulence to eggplant ‘Surya’ (E4) and virulence to pepper Perennial (P8). Interestingly, this host-specific differential function has been reported for the *ripP1* homolog in *Xanthomonas euvesicatoria*, *XopJ* (formerly AvrXv4), whose protein displays avirulence function on tomato (Astua-Monge et al. 2000) but virulence function on pepper, reducing the salicylic acid accumulation

TABLE 9. Correspondence between phenotype-genotype associations inferred from comparative genomic hybridization (CGH) data (step 1) and from full-length polymerase chain reaction (PCR) data (step 3) for the 25 type III effector and effector-like genes^a

Gene code	Rip name/other name	Dingras (E1)		AG91-25 (E6)		Hawaii7996 (T5)	
		CGH	PCR	CGH	PCR	CGH	PCR
BA02498	–	V	–	–	–	–	–
BA07003	–	V	–	–	–	–	V
PT01265	–	V	V	V	–	–	–
PT03558	–	V	–	–	V	–	V
PT07001	–	V	V	V	–	–	–
RSc1723	–	A	–	–	–	–	–
RSp0854	<i>HrpZ</i>	A	–	–	–	–	–
RSp0099	<i>ripA2</i>	A	–	–	–	–	–
RSp0847	<i>ripA4</i>	A	–	A	–	–	–
PT01391	<i>ripA5_2</i>	–	V	V	V	–	V
RSp1384	<i>RipAS</i>	–	A	A	–	–	–
RSp1460	<i>RipAU</i>	–	–	A	A	–	A
RSp0572	<i>ripAX2</i>	–	–	–	A	A	A
RSp1582	<i>ripAZ1</i>	–	–	A	–	–	–
PT04834	<i>ripE2</i>	V	V	V	–	–	–
RSp0028	<i>ripG3</i>	–	–	A	A	–	A
RSc1800	<i>ripG4</i>	A	–	–	–	–	–
RSc1801	<i>ripG5</i>	A	–	–	–	–	A
RSp1130	<i>RipN</i>	A	A	A	–	–	A
RSc0826	<i>ripP1</i>	–	–	A	A	–	A
RSc0868	<i>ripP2</i>	–	–	A	A	–	A
RSp0296	<i>ripS5</i>	A	–	–	–	–	A
PT03560	<i>RipU</i>	–	–	–	V	V	V
PT01326	<i>ripV2</i>	–	–	–	–	V	V
RSc2775	<i>RipW</i>	A	–	–	–	–	–

^a Phenotype-CGH associations are summarized in Table 6, whereas phenotype-PCR data associations are summarized in Table 8 and Figure 3. Genes marked in bold are identified from full-length PCR, or from both approaches. A, associated with avirulence; V, associated with virulence.

(Ustun et al. 2013). Conversely, *ripAF1* was associated with virulence to eggplants SM6 and Surya and avirulent to pepper Perennial. Again, these findings may support the view of type III effectors as dual interactors, contributing to virulence or overall bacterial fitness within a species or a species cultivar, and specifically recognized by other cultivars to trigger defense responses (Cook et al. 2015).

Future research. This study should be regarded as a first step toward decomposing the molecular bases of solanaceous–*R. solanacearum* interactions. As previously stated (Kirzinger and Stavrinides 2012), “changes in host specificity can range from the smallest to the largest genetic change,” including SNPs, residue change, intragenic or total gene insertion/deletions, and gene repertoire up to genomic island. In this study, we considered only the repertoire and gene scales. Future research is now needed at the sequence level to identify regions or residues that may be critical for detection by the plant (previously named avirulence) or for its virulence function. The alleles of the effector short-list that we identified will be now monitored in natural *R. solanacearum* populations, to assess their distribution, diversity, and the type of selection they are subjected to, to complete the Clarke’s inventory of conserved effectors (Clarke et al. 2015).

In future research, it will be important also to validate the avirulence function of these candidates by knock-out and gain-of-function experiments (thus following the Falush-Bowden approach [Falush and Bowden 2006]), and using complementary functional screens that will help elucidate the actual mechanism of their recognition by plants (in which organ, at which pathogenesis stage). Once such a validation is completed, future research should also focus on the plant targets of these bacterial proteins. Finally, a large field of research is open for deciphering the interaction networks (synergies, antagonisms) involving type III effectors and the architecture of *Solanaceae* innate immunity.

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