Genome-Wide Identification of a Large Repertoire of *Ralstonia solanacearum* Type III Effector Proteins by a New Functional Screen

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The gram-negative plant-pathogenic bacterium *Ralstonia solanacearum* utilizes the hypersensitive response and pathogenicity (Hrp) type III secretion system (T3SS) to cause disease in plants. To determine the entire repertoire of effector proteins possessed by *R. solanacearum* RS1000, we constructed a transposon carrying a calmodulin-dependent adenylate cyclase reporter that can be used to specifically detect *rip* (*Ralstonia* protein injected into plant cells) genes by monitoring the cAMP level in plant leaves inoculated with insertion mutants. From the new functional screen using this transposon, we identified 38 new Rip proteins translocated into plant cells via the Hrp T3SS. In addition, most of the 34 known effectors of RS1000 could be detected by the screen, except for three effectors that appear to be small in size or only weakly expressed. Finally, we identified 72 Rips in RS1000, which include 68 effector proteins classified into over 50 families and four extracellular components of the Hrp T3SS. Interestingly, one-third of the effectors are specific to *R. solanacearum*. Many effector proteins contain various repeated amino acid sequences or known enzyme motifs. We also show that most of the *R. solanacearum* effector proteins, but not Hrp extracellular components, require an Hrp-associated protein, HpaB, for their effective translocation into plant cells.

Many gram-negative pathogenic bacteria utilize the type III secretion system (T3SS) to interact with their respective hosts (Cornelis and Van Gijsegem 2000; Mota and Cornelis 2005; Galán and Wolf-Watz 2006). In most gram-negative plant-pathogenic bacteria, the virulence-associated T3SS is present and called hypersensitive response and pathogenicity (Hrp) because defects in the T3SS result in the loss of both the ability to induce hypersensitive response (HR) in nonhost plants and pathogenicity in host plants (Alfano and Collmer 1997). The Hrp T3SS acts as a specialized injection system of virulence factors, the so-called effector proteins, into host plant cells. Recent research advances have begun to clarify the role of effector proteins inside host cells; that is, the modification of various plant cellular functions to promote the proliferation of the pathogen (Alfano and Collmer 2004; Mudgett 2005; Jones and Dangl 2006; Gohre and Robatzek 2008).

As a first step toward understanding how bacterial pathogens manipulate plant cellular functions during infection, it is important to determine the complete repertoire of effector proteins possessed by a certain plant pathogen and to identify the function of individual effectors in host plant cells. For this purpose, attempts to catalog type III effector repertoires in plant-pathogenic bacteria are ongoing. To date, four strategies have mainly been used to identify effector genes. The first strategy is the isolation of pathogenicity-related genes belonging to the *hrp* regulon. This is based on the finding that the expressions of many type III effector genes from phytopathogenic bacteria are co-regulated with those of *hrp* genes by common regulatory proteins. Such candidate effector genes were isolated by genetic screening (Noël et al. 2001; Fouts et al. 2002; Mukaihara et al. 2004; Chang et al. 2005), by whole-genome computer search for consensus sequences of upstream promoters (Zwiesler-Vollick et al. 2002; Cunnac et al. 2004) or by whole-genome microarray analyses (Occhialini et al. 2005; Ferreira et al. 2006). True effector genes were selected from these candidates by validating their products for the ability to be translocated into plant cells, mainly using a *Bordetella pertussis* calmodulin-dependent adenylate cyclase (Cya) reporter (Sory and Cornelis 1994; Cunnac et al. 2004; Schechter et al. 2004). By comparing the N-terminal amino acid sequences of effector proteins identified from such studies, several common characteristics of the N-terminal type III secretion signal have been proposed in *Pseudomonas syringae* pv. *tomato* DC3000. This gave rise to the second strategy to screen the candidate effector genes; that is, the identification of genes containing probable type III secretion signals (Petnicki-Ocwieja et al. 2002). The accumulation of information on effector genes in plant- and animal-pathogenic bacteria enabled the development of the third strategy; that is, the isolation of candidate effector genes that show homology to other effector genes. Multiple approaches in combination with the abovementioned strategies enabled the identification of dozens of new effector proteins in genome-sequenced pathogens (Lindeberg et al. 2006; Schechter et al. 2006; Vinatzer et al. 2006; Almeida et al. 2009; Furutani et al. 2009). As the fourth strategy in another line of study, several functional screening systems that can directly identify type III effector genes through an in planta translocation activity of a product have been constructed in *P. syringae* and *Xanthomonas campestris*. In such systems, effector genes were isolated from random insertions of transposons containing avirulence (*avr*) genes lacking the type III secretion signal by monitoring the restoration of Avr-induced HR on plants (Guttman et al. 2002; Roden et al. 2004).

Ralstonia solanacearum is a gram-negative soilborne bacterium that causes bacterial wilt disease in more than 200 plant species, including economically important crops such as tomato,

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potato, tobacco, banana, and eggplant (Hayward 1991). This pathogen enters plant roots, invades xylem vessels, proliferates and spreads throughout the vascular system, and produces a copious amount of extracellular polysaccharides (EPS) that block water transport, resulting in the wilting of infected plants (Schell 2000). As in many other phytopathogenic bacteria, the Hrp T3SS is essential for the growth of *R. solanacearum* in host plants (Boucher et al. 1987). Defects in the Hrp T3SS completely abolish the pathogenicity of the pathogen, whereas mutation in a single effector gene shows little or no effect in most cases (Cunnac et al. 2004; Mukaihara et al. 2004), suggesting that many functionally redundant effector proteins are delivered into plant cells via the Hrp T3SS.

In *R. solanacearum*, expressions of most of the *hrp* genes and the known effector genes are controlled by an AraC-type transcriptional activator, HrpB (Genin et al. 1992). In a previous study, using a genetic screen, we identified 30 *hpx* (*hrpB*-dependent expression) genes and well-known *popA*, *popB*, and *popC* genes as candidate effector genes in *R. solanacearum* RS1000 (Mukaihara et al. 2004). Recently, we have cloned 11 additional candidate effector genes that share homology with known *hpx* genes from RS1000. Using a Cya reporter system, we investigated the translocation of these 44 gene products

into plant cells via the Hrp T3SS, and identified 34 effector genes (Mukaihara and Tamura 2009). However, a large number of potential effector genes are considered to still exist in *R. solanacearum* because our search for *hrpB*-regulated genes is not saturated (Mukaihara et al. 2004) and because a genome-wide microarray analysis identified 143 *hrpB* upregulated genes in *R. solanacearum* GMI1000 (Occhialini et al. 2005). The genome sequence of RS1000 has not yet been analyzed; hence, we could not apply any bioinformatics- or microarray-based prediction of candidate effector genes, as performed in the two genome-sequenced strains GMI1000 (Salanoubat et al. 2002) and UW551 (Gabriel et al. 2006), to this strain. The objective of this study was to construct a new functional screening system that can detect type III effector genes at the genome-wide level even in a genome-unsequenced strain.

In a Cya reporter assay on plants, the translocation of effector proteins into plant cells was clearly detected on the basis of the Cya activity as a high production of cAMP, which increased 100- to 10,000-fold compared with the background level, even though the expression levels of effector proteins were low (Mukaihara and Tamura 2009). This is caused by the very low concentration of cAMP in higher plant tissues compared with animal tissues (Spiteri et al. 1989). Such a high sensitivity of the Cya reporter in plant systems led us to the idea of using a Cya reporter instead of an Avr reporter for the functional screening of effector genes. In this study, we constructed a transposon containing a '*cya*' gene for searching *R. solanacearum* genes containing an in planta translocation signal. Using the new screening system, we identified a large repertoire of type III effector proteins translocated into plant cells via the Hrp T3SS. We also show that most of the *R. solanacearum* effector proteins require an Hrp-associated protein, HpaB, for their effective translocation into plant cells.

RESULTS

A functional screen to isolate *R. solanacearum* genes encoding proteins translocated into plant cells.

Using the plasmid pTNP106, we constructed a library of mini-Tn5 '*cya*' insertions in *R. solanacearum* RS1085, a nalidixic acid-resistant (Nal^r) EPS⁻ derivative of the wild-type strain RS1000. RS1085 is deficient in the production of EPS and, therefore, can form more than 100 single colonies on a selective plate. The transposon mini-Tn5 '*cya*' contains an N-terminal-truncated '*cya*' gene associated with the O-end and the kanamycin-resistant gene (Kan^r) for the selection of insertion mutants (Fig. 1A). When mini-Tn5 '*cya*' is transposed into the genome of RS1085 from pTNP106 and inserted in frame into an open reading frame (ORF) under the control of an active promoter, the insertion mutant can express a Cya fusion protein. Mutants expressing a Cya fusion protein translocated into plant cells were screened by the following plant assay. First, cultures of a set of 96 insertion mutants were combined as a pool, and the mixture was inoculated into a host eggplant leaf. Then, leaf disks were taken from the leaf 15 h after inoculation, and the cAMP level in the sample was measured. In all, 8,064 insertion mutants were assayed as 84 pools, and several pools showed a marked increase in the cAMP level (more than 50 fmol/well) in the inoculated leaves compared with other samples (Fig. 1B). These pools (boxed in red) were isolated as positive pools because they were considered to contain a positive mutant expressing a Cya fusion protein translocated into plant cells. Next, the 96 mutants in a positive pool were divided into several minigroups and the plant assay was repeated until a single positive mutant was specified in the pool. In this study, we assayed approximately 105,000 insertion mutants as 1,092 pools on eggplant leaves and finally isolated 314 positive mutants.

Fig. 1. Calmodulin-dependent adenylate cyclase (Cya)-based functional screen for genes encoding proteins translocated into plant cells. **A**, Structure of mini-Tn5 '*cya*' transposon. The filled boxes indicate inner (I) and outer (O) ends of IS50, and the thick line between them indicates a transposon sequence. The 5'-truncated '*cya*' gene is represented by a filled arrow. Open arrows represent antibiotic-resistant genes. An open circle represents the origin (ori) of pMB1 used for plasmid rescue. The thin line shows the genome of *Ralstonia solanacearum*. A hatched box represents an open reading frame. **B**, Monitoring of the cAMP levels of plant samples inoculated with a pool of 96 insertion mutants. Numbers on the top wells indicate the concentration of cAMP standards (0 to 3,200 fmol/well). B and NSB indicate blank and nonspecific binding, respectively. Wells that show an increased cAMP level (more than 50 fmol/well) are boxed in red.

Identification of *rip* genes.

Each of the genomic DNAs from the 314 positive mutants was purified and the DNA region surrounding the transposon was rescued as described in Materials and Methods. Sequence analysis revealed that 308 of these mutants were independent insertions. These mutants were designated A1 to A308. Of these, 186 transposons were inserted into known *hpx* and *pop* genes for which the translocations of their protein products into plant cells have been demonstrated. To date, we have reported 34 type III effector proteins encoded by the *hpx* and *pop* genes in *R. solanacearum* RS1000 (Mukaihara and Tamura 2009). In this study, 31 of them were detected by the screen (Table 1), indicating that our new functional screening system can efficiently trap type III effector genes. The other 122 transposons were inserted into new loci, and 38 new genes were identified (Table 2). These genes were designated *rip* (*Ralstonia* protein injected into plant cells), associated with the first designation of effector genes in *R. solanacearum* (Cunnac et al. 2004). The above 34 *hpx* and *pop* genes were simultaneously renamed using the designation of *rip* (Table 1). Most of the *rip* genes were trapped by more than two independent transposon insertions (Tables 1 and 2), indicating that our screen for *rip* genes reached a near-saturation level.

hrpB-dependent expression of new *rip* genes.

Sequence analysis revealed that most of the 38 newly identified *rip* genes contained a perfect or imperfect plant-inducible promoter (PIP) box motif (consensus 5'-TTCGB-N₁₅-TTCGB-3'), a recognition sequence by the HrpB transcriptional activator,

on the putative promoter region (Table 3). To examine whether these genes are actually regulated by HrpB, we compared the expression level of the Cya fusion protein between the *hrpB*^c (constitutively expressing *hrpB*) and Δ *hrpB* strains. The Cya activities of 31 *rip* genes, which have a PIP box motif in the putative promoter region, increased 4- to 80-fold in the *hrpB*^c background compared with the Δ *hrpB* background (Table 3). In five *rip* genes (namely, *rip3*, *rip7*, *rip22*, *rip69*, and *rip70*), which have a disrupted or no PIP box motif, the Cya activities were similar in both *hrpB*^c and Δ *hrpB* backgrounds but the expression levels were very low. Two *rip* genes (namely, *rip4* and *rip72*), showed the *hrpB*-dependent expression of the Cya fusion whereas no PIP box motif was found in the upstream region of these genes. These results indicated that the expressions of most of the new *rip* genes were regulated by HrpB.

The new *rip* gene products were translocated into plant cells in an Hrp-T3SS-dependent manner.

To examine whether the new *rip* gene products were translocated into plant cells via the Hrp T3SS, we constructed Δ *hrpY* strains expressing the Cya fusion protein. The *hrpY* gene encodes an Hrp pilin protein, HrpY (Van Gijsegem et al. 2000). In the Δ *hrpY* mutant, *hrpB* is still active but functional Hrp complexes are not produced. The *hrp*⁺ and Δ *hrpY* strains were inoculated into host eggplant leaves, and the cAMP level in the inoculated leaves was measured 12 h after inoculation. Interestingly, in all of the 38 new *rip* genes, the cAMP level did not increase in the Δ *hrpY* background although it increased 100- to 4,600-fold in the *hrp*⁺ background compared with the

Table 1. Detection of 34 known type III effector genes in *Ralstonia solanacearum* RS1000

Gene	mini-Tn5'cya fusion ^a	Protein size (aa)	Insertion site (aa) ^b
<i>hpx2</i> (<i>rip50</i>)	A6*, A83	481	227
<i>hpx4</i> (<i>rip45</i>)	A7, A8, A45, A49, A89, A97, A99, A117, A151, A190, A192*, A196, A226, A261, A286, A289, A301	1,335	281
<i>hpx8</i> (<i>rip68</i>)	A130*, A237	228	53
<i>hpx9</i> (<i>rip66</i>)	A126, A193*	878	350
<i>hpx10</i> (<i>rip56</i>)	A4, A11, A28, A56, A72, A78, A93, A119, A129, A148*, A183, A201, A202, A221, A239, A241, A243, A277, A298	1,156	113
<i>hpx11</i> (<i>rip2</i>)	A173, A250*	455	117
<i>hpx13</i> (<i>rip13</i>)	A62, A86*, A162, A240, A283	621	145
<i>hpx14</i> (<i>rip14</i>)	A10, A85*, A145, A267	647	85
<i>hpx15</i> (<i>rip17</i>)	A209*, A260	462	168
<i>hpx16</i> (<i>rip18</i>)	Not detected	538	...
<i>hpx17</i> (<i>rip19</i>)	A58, A276*	720	607
<i>hpx18</i> (<i>rip21</i>)	A214*	306	204
<i>hpx20</i> (<i>rip37</i>)	A16, A27, A37, A44, A55*, A115, A137, A271	1,035	192
<i>hpx21</i> (<i>rip55</i>)	A91*, A144, A197, A207, A227, A278	411	87
<i>hpx23</i> (<i>rip63</i>)	A77, A292*	518	188
<i>hpx24</i> (<i>rip64</i>)	A59*, A293	1,702	1,518
<i>hpx25</i> (<i>rip34</i>)	A36*, A131, A188, A254, A264, A265, A281, A295	641	43
<i>hpx26</i> (<i>rip58</i>)	A138*, A255	474	162
<i>hpx27</i> (<i>rip39</i>)	A165*, A172, A218, A231	830	98
<i>hpx28</i> (<i>rip23</i>)	A19*, A153, A156, A175, A242	809	685
<i>hpx29</i> (<i>rip12</i>)	A48, A90, A163, A206*, A225	514	242
<i>hpx30</i> (<i>rip20</i>)	A17, A43, A71, A98*, A102, A133, A135, A154, A208, A217, A236, A245, A248, A304	2,497	239
<i>hpx31</i> (<i>rip29</i>)	A32, A51, A73, A118, A120, A182, A186, A194, A238*, A270	1,127	105
<i>hpx32</i> (<i>rip44</i>)	A140*, A251	1,238	542
<i>hpx33</i> (<i>rip43</i>)	A23, A81, A101, A109, A110, A149, A152, A155*, A203, A259	1,375	144
<i>hpx34</i> (<i>rip33</i>)	A31, A57, A124, A204*, A220	2,339	443
<i>hpx35</i> (<i>rip54</i>)	A30, A50, A79*, A112, A249, A262	2,208	1,168
<i>hpx36</i> (<i>rip65</i>)	A96, A123, A146, A167, A198, A288*, A300	2,504	743
<i>hpx37</i> (<i>rip27</i>)	A13, A34, A41, A61, A65, A74, A82, A84, A113, A116*, A127, A132, A147, A158, A168, A170, A174, A181, A199, A212, A223, A244, A246, A299, A307	2,353	331
<i>popA</i> (<i>rip49</i>)	A274*	330	282
<i>popB</i> (<i>rip48</i>)	Not detected	174	...
<i>popC</i> (<i>rip47</i>)	A53*, A215, A272, A287	984	198
<i>gala1</i> (<i>rip53</i>)	Not detected	493	...
<i>gala3</i> (<i>rip28</i>)	A63*, A296	607	450

^a Smallest fusion in the multiple isolates is indicated by an asterisk.

^b Number of amino acids from start codon to insertion site of transposon in smallest fusion.

background level (Table 4). This result clearly indicates that all 38 *rip* gene products were translocated into plant cells via the Hrp T3SS. Adding the 38 Rips to the 34 previously identified Rips (Mukaihara and Tamura 2009), strain RS1000 was found to possess at least 72 Rips as Hrp T3SS substrate proteins (Table 5). It has been shown that the type III secretion signal is located within the first 50 to 100 N-terminal amino acids of the effector protein. All of the Tn insertion mutants isolated in this study showed a fusion of the Cya reporter after the first 41 N-terminal amino acids of the protein (Tables 1 and 2).

Sequence features of new Rip proteins.

The sequences of the 38 newly identified Rip proteins were compared with published sequences on databases (Table 5). All of the known *R. solanacearum* Hrp-secreted type III effector proteins that have been reported to date but have not yet been identified from RS1000—namely, AvrA (Meyer et al. 2006), PopP1 (Lavie et al. 2002), PopP2 (Deslandes et al. 2003), and RipT (Cunnac et al. 2004)—were detected using the screen. This result also supports the abovementioned conclusion that the identification of RS1000 *rip* genes is nearly saturated in this screen. Eight of the new Rips (namely, Rip24, Rip26, Rip30, Rip32, Rip35, Rip36, Rip40, and Rip62) were found to share homology with known type III effector proteins from other plant-pathogenic bacteria, designated Hrp outer protein (Hop) from *P. syringae* and *Xanthomonas* outer protein

(Xop) from *Xanthomonas* spp. Interestingly, several Rips contained eukaryotic protein motifs, such as ankyrin repeats (Rip1, Rip3, and Rip60), Ser/Thr acetyltransferase (Rip4, Rip7, Rip8, and Rip9), Ser/Thr kinase (Rip60), and pentatricopeptide (PPR) repeats (Rip31). Rip38 shared homology with class III lipase. Four Rips (namely, Rip24, Rip46, Rip52, and Rip70) were considered to be extracellular components of the Hrp secretion apparatus because they are homologues of the HrpW harpin from *P. syringae* and *Erwinia amylovora* (Charkowski et al. 1998; Kim and Beer 1998), the Hrp pilin protein HrpY (Van Gijsegem et al. 2000), and the translocon proteins PopF1 and PopF2 (Meyer et al. 2006), respectively.

Most *R. solanacearum* type III effector proteins required Hrp-associated protein HpaB for effective translocation into plant cells.

In a previous study, we identified an *hrpB*-regulated gene, *hpaB*, in the *hrp* cluster (Mukaihara et al. 2004). The *hpaB*-defective mutants of *R. solanacearum* showed a typical Hrp-deficient phenotype, the loss of HR induction and pathogenicity in plants, although Hrp pili were still produced on the cell surface of the mutant. Therefore, we considered that defects in *hpaB* may affect the translocation of effector proteins into plant cells via the Hrp T3SS. To verify this hypothesis by in planta Cya activity assay, we examined the translocation of the 72 abovementioned Rip proteins into plant cells in the $\Delta hpaB$

Table 2. Thirty-eight newly isolated *rip* (*Ralstonia* protein injected into plant cells) genes in this study

Gene	DDBJ accession no. ^a	mini-Tn5'cya fusion ^b	Protein size (aa)	Insertion site (aa) ^c
<i>rip1</i>	AB516324	A200*, A269	430	118
<i>rip3</i>	AB516325	A40*	162	67
<i>rip4</i>	AB516326	A38, A134, A166*, A169, A177, A294	311	169
<i>rip5</i> (<i>avrA</i>)	AB302266	A150*	267	219
<i>rip6</i>	AB516327	A256*	98	67
<i>rip7</i> (<i>popP1</i>)	AB302267	A69*, A143	368	215
<i>rip8</i> (<i>popP2-1</i>)	AB302268	A20, A39*, A105, A176, A228, A282	488	55
<i>rip9</i> (<i>popP2-2</i>)	AB516328	A191*	240	86
<i>rip10</i>	AB516329	A1*	157	132
<i>rip11</i>	AB516330	A2*, A5	96	49
<i>rip15</i>	AB516331	A24, A26, A29, A35, A52, A68, A76*, A103, A161, A235, A308	765	53
<i>rip16</i>	AB516332	A70, A114*, A159, A224, A285	584	89
<i>rip22</i>	AB516348	A189*	188	175
<i>rip24</i>	AB302275	A279*	380	372
<i>rip25</i> (<i>ripT</i>)	AB302269	A75*, A266	297	41
<i>rip26</i>	AB302270	A9, A14, A15, A21*, A66, A80, A88, A95, A108, A210, A229, A273	425	99
<i>rip30</i>	AB516333	A64*, A252	719	530
<i>rip31</i>	AB516334	A33, A222*, A302	1,336	632
<i>rip32</i>	AB516335	A106, A141*, A219, A290	742	50
<i>rip35</i>	AB302272	A128, A284*	451	372
<i>rip36</i>	AB302273	A216*, A305	218	165
<i>rip38</i>	AB516336	A42*, A100, A185, A232	449	102
<i>rip40</i>	AB302271	A12, A22*, A25, A87, A136, A213, A253	292	59
<i>rip41</i>	AB516337	A160, A233*, A263	203	79
<i>rip42</i>	AB516337	A184*	189	168
<i>rip46</i> (<i>hpaZ</i>)	AB167955	A47*, A230	75	49
<i>rip51</i>	AB516338	A46, A111, A179*, A180, A291	659	281
<i>rip52</i> (<i>popF2</i>)	AB516339	A60, A122, A187*, A247	740	203
<i>rip57</i>	AB516340	A94, A121, A164*, A205, A275	1,364	102
<i>rip59</i>	AB516341	A107, A268*	292	180
<i>rip60</i>	AB516342	A18*, A54, A125, A142, A171, A178, A258	763	119
<i>rip61</i>	AB516343	A104*, A211, A257	605	100
<i>rip62</i>	AB302274	A67, A157*	822	419
<i>rip67</i>	AB516349	A195*, A306	197	121
<i>rip69</i>	AB516344	A280, A303*	448	323
<i>rip70</i> (<i>popF1</i>)	AB516345	A234*	726	702
<i>rip71</i>	AB516346	A139*, A297	255	163
<i>rip72</i>	AB516347	A3*, A92	325	92

^a DDBJ = DNA Data Bank of Japan.

^b Smallest fusion in the multiple isolates is indicated by an asterisk.

^c Number of amino acids from start codon to insertion site of transposon in smallest fusion.

background. In 66 Rip proteins, the cAMP level in inoculated leaves markedly decreased in the $\Delta hpaB$ mutants (Table 4). In contrast, in the remaining six Rips—namely, Rip24, Rip46 (HpaZ), Rip48 (PopB), Rip49 (PopA), Rip52 (PopF2), and Rip70 (PopF1)—cAMP level even increased three to 23-fold in the $\Delta hpaB$ mutants (Table 4). This result indicates that most of the *R. solanacearum* effector proteins require HpaB for their effective translocation into plant cells via the Hrp T3SS.

Comparison of repertoire of Rip proteins among *R. solanacearum* strains.

We compared amino acid sequences of the 72 Rip proteins from RS1000 (race 1, biovar, 4 phylotype I) with those from two genome-sequenced strains GMI1000 (race 1, biovar 3, phylotype I) and UW551 (race 3, biovar 2, phylotype II). Most of the Rip proteins from RS1000 showed more than 95% identity to those from GMI1000 but much lower similarities to those from UW551 (Table 5). The surrounding genes of most of the *rip* genes showed the same gene order as those of GMI1000 within the sequenced range (data not shown). These results indicate that the two phylotype I strains RS1000 and GMI1000 are closely related at the DNA level. Despite the high similarity to

GMI1000, several effector genes of RS1000 showed mutations, such as hyperaccumulation of point mutation (*rip13*), frame shift (*rip4*, *rip9*, and *rip71*), deletion (*rip3*, *rip5*, *rip19*, and *rip53*), and insertion of an insertion element (IS) sequence (*rip12*, *rip22*, *rip60*, and *rip67*). On the other hand, *rip60* in GMI1000 was disrupted by IS insertion. Genes *rip38* and *rip59* were not found in the genome of GMI1000 but existed in that of UW551. Gene *rip38* was located between RSp0720 and RSp0721 on the genome of RS1000, and the gene order is conserved in UW551 (data not shown). This may indicate that *rip38* is deleted in the genome of GMI1000. Gene *rip59* was replaced with another gene, RSp1212, in the genome of GMI1000 (data not shown). Interestingly, RSp1212 was an *hrpB*-regulated gene containing a PIP-box promoter and predicted to encode a type III effector protein in GMI1000 (Occhialini et al. 2005). Gene *rip10* was not found in the genome of GMI1000 or UW551 and was considered to be specific in RS1000.

DISCUSSION

In this study, using a Cya reporter instead of an Avr reporter, we constructed a new functional screening system to isolate

Table 3. In vitro adenylate cyclase activity of cell lysates from *Ralstonia solanacearum* *hrpB*^c and $\Delta hrpB$ strains containing 'cya fusion in new *rip* (*Ralstonia* protein injected into plant cells) genes

Gene	Probable PIP box and -10 sequence ^a		'cya fusion	cAMP level ^b			Expressed ^c
	Position	Nucleotide sequence		<i>hrpB</i> ^c	$\Delta hrpB$	<i>hrpB</i> ^c / $\Delta hrpB$	
<i>rip1</i>	-100	TTCGT -N16- TTCGC -N30-GAGTCT	A269	10.5 ± 3.94	0.12 ± 0.04	87.5	Yes
<i>rip3</i>	...	Not found	A40	0.08 ± 0.01	0.07 ± 0.02	1.1	No
<i>rip4</i>	...	Not found	A134	1.90 ± 0.46	0.08 ± 0.04	23.8	Yes
<i>rip5</i>	-97	TTCGC -N15- TTCGC -N31-AATACT	A150	26.6 ± 3.92	0.09 ± 0.01	296	Yes
<i>rip6</i>	-557	TTCGG -N15- TTCGG -N32-TATTTT	A256	0.32 ± 0.04	0.06 ± 0.01	5.3	Yes
<i>rip7</i>	...	IS insertion in the PIP box motif	A69	0.18 ± 0.02	0.14 ± 0.01	1.3	No
<i>rip8</i>	-152	TTCGG -N15- TTCGC -N31-TAAGCT	A176	120 ± 23.9	6.45 ± 0.78	18.6	Yes
<i>rip9</i>	-302	TTCGG -N15- TTCGC -N31-TATTGT	A191	27.5 ± 5.02	0.20 ± 0.05	138	Yes
<i>rip10</i>	-238	TTCGC -N15- TTCGC -N31-TAAGCT	A1	60.4 ± 12.2	0.22 ± 0.04	275	Yes
<i>rip11</i>	-87	TTCGC -N15- TTCGC -N30-AACAAT	A2	78.6 ± 6.83	0.18 ± 0.05	437	Yes
<i>rip15</i>	-182	TACGC -N15- TTCGC -N31-CACAGT	A24	0.84 ± 0.08	0.07 ± 0.02	12.0	Yes
<i>rip16</i>	-597	TTCGC -N15- TTCGC -N31-TATGGT	A224	38.4 ± 6.72	0.10 ± 0.02	384	Yes
<i>rip22</i>	...	Not found	A189	0.25 ± 0.12	0.15 ± 0.05	1.7	No
<i>rip24</i>	-123	TTCGT -N15- TGCGG -N32-TACAAC	A279	750 ± 139	4.37 ± 0.76	172	Yes
<i>rip25</i>	-161	TTCGT -N15- TTCGG -N32-TACGCT	A75	8.17 ± 1.61	0.11 ± 0.02	74.3	Yes
<i>rip26</i>	-127	TTCGG -N15- TTCGC -N31-TATTGT	A15	131 ± 21.9	2.24 ± 0.44	58.5	Yes
<i>rip30</i>	-173	TTCGC -N15- TTCGC -N31-CATAGT	A64	4.64 ± 1.76	0.13 ± 0.03	35.7	Yes
<i>rip31</i>	-177	ATCGC -N15- TTCGC -N31-TATAAA	A33	2.39 ± 1.09	0.20 ± 0.08	12.0	Yes
<i>rip32</i>	-239	TTCGC -N15- TTCGC -N32-CATACT	A106	0.92 ± 0.07	0.11 ± 0.03	8.4	Yes
<i>rip35</i>	-251	TTCGC -N15- TTCGC -N31-TATCGT	A128	63.6 ± 14.6	0.81 ± 0.05	78.5	Yes
<i>rip36</i>	-126	TTCGC -N15- TTCGC -N31-TAGCAT	A216	37.9 ± 7.60	0.21 ± 0.02	180	Yes
<i>rip38</i>	-142	TTCGC -N15- TTCGT -N32-TAGATT	A185	2.94 ± 1.15	0.18 ± 0.05	16.3	Yes
<i>rip40</i>	-305	TTCGC -N15- TTCGC -N34-CAATCT	A25	3.75 ± 0.68	0.04 ± 0.01	93.8	Yes
<i>rip41</i>	-1,126	TTCGG -N15- TTCGC -N31-TAGGAT	A160	0.70 ± 0.04	0.09 ± 0.03	7.8	Yes
<i>rip42</i>	-92	TTCGG -N15- TTCGC -N31-TAGGAT	A184	84.8 ± 31.5	0.28 ± 0.06	303	Yes
<i>rip46</i>	-627	TTCGT -N15- TTCGG -N32-CATCAT	A230	568 ± 135	0.23 ± 0.04	247	Yes
<i>rip51</i>	-104	TTCGC -N15- TTCGC -N32-CATGCT	A111	10.3 ± 5.06	0.14 ± 0.06	73.6	Yes
<i>rip52</i>	-114	TTCGC -N15- TTCGC -N32-TAGCCT	A122	6.85 ± 2.25	0.28 ± 0.10	24.5	Yes
<i>rip57</i>	-94	TTCGC -N15- TTCGC -N32-TAGCCT	A94	2.26 ± 1.00	0.16 ± 0.04	14.1	Yes
<i>rip59</i>	-135	TTCGT -N15- TTCGG -N32-TACGGT	A268	1.65 ± 0.48	0.14 ± 0.05	11.8	Yes
<i>rip60</i>	-178	TTCGG -N15- TTCGG -N31-TACCGT	A178	1.01 ± 0.24	0.10 ± 0.04	10.1	Yes
<i>rip61</i>	-93	CTCGC -N15- TTCGC -N32-TAAAAA	A211	2.32 ± 1.22	0.17 ± 0.03	13.6	Yes
<i>rip62</i>	-540	TTCGC -N15- TACGG -N31-TATCAT	A67	2.28 ± 0.36	0.17 ± 0.01	13.4	Yes
<i>rip67</i>	-117	TTCGG -N15- TACGC -N31-TATATT	A195	24.3 ± 6.08	0.16 ± 0.02	152	Yes
<i>rip69</i>	...	Not found	A280	0.35 ± 0.05	0.18 ± 0.02	1.9	No
<i>rip70</i>	...	Not found	A234	0.06 ± 0.02	0.18 ± 0.04	0.4	No
<i>rip71</i>	-98	TTCGC -N15- TACCG -N33-CAAGGA	A139	0.43 ± 0.07	0.11 ± 0.02	3.9	Yes?
<i>rip72</i>	...	Not found	A92	1.66 ± 0.38	0.12 ± 0.04	13.8	Yes

^a Position indicates position of the first nucleotide (T) of the first TTCGB motif of a plant-inducible promoter (PIP) box (consensus 5'-TTCGB-N15-TTCGB-3') relative to the start codon of an open reading frame. Conserved nucleotides of a PIP box are shown in bold. N means the number of spacer nucleotides between the first and second TTCGB motifs of a PIP box or between the second motif of a PIP box and a -10 sequence (consensus 5'-YANNRT-3').

^b Adenylate cyclase activity (nmol cAMP per milligram of total soluble protein) is shown as the mean ± standard deviation of values from three independent experiments.

^c *hrpB*-dependent expression.

type III effector genes in the *R. solanacearum* genome-unsequenced strain RS1000. We screened approximately 105,000 Tn insertion mutants and identified 69 *rip* genes encoding proteins translocated into plant cells. To date, two Avr-reporter-based functional screening systems for effector genes have been reported. Thirteen effector genes were identified from 75,000 fusions of the AvrRpt2⁸¹⁻²⁵⁵ reporter in *P. syringae* pv. *maculicola* (Guttman et al. 2002). Similarly, seven effector genes were identified from 14,000 fusions of the AvrBs2⁶²⁻⁵⁷⁴ reporter in *X. campestris* pv. *vesicatoria* (Roden et al. 2004). In our Cya-reporter-based screen, 314 positive mutants were isolated by monitoring the increased production of cAMP in inoculated leaves; thus, the frequency of detection of positive mutants per total number of mutants was estimated to be 1/330. In the abovementioned two Avr-reporter-based screens, the frequencies of detecting HR-positive mutants per total number of mutants were 1:3,000 and 1:2,000 (Guttman et al. 2002; Roden et al. 2004). Although the genome size and the number of effector genes would vary among the pathogens, the efficiency of isolating positive mutants using the Cya-based screen is six to nine times higher than those using the Avr-based screens. The high sensitivity of the Cya reporter makes our system a high-throughput system because a pool of 96 insertion mutants could be tested for their translocation activity in

one assay in the Cya-based system whereas a pool of only eight insertion mutants could be tested in Avr-based systems.

Most of the *rip* genes detected by the screen had more than two independent transposon insertions (Tables 1 and 2). This indicates that our screen for *rip* genes in RS1000 reached a near-saturation level. In a previous work, we identified 34 effector genes in RS1000 (Mukaihara and Tamura 2009). In this study, three genes—namely, *hpx16* (*rip18*), *popB* (*rip48*), and *galal* (*rip53*)—could not be detected using the screen (Table 1). The ORF of *popB* is very small and the expression level of *galal* is very low (Mukaihara and Tamura 2009). Therefore, it is possible that we have missed some effector genes that are small in size or expressing at very low levels. Nevertheless, we identified 72 Rip proteins in RS1000, and 68 of these are considered to be type III effector proteins, because 4 Rips are probably extracellular components of the Hrp type III secretion apparatus (Table 5). Although genome-based prediction led to the identification of 28 confirmed and 46 candidate effector proteins in *R. solanacearum* GMI1000 (Poueymiro and Genin 2009), in this study, we provide the first experimental validation of a near-complete effector repertoire of this pathogen. As far as we know, *R. solanacearum* possesses the largest type III effector repertoire among bacterial pathogens whose repertoire has

Table 4. cAMP levels in plant leaves inoculated with *Ralstonia solanacearum* *hrp*⁺, *ΔhrpY*, and *ΔhpaB* strains expressing the calmodulin-dependent adenylate cyclase (Cya) fusion protein in the wild-type *hrpB* background

Gene ^b	'cya fusion	cAMP level (fold increase) ^a			Translocation ^c	HpaB requirement ^d
		<i>hrp</i> ⁺	<i>ΔhrpY</i>	<i>ΔhpaB</i>		
None	—	0.02 ± 0.004 (1.0)	0.02 ± 0.004 (1.0)	0.02 ± 0.004 (1.0)	—	—
<i>rip1</i>	A269	4.07 ± 0.45 (204)	0.03 ± 0.002 (1.5)	0.03 ± 0.002 (1.5)	Yes	Yes
<i>rip2</i> (<i>hpx11</i>)	<i>hpx11</i> '-'cya	83.0 ± 14.9 (4,150)	0.06 ± 0.01 (3.0)	0.05 ± 0.01 (2.5)	Yes	Yes
<i>rip3</i>	A40	71.7 ± 5.80 (3,585)	0.02 ± 0.002 (1.0)	0.57 ± 0.06 (28.5)	Yes	Yes
<i>rip4</i>	A134	16.5 ± 0.63 (825)	0.02 ± 0.002 (1.0)	0.08 ± 0.02 (4.0)	Yes	Yes
<i>rip5</i> (<i>avrA</i>)	A150	92.8 ± 11.0 (4,640)	0.05 ± 0.01 (2.5)	0.06 ± 0.01 (3.0)	Yes	Yes
<i>rip6</i>	A256	3.20 ± 0.18 (160)	0.02 ± 0.002 (2.0)	0.02 ± 0.004 (1.0)	Yes	Yes
<i>rip7</i> (<i>popP1</i>)	A69	8.71 ± 1.37 (436)	0.04 ± 0.01 (2.0)	0.05 ± 0.01 (2.5)	Yes	Yes
<i>rip8</i> (<i>popP2-1</i>)	A176	86.5 ± 16.1 (4,325)	0.05 ± 0.01 (2.5)	1.92 ± 0.37 (96.0)	Yes	Yes
<i>rip9</i> (<i>popP2-2</i>)	A191	3.25 ± 0.11 (163)	0.001 ± 0.001 (0.5)	0.04 ± 0.006 (2.0)	Yes	Yes
<i>rip10</i>	A1	83.5 ± 12.5 (4,175)	0.02 ± 0.001 (1.0)	3.41 ± 0.51 (170)	Yes	Yes
<i>rip11</i>	A2	65.2 ± 2.19 (3,260)	0.04 ± 0.01 (2.0)	1.09 ± 0.27 (54.5)	Yes	Yes
<i>rip12</i> (<i>hpx29</i>)	<i>hpx29</i> '-'cya	98.1 ± 8.37 (4,905)	0.02 ± 0.004 (1.0)	0.02 ± 0.01 (1.0)	Yes	Yes
<i>rip13</i> (<i>hpx13</i>)	<i>hpx13</i> '-'cya	54.0 ± 7.43 (2,700)	0.03 ± 0.01 (1.5)	0.04 ± 0.01 (2.0)	Yes	Yes
<i>rip14</i> (<i>hpx14</i>)	<i>hpx14</i> '-'cya	187 ± 15.1 (9,350)	0.02 ± 0.002 (1.0)	0.55 ± 0.16 (27.5)	Yes	Yes
<i>rip15</i>	A24	64.3 ± 6.20 (3,215)	0.02 ± 0.002 (1.0)	0.16 ± 0.03 (8.0)	Yes	Yes
<i>rip16</i>	A224	78.7 ± 13.1 (3,935)	0.03 ± 0.002 (1.5)	1.35 ± 0.25 (67.5)	Yes	Yes
<i>rip17</i> (<i>hpx15</i>)	<i>hpx15</i> '-'cya	27.5 ± 5.44 (1,375)	0.02 ± 0.002 (1.0)	0.04 ± 0.01 (2.0)	Yes	Yes
<i>rip18</i> (<i>hpx16</i>)	<i>hpx16</i> '-'cya	4.53 ± 0.76 (227)	0.03 ± 0.003 (1.5)	0.03 ± 0.01 (1.5)	Yes	Yes
<i>rip19</i> (<i>hpx17</i>)	<i>hpx17</i> '-'cya	11.3 ± 3.61 (565)	0.07 ± 0.02 (3.5)	0.05 ± 0.01 (2.5)	Yes	Yes
<i>rip20</i> (<i>hpx30</i>)	<i>hpx30</i> '-'cya	40.7 ± 5.72 (2,035)	0.01 ± 0.002 (0.5)	0.11 ± 0.02 (5.5)	Yes	Yes
<i>rip21</i> (<i>hpx18</i>)	<i>hpx18</i> '-'cya	75.7 ± 18.2 (3,785)	0.03 ± 0.01 (1.5)	0.18 ± 0.04 (9.0)	Yes	Yes
<i>rip22</i>	A189	1.68 ± 0.14 (84.0)	0.02 ± 0.003 (1.0)	0.05 ± 0.01 (1.0)	Yes	Yes
<i>rip23</i> (<i>hpx28</i>)	<i>hpx28</i> '-'cya	71.5 ± 4.90 (3,575)	0.02 ± 0.003 (1.0)	0.05 ± 0.02 (2.5)	Yes	Yes
<i>rip24</i>	A279	3.93 ± 0.61 (197)	0.04 ± 0.01 (2.0)	21.8 ± 5.57 (1090)	Yes	No
<i>rip25</i> (<i>ripT</i>)	A75	88.3 ± 12.6 (4,415)	0.04 ± 0.01 (2.0)	0.21 ± 0.02 (10.5)	Yes	Yes
<i>rip26</i>	A15	81.2 ± 14.2 (4,060)	0.04 ± 0.01 (2.0)	0.14 ± 0.03 (7.0)	Yes	Yes
<i>rip27</i> (<i>hpx37</i>)	<i>hpx37</i> '-'cya	3.27 ± 1.20 (164)	0.05 ± 0.01 (2.5)	0.04 ± 0.02 (2.0)	Yes	Yes
<i>rip28</i> (<i>galal</i>)	<i>galal</i> '-'cya	71.5 ± 8.70 (3,575)	0.03 ± 0.002 (1.5)	0.05 ± 0.01 (2.5)	Yes	Yes
<i>rip29</i> (<i>hpx31</i>)	<i>hpx31</i> '-'cya	24.5 ± 10.2 (1,225)	0.03 ± 0.002 (1.5)	0.03 ± 0.01 (1.5)	Yes	Yes
<i>rip30</i>	A64	49.4 ± 4.95 (2,470)	0.02 ± 0.002 (1.0)	0.55 ± 0.03 (27.5)	Yes	Yes
<i>rip31</i>	A33	52.1 ± 2.71 (2,605)	0.02 ± 0.003 (1.0)	0.20 ± 0.04 (10.0)	Yes	Yes
<i>rip32</i>	A106	12.8 ± 1.91 (640)	0.02 ± 0.001 (1.0)	0.03 ± 0.006 (1.5)	Yes	Yes

(continued on next page)

^a Adenylate cyclase activity (nmol cAMP per milligram of total soluble protein) was measured and is shown as the mean ± standard deviation of values from three independent experiments. Fold increase indicates the ratio of activity of a strain expressing the Cya fusion protein to that of the parental strain that does not express Cya.

^b cAMP levels in the *hrp*⁺ and *ΔhrpY* backgrounds of 34 known *rip* (*Ralstonia* protein injected into plant cells) genes were previously reported (Mukaihara and Tamura 2009).

^c Hrp-dependent translocation into plant cells.

^d HpaB requirement in translocation.

been analyzed at the genome-wide level. The large repertoire of type III effectors may account for the unusually broad host range that covers more than 200 plant species over several botanical families of *R. solanacearum*.

We previously reported three effector families composed of more than four members: namely, the Hpx4, Hpx30, and GALA families in RS1000 (Mukaihara and Tamura 2009). In the present study, we identified five additional effector families with amplified gene number: namely, the Hpx8 (Rip68 and Rip72), Rip6 (Rip6 and Rip11), Rip61 (Rip61 and Rip69), XopP (Rip30 and Rip32), and YopJ/AvrRxv (Rip7, Rip8, and Rip9) families (Table 5). Twenty-five effectors belong to the abovementioned eight effector families, and the currently validated 68 *R. solanacearum* effectors are found to fall into over 50 families at the protein level.

Among 68 *R. solanacearum* effectors, homologues of 45 effectors can be found in other plant- and animal-pathogenic bacteria, although their translocation activities into host cells and their roles in pathogenicity remain unclear (Table 5). This, in turn, indicates that one-third of all the effectors are specific to *R. solanacearum* strains. Eighteen effectors are homologues of type III effector proteins from other plant and animal pathogens, such as AvrBs3, HopD1, HopF2, HopG1, HopH1, HopQ1, HopR1, HopX1, HopAA1-1, HopAV1, IpaH, XopC, XopP, YopJ/AvrRxv, and YopT/HopC1 (Table 5). Some of the abovementioned effectors have been reported to suppress host defense responses or to stimulate host cellular processes dur-

ing infection for the benefit of bacterial proliferation in respective hosts (Shao et al. 2002; Jamir et al. 2004; Kay et al. 2007; Li et al. 2005; Mukherjee et al. 2006; Rohde et al. 2007; Munkvold et al. 2008; Cunnac et al. 2009; Kvitko et al. 2009). It should be examined in a future work whether *R. solanacearum* effectors would have similar activities in plant cells.

It is noteworthy that one-third of the RS1000 effector proteins contain various repeated amino acid sequences (ankyrin repeats, AvrBs3 repeats, leucine-rich repeats, pentatricopeptide repeats, and SKWP repeats) or known enzyme motifs (class III lipase, cysteine proteinase, E3 ubiquitin ligase, nucleoside hydrolase, Nudix hydrolase, Ser/Thr acetyltransferase, and Ser/Thr kinase) (Table 5). It is possible that the targets of these effectors are proteins, lipids, nucleoside derivatives, and DNA or RNA molecules in host plant cells. None of the host factors targeted by *R. solanacearum* effectors have been clarified to date, except PopP2. It has recently been shown that PopP2 physically interacts with *Arabidopsis* cysteine proteinase RD19, an essential host factor for PopP2-triggered RRS1-R-mediated resistance, although the role of RD19 in plant defense responses remains unclear (Bernoux et al. 2008). In addition to the analysis of effector activities in plant cells, the identification of target plant factors would clarify the molecular mechanisms by which effector proteins promote the growth of *R. solanacearum* in host plants.

Among 72 *rip* genes of RS1000, 27 (37%) are located on the chromosome and 45 (63%) are on the megaplasmid, indi-

Table 4. (continued from preceding page)

Gene ^b	'cya fusion	cAMP level (fold increase) ^a			Translocation ^c	HpaB requirement ^d
		<i>hrp</i> ⁺	Δ <i>hrpY</i>	Δ <i>hpaB</i>		
<i>rip33</i> (<i>hpx34</i>)	<i>hpx34</i> '-'cya	13.7 ± 2.67 (685)	0.04 ± 0.01 (2.0)	0.04 ± 0.01 (2.0)	Yes	Yes
<i>rip34</i> (<i>hpx25</i>)	<i>hpx25</i> '-'cya	144 ± 21.3 (7,200)	0.04 ± 0.01 (2.0)	0.18 ± 0.07 (9.0)	Yes	Yes
<i>rip35</i>	A128	49.9 ± 4.00 (2,495)	0.04 ± 0.01 (2.0)	0.03 ± 0.004 (1.5)	Yes	Yes
<i>rip36</i>	A216	85.9 ± 9.14 (4,295)	0.05 ± 0.01 (2.5)	0.44 ± 0.10 (22.0)	Yes	Yes
<i>rip37</i> (<i>hpx20</i>)	<i>hpx20</i> '-'cya	89.5 ± 8.32 (4,475)	0.02 ± 0.002 (1.0)	0.04 ± 0.01 (2.0)	Yes	Yes
<i>rip38</i>	A185	13.3 ± 2.67 (665)	0.01 ± 0.001 (0.5)	0.03 ± 0.008 (1.5)	Yes	Yes
<i>rip39</i> (<i>hpx27</i>)	<i>hpx27</i> '-'cya	75.2 ± 5.54 (3,760)	0.02 ± 0.002 (1.0)	0.63 ± 0.16 (31.5)	Yes	Yes
<i>rip40</i>	A25	87.4 ± 7.61 (4,370)	0.04 ± 0.01 (2.0)	0.90 ± 0.19 (45.0)	Yes	Yes
<i>rip41</i>	A160	39.1 ± 7.30 (1,955)	0.02 ± 0.002 (1.0)	0.30 ± 0.02 (15.0)	Yes	Yes
<i>rip42</i>	A184	73.5 ± 4.38 (3,675)	0.01 ± 0.001 (0.5)	0.37 ± 0.06 (18.5)	Yes	Yes
<i>rip43</i> (<i>hpx33</i>)	<i>hpx33</i> '-'cya	21.9 ± 3.79 (1,095)	0.05 ± 0.02 (2.5)	0.07 ± 0.01 (3.5)	Yes	Yes
<i>rip44</i> (<i>hpx32</i>)	<i>hpx32</i> '-'cya	13.6 ± 2.30 (680)	0.04 ± 0.01 (2.0)	0.04 ± 0.01 (2.0)	Yes	Yes
<i>rip45</i> (<i>hpx4</i>)	<i>hpx4</i> '-'cya	25.7 ± 2.98 (1,285)	0.01 ± 0.002 (0.5)	0.04 ± 0.01 (2.0)	Yes	Yes
<i>rip46</i> (<i>hpaZ</i>)	<i>hpaZ</i> '-'cya	15.0 ± 1.70 (750)	0.04 ± 0.01 (2.0)	45.5 ± 4.31 (2275)	Yes	No
<i>rip47</i> (<i>popC</i>)	<i>popC</i> '-'cya	122 ± 13.7 (6,100)	0.06 ± 0.01 (3.0)	0.03 ± 0.002 (1.5)	Yes	Yes
<i>rip48</i> (<i>popB</i>)	<i>popB</i> '-'cya	46.9 ± 7.08 (2,345)	0.16 ± 0.04 (8.0)	207 ± 17.0 (10,350)	Yes	No
<i>rip49</i> (<i>popA</i>)	<i>popA</i> '-'cya	49.4 ± 5.20 (2,470)	0.25 ± 0.03 (12.5)	275 ± 32.5 (13,750)	Yes	No
<i>rip50</i> (<i>hpx2</i>)	<i>hpx2</i> '-'cya	75.3 ± 3.81 (3,765)	0.02 ± 0.002 (1.0)	0.04 ± 0.01 (2.0)	Yes	Yes
<i>rip51</i>	A111	68.3 ± 16.7 (3,415)	0.02 ± 0.002 (1.0)	0.42 ± 0.06 (21.0)	Yes	Yes
<i>rip52</i> (<i>popF2</i>)	A122	2.10 ± 0.30 (105)	0.02 ± 0.002 (1.0)	48.8 ± 5.40 (2,440)	Yes	No
<i>rip53</i> (<i>gala1</i>)	<i>gala1</i> '-'cya	4.23 ± 1.50 (212)	0.02 ± 0.01 (1.0)	0.01 ± 0.005 (0.5)	Yes	Yes
<i>rip54</i> (<i>hpx35</i>)	<i>hpx35</i> '-'cya	16.2 ± 1.42 (810)	0.03 ± 0.01 (1.5)	0.03 ± 0.01 (1.5)	Yes	Yes
<i>rip55</i> (<i>hpx21</i>)	<i>hpx21</i> '-'cya	14.6 ± 2.78 (730)	0.02 ± 0.01 (1.0)	0.02 ± 0.01 (1.0)	Yes	Yes
<i>rip56</i> (<i>hpx10</i>)	<i>hpx10</i> '-'cya	57.6 ± 4.29 (2,880)	0.04 ± 0.001 (2.0)	0.07 ± 0.01 (3.5)	Yes	Yes
<i>rip57</i>	A94	17.4 ± 2.83 (870)	0.02 ± 0.003 (1.0)	0.11 ± 0.03 (5.5)	Yes	Yes
<i>rip58</i> (<i>hpx26</i>)	<i>hpx26</i> '-'cya	79.2 ± 18.9 (3,960)	0.03 ± 0.01 (1.5)	0.02 ± 0.003 (1.0)	Yes	Yes
<i>rip59</i>	A268	51.8 ± 11.8 (2,590)	0.02 ± 0.002 (1.0)	0.67 ± 0.06 (33.5)	Yes	Yes
<i>rip60</i>	A178	19.9 ± 4.03 (995)	0.02 ± 0.002 (1.0)	0.60 ± 0.04 (30.0)	Yes	Yes
<i>rip61</i>	A211	53.0 ± 4.17 (2,650)	0.01 ± 0.001 (0.5)	0.35 ± 0.06 (17.5)	Yes	Yes
<i>rip62</i>	A67	1.36 ± 0.22 (68.0)	0.03 ± 0.004 (1.5)	0.03 ± 0.004 (1.5)	Yes	Yes
<i>rip63</i> (<i>hpx23</i>)	<i>hpx23</i> '-'cya	97.2 ± 19.2 (4,860)	0.02 ± 0.003 (1.0)	0.01 ± 0.002 (0.5)	Yes	Yes
<i>rip64</i> (<i>hpx24</i>)	<i>hpx24</i> '-'cya	4.24 ± 1.11 (212)	0.03 ± 0.01 (1.5)	0.01 ± 0.005 (0.5)	Yes	Yes
<i>rip65</i> (<i>hpx36</i>)	<i>hpx36</i> '-'cya	10.8 ± 1.18 (540)	0.05 ± 0.01 (2.5)	0.04 ± 0.01 (2.0)	Yes	Yes
<i>rip66</i> (<i>hpx9</i>)	<i>hpx9</i> '-'cya	59.5 ± 2.40 (2,975)	0.01 ± 0.005 (0.5)	0.07 ± 0.02 (3.5)	Yes	Yes
<i>rip67</i>	A195	27.0 ± 5.35 (1,350)	0.01 ± 0.002 (0.5)	0.44 ± 0.03 (22.0)	Yes	Yes
<i>rip68</i> (<i>hpx8</i>)	<i>hpx8</i> '-'cya	18.3 ± 1.87 (915)	0.02 ± 0.003 (1.0)	0.04 ± 0.01 (1.0)	Yes	Yes
<i>rip69</i>	A280	11.0 ± 1.86 (550)	0.02 ± 0.002 (1.0)	0.10 ± 0.02 (5.0)	Yes	Yes
<i>rip70</i> (<i>popF1</i>)	A234	4.73 ± 0.84 (237)	0.02 ± 0.003 (1.0)	22.2 ± 3.04 (1110)	Yes	No
<i>rip71</i>	A139	29.6 ± 0.92 (1,480)	0.03 ± 0.002 (1.5)	0.29 ± 0.06 (14.5)	Yes	Yes
<i>rip72</i>	A92	15.7 ± 2.52 (785)	0.02 ± 0.002 (1.0)	0.48 ± 0.07 (24.0)	Yes	Yes

cating that *rip* genes of *R. solanacearum* are widely distributed in the genome of this bacterium without forming large gene clusters (Table 5). Comparison of *rip* genes and their surrounding genes between RS1000 and GMI1000 revealed that the two phylotype I strains are closely related at the DNA level (Table 5). The type III effector repertoires were nearly the same between the two strains, although 11 RS1000 effector genes had mutations, such as frameshift, large internal deletion, and IS insertion. Although their products are translocated into plant cells via the Hrp T3SS (Table 4), these effectors may lose their original activities because they are truncated forms. On the other hand, three *rip* genes were not found in GMI1000, two of which are deleted or replaced with other probable effector genes (data not shown). Type III effectors recognized as Avr proteins are known to restrict the host range of plant pathogens (Alfano and Collmer 2004; Jones and Dangl 2006). Mutations in the type III effector gene *hopQ1-1* of *P. syringae* pv. *tomato* DC3000 enabled this strain to cause disease in the nonhost *Nicotiana benthamiana* (Wei et al. 2007). In *R. solanacearum* GMI1000, a combinatorial disruption of two effector genes, *avrA* and *popP1*, enabled this strain to cause disease in *N. tabacum* (Poueymire et al. 2009). There-

fore, the abovementioned mutations in effector genes might be involved in pathogenic adaptation in certain host plants of this strain by escaping effector recognition.

The Hrp-dependent translocation of 66 effector proteins into plant cells was abolished by the $\Delta hpaB$ mutation (Table 4). Such defects in effector translocation seem to underlie the Hrp-defective phenotype observed in the $\Delta hpaB$ mutant (Mukaihara et al. 2004). HpaB is a small (17-kDa), acidic (isoelectric point = 4.20), and intracellular (T. Mukaihara, *unpublished data*) protein, which is encoded within an *hrp* gene cluster (Mukaihara et al. 2004). Such characteristics well correspond to those of class IB chaperones in T3SSs (Parsot et al. 2003). It has been shown that HpaB from *X. campestris* pv. *vesicatoria* and *X. oryzae* pv. *oryzae*, which share high homology to that from *R. solanacearum*, controls the secretion of at least four *X. campestris* pv. *vesicatoria* and 15 *X. oryzae* pv. *oryzae* effectors (Büttner et al. 2004; Furutani et al. 2009). Although the function of HpaB in *R. solanacearum* should be further investigated in the future, it seems to act as a global effector chaperone for most effector proteins in *R. solanacearum*. The six remaining Rip proteins, including four extracellular components of the Hrp T3SS, were translocated into plant

Table 5. Seventy-two currently identified *rip* (*Ralstonia* protein injected into plant cells) genes encoding type III effectors and related proteins in RS1000

Type III effector gene	Corresponding open reading frame (% identity of product) in		Comment, type III effector protein, or bacterial species ^a
	GMI1000	UW551	
Type III effector gene			
<i>rip1</i>	RSc0041 (99.5)	RRSL_02040 (84.9)	Ankyrin repeat domain
<i>rip2</i> (<i>hpx11</i>)	RSc0245 (99.3)	RRSL_02573 (72.7)	HopQ1 family, inocine-uridine-preferring nucleoside hydrolase domain, Aac, Psp, Pst, Xac, Xcc, Xcv, Xoo
<i>rip3</i>	RSc0257 (50.1)	RRSL_01439 (46.5)	Two independent deletions in ankyrin repeat domain
<i>rip4</i>	RSc0321 (98.4)	RRSL_01625 (49.1)	Frameshift mutation, Ser/Thr acetyltransferase domain
<i>rip5</i> (<i>avrA</i>)	RSc0608 (58.8)	RRSL_01581 (73.1)	No known motifs
<i>rip6</i>	RSc0824 (100)	...	Rip6 family, no known motifs
<i>rip7</i> (<i>popP1</i>)	RSc0826 (95.1)	...	YopJ/AvrRxv family, Ser/Thr acetyltransferase domain, Aac, Pss, Sal, Xcv, Yer
<i>rip8</i> (<i>popP2-1</i>)	RSc0868 (98.6)	...	YopJ/AvrRxv family, Ser/Thr acetyltransferase domain, Aac, Pss, Sal, Xcv, Yer
<i>rip9</i> (<i>popP2-2</i>)	RSc0868 (94.4)	...	Frameshift mutation, YopJ/AvrRxv family, Ser/Thr acetyltransferase domain, Aac, Pss, Sal, Xcv, Yer
<i>rip10</i>	...	...	No known motifs, Xcc
<i>rip11</i>	RSc0895 (97.9)	...	Rip6 family, no known motifs
<i>rip12</i> (<i>hpx29</i>)	RSc1349 (99.0)	RRSL_01554 (66.6)	IS insertion, IpaH/SspH family, E3 ubiquitin ligase domain, Sal, Shi
<i>rip13</i> (<i>hpx13</i>)	RSc1356 (80.9)	RRSL_01561 (69.7)	Hypervariation, GALA family, type I/GALA LRR domain, Aac, Lp
<i>rip14</i> (<i>hpx14</i>)	RSc1357 (97.2)	RRSL_01562 (63.1)	GALA family, type I/GALA LRR domain, Aac, Lp
<i>rip15</i>	RSc1386 (98.7)	RRSL_00426 (57.2)	No known motifs, Aac, Xac, Xcc, Xcv
<i>rip16</i>	RSc1475 (99.0)	RRSL_00705 (72.7)	No known motifs, Aac
<i>rip17</i> (<i>hpx15</i>)	RSc1800 (99.0)	RRSL_00532 (56.6)	GALA family, type I/GALA LRR domain, Aac, Lp
<i>rip18</i> (<i>hpx16</i>)	RSc1801 (98.4)	RRSL_00531 (67.0)	GALA family, type I/GALA LRR domain, Aac, Lp
<i>rip19</i> (<i>hpx17</i>)	RSc1815 (57.0)	...	Large deletion in central repeat domain, AvrBs3 family, AvrBs3 repeats, Xac, Xcc, Xcv, Xoo
<i>rip20</i> (<i>hpx30</i>)	RSc1839 (96.8)	RRSL_03921 (62.8)	Hpx30 family, SKWP repeat domain, Ml, Psp, Rg, Xac, Xcv, Xoo
<i>rip21</i> (<i>hpx18</i>)	RSc2101 (99.3)	RRSL_01260 (69.0)	No known motifs
<i>rip22</i>	RSc2132 (98.4)	RRSL_04062 (63.7)	IS insertion, no known motifs
<i>rip23</i> (<i>hpx28</i>)	RSc2359 (98.6)	...	No known motifs
<i>rip25</i> (<i>ripT</i>)	RSc3212 (99.7)	...	YopT/HopC1 family, cysteine proteinase domain, Ea, Psp, Pst, Yer
<i>rip26</i>	RSc3369 (99.1)	RRSL_00852 (84.6)	HopX1 family, no known motifs, Pss, Pst, Xac, Xcc, Xcv
<i>rip27</i> (<i>hpx37</i>)	RSc3401 (99.2)	RRSL_04182 (87.7)	Hpx30 family, SKWP repeat domain, Ml, Psp, Rg, Xac, Xcv, Xoo
<i>rip28</i> (<i>gala3</i>)	RSp0028 (96.4)	RRSL_00752 (55.9)	GALA family, type I/GALA LRR domain, Aac, Lp
<i>rip29</i> (<i>hpx31</i>)	RSp0099 (99.4)	RRSL_03418 (78.2)	Hpx4 family, no known motifs, Bg
<i>rip30</i>	RSp0160 (98.9)	RRSL_00041 (81.7)	XopP family, no known motifs, Xac, Xcc, Xcv, Xoo
<i>rip31</i>	RSp0193 (94.9)	...	Pentatricopeptide repeat (PPR) domain
<i>rip32</i>	RSp0215 (97.1)	RRSL_03559 (63.2)	XopP family, no known motifs, Xac, Xcc, Xcv, Xoo
<i>rip33</i> (<i>hpx34</i>)	RSp0296 (98.8)	RRSL_04182 (70.5)	Hpx30 family, SKWP repeat domain, Ml, Psp, Rg, Xac, Xcv, Xoo

(continued on next page)

^a Specific comment, similar type III effector protein, or bacterial species containing similar gene. Species abbreviations: Aac, *Acidovorax avenae* subsp. *citrulli*; Bg, *Burkholderia glumae*; Ea, *Erwinia amylovora*; Lp, *Legionella pneumophila*; Ml, *Mesorhizobium loti*; Psp, *Pseudomonas syringae* pv. *phaseolicola*; Pss, *P. syringae* pv. *syringae*; Pst, *P. syringae* pv. *tomato*; Rg, *Rickettsiella grylli*; Sal, *Salmonella* sp.; Shi, *Shigella* sp.; Xac, *Xanthomonas axonopodis* pv. *citri*; Xcc, *X. campestris* pv. *campestris*; Xcv, *X. campestris* pv. *vesicatoria*; Xoo, *X. oryzae* pv. *oryzae*; Yer, *Yersinia* sp.

^b Hrp type III secretion system (T3SS) extracellular component gene.

cells via the Hrp T3SS in an HpaB-independent manner, and their levels even increased in the $\Delta hpaB$ background (Table 4). This indicates that HpaB-independent effectors are preferentially translocated into plant cells in the $\Delta hpaB$ background where the translocation of a large number of HpaB-dependent effectors is inefficient. It is interesting that, as in *Xanthomonas* spp., HpaB is dispensable for the translocation of noneffectors, including the harpin, Hrp pilin, and translocon proteins of *R. solanacearum*.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions.

The bacterial strains used in this study are listed in Table 6. All of the *R. solanacearum* strains are derivatives of RS1085 (Mukaihara et al. 2004), which is a spontaneous Nal^r EPS⁻ derivative of the wild-type strain RS1000 (race 1, biovar 4, phylotype I) (Mukaihara et al. 2004). *Escherichia coli* JM109 (Sambrook et al. 1989) was used for plasmid construction. S17-1 (Simon et al. 1983) was used as a donor for oriT/mob plasmids in conjugation experiments. *R. solanacearum* strains were grown at 28°C in all experiments. BG medium (Boucher

et al. 1985) was used for standard cultivation. The composition of an hrp-inducing medium has been described (Tamura et al. 2005). *E. coli* strains were grown in Luria-Bertani medium (Sambrook et al. 1989) at 37°C. Antibiotics were added to the media as described previously (Mukaihara et al. 2004).

Construction of transposon vector for screening.

Effector-probing transposon mini-Tn5'*cya* was constructed from pBSL204 (Alexeyev et al. 1995) and pARO-HA-'Cya (Murata et al. 2006) as follows. pARO-HA-'Cya was linearized with *Xba*I and inserted into the *Spe*I site of pBSL204. The resulting plasmid was then digested with *Cla*I to exclude the 3.3-kb *Cla*I fragment containing *oriT/mob* from pARO-HA-'Cya and self-ligated to yield pTNP106. pTNP106 harbors a transposon mini-Tn5'*cya*, in which the 5'-truncated '*cya* gene is associated with the O-end (Fig. 1A).

Transposon insertion and screening of *R. solanacearum* rip genes encoding proteins translocated into plant cells.

R. solanacearum RS1085 and *E. coli* S17-1 harboring pTNP106 carrying mini-Tn5'*cya* were mated on a nitrocellu-

Table 5. (continued from preceding page)

Type III effector gene	Corresponding open reading frame (% identity of product) in		Comment, type III effector protein, or bacterial species ^a
	GMI1000	UW551	
<i>rip34</i> (<i>hpx25</i>)	RSp0304 (97.5)	RRSL_00947 (62.8)	HopD1 family, no known motifs, Aac, Psp, Pst, Xcv
<i>rip35</i>	RSp0323 (98.7)	RRSL_00926 (83.2)	HopG1 family, no known motifs, Aac, Psp, Pst, Xac, Xcc
<i>rip36</i>	RSp0572 (100)	...	HopH1 family, no known motifs, Aac, Pss, Pst, Xcc, Xcv, Xoo
<i>rip37</i> (<i>hpx20</i>)	RSp0672 (97.8)	RRSL_02264 (69.0)	GALA family, type I/GALA LRR domain, Aac, Lp
<i>rip38</i>	...	RRSL_02221 (77.2)	Class III lipase domain, Aac, Xac, Xcv, Xoo
<i>rip39</i> (<i>hpx27</i>)	RSp0732 (99.5)	RRSL_02213 (85.8)	HopAV1 family, no known motifs, Psp
<i>rip40</i>	RSp0822 (96.3)	...	HopF2 family, no known motifs, Aac, Psp, Pst
<i>rip41</i>	RSp0838 (99.0)	RRSL_01021 (79.2)	No known motifs, Bg
<i>rip42</i>	RSp0839 (88.1)	RRSL_01019 (29.7)	No known motifs
<i>rip43</i> (<i>hpx33</i>)	RSp0845 (99.5)	RRSL_01013 (71.3)	No known motifs
<i>rip44</i> (<i>hpx32</i>)	RSp0846 (98.5)	...	Hpx4 family, no known motifs, Bg
<i>rip45</i> (<i>hpx4</i>)	RSp0847 (98.3)	...	Hpx4 family, no known motifs, Bg
<i>rip47</i> (<i>popC</i>)	RSp0875 (99.7)	RRSL_02441 (50.2)	Type II LRR domain, Xcv
<i>rip48</i> (<i>popB</i>)	RSp0876 (98.9)	RRSL_02442 (69.9)	No known motifs
<i>rip49</i> (<i>popA</i>)	RSp0877 (94.2)	RRSL_02443 (60.8)	No known motifs
<i>rip50</i> (<i>hpx2</i>)	RSp0879 (98.3)	RRSL_03105 (51.1)	No known motifs
<i>rip51</i>	RSp0885 (98.3)	RRSL_03113 (68.2)	No known motifs, Aac
<i>rip53</i> (<i>gala1</i>)	RSp0914 (80.1)	...	Two independent deletions in LRR, GALA family, type I/GALA LRR domain, Aac, Lp
<i>rip54</i> (<i>hpx35</i>)	RSp0930 (98.6)	RRSL_00237 (78.3)	Hpx30 family, SKWP repeat domain, Ml, Psp, Rg, Xac, Xcv, Xoo
<i>rip55</i> (<i>hpx21</i>)	RSp1022 (96.2)	RRSL_01066 (59.7)	No known motifs, Aac
<i>rip56</i> (<i>hpx10</i>)	RSp1024 (98.2)	RRSL_01071 (77.9)	Hpx4 family, no known motifs, Bg
<i>rip57</i>	RSp1031 (98.6)	RRSL_00271 (49.3)	No known motifs
<i>rip58</i> (<i>hpx26</i>)	RSp1130 (97.3)	RRSL_04736 (76.8)	Nudix hydrolase domain
<i>rip59</i>	...	RRSL_04660 (74.8)	No known motifs
<i>rip60</i>	RSp1218 (99.4), RSp1215 (98.7)	RRSL_04655 (78.3)	IS insertion in GMI1000, Ser/Thr kinase domain, ankyrin repeat domain
<i>rip61</i>	RSp1236 (98.2)	RRSL_01136 (63.8)	Rip61 family, no known motifs
<i>rip62</i>	RSp1239 (98.8)	RRSL_03371 (71.8)	XopC family, no known motifs, Aac, Xac, Xcc, Xcv, Xoo
<i>rip63</i> (<i>hpx23</i>)	RSp1277 (99.4)	RRSL_02855 (75.1)	HopAA1-1 family, no known motifs, Pss, Pst
<i>rip64</i> (<i>hpx24</i>)	RSp1281 (99.2)	RRSL_03375 (79.8)	HopR1 family, no known motifs, Psp, Pst, Xcc
<i>rip65</i> (<i>hpx36</i>)	RSp1374 (98.9)	...	Hpx30 family, SKWP repeat domain, Ml, Psp, Rg, Xac, Xcv, Xoo
<i>rip66</i> (<i>hpx9</i>)	RSp1384 (99.0)	RRSL_00571 (51.2)	No known motifs
<i>rip67</i>	RSp1388 (94.1)	RRSL_00576 (42.2)	IS insertion, no known motifs
<i>rip68</i> (<i>hpx8</i>)	RSp1460 (98.7)	RRSL_02518 (39.6)	Hpx8 family, no known motifs, Aac, Xac, Xcv, Xoo
<i>rip69</i>	RSp1475 (96.2)	...	Rip61 family, no known motifs
<i>rip71</i>	RSp1582 (90.5)	...	Frameshift mutation, no known motifs, Lp
<i>rip72</i>	RSp1601 (97.5)	RRSL_02518 (61.8)	Hpx8 family, no known motifs, Aac, Xac, Xcv, Xoo
Hrp T3SS ^b			
<i>rip24</i>	RSc2775 (99.5)	RRSL_02682 (80.1)	HrpW family, harpin-like protein, pectate lyase domain, Aac, Ea, Pss, Xac, Xcc
<i>rip46</i> (<i>hpaZ</i>)	RSp0854 (97.3)	RRSL_00507 (62.7)	Similar to Hrp pilin protein HrpY
<i>rip52</i> (<i>popF2</i>)	RSp0900 (98.8)	RRSL_04777 (81.4)	HrpF/XopX family, T3SS translocator, Ml, Xac, Xcc, Xcv, Xoo
<i>rip70</i> (<i>popF1</i>)	RSp1555 (99.0)	RRSL_04777 (78.2)	HrpF/XopX family, T3SS translocator, Ml, Xac, Xcc, Xcv, Xoo

Table 6. Bacterial strains

Strain	Description	Reference
<i>Ralstonia solanacearum</i>		
RS1085	Nal ^r Δ epsP	Mukaihara et al. 2004
RS1204	RS1085 Δ (hrpB-hrcT)	Murata et al. 2006
RS1212	RS1085 Δ hpaB	Mukaihara et al. 2004
RS1239	RS1085 RSc2836::mini-Tn5hrpB (constitutively expressing hrpB [hrpB ^c])	Tamura et al. 2005
RS1254	RS1085 Δ hrpY	Mukaihara et al. 2004
<i>Escherichia coli</i>		
JM109	recA1 supE44 endA1 hsdR17 gyrA96 relA1 Δ (lac-proAB) F' (traD36 proAB ⁺ lacI ^f lacZAM15)	Sambrook et al. 1989
S17-1	thi pro hsdR ⁻ hsdM ⁺ recA (chr::RP4-2-Tc::Mu-Km::Tn7)	Simon et al. 1983

lose membrane filter on BG plates at 28°C overnight. Samples were spread and incubated on BG plates containing kanamycin (20 µg/ml) and nalidixic acid (30 µg/ml) at 28°C. Antibiotic-resistant transconjugants appeared after 3 days of incubation. Single colonies were inoculated into 96-well deep plates containing 0.5 ml of BG medium containing nalidixic acid (10 µg/ml) using toothpicks. To avoid the isolation of siblings, only 96 transconjugants were isolated from a selection plate of each mating mixture. The 96-well deep plates were incubated at 28°C overnight with agitation at 1,300 rpm using TAITEC BioShaker (TAITEC, Saitama, Japan). To preserve the original plates, 50 µl of an overnight culture in each well was transferred into a new 96-well microplate and mixed with an equal amount of 30% glycerol (final concentration, 15% glycerol) for storing at -80°C. For screening, a set of overnight cultures of 96 transconjugants from one plate was combined as a pool, centrifuged, washed once with distilled water (DW) and resuspended in DW at an optical density at 600 nm = 0.5 (approximately 5×10^8 CFU/ml). The mixed bacterial suspension was inoculated into fully expanded leaves of 6-week-old susceptible eggplants using a needleless syringe, and the inoculated plants were kept in a greenhouse at 28°C. Two leaf disks (0.8 cm² each) were taken 15 h after inoculation, frozen in liquid nitrogen, and kept at -80°C. Extracts of plant samples were prepared as previously described (Murata et al. 2006). Using an aliquot of a 10-µl plant extract, cAMP level was measured using a cAMP enzyme immunoassay kit (Biotrack; GE healthcare, Buckinghamshire, U.K.). Pools that showed a marked increase in the cAMP level (more than 50 fmol/well) in the inoculated leaves compared with other samples were chosen as positive pools. Under the experimental condition that we used, 70 to 75% of pools showed a cAMP level below the detection limit (less than 12.5 fmol/well). *R. solanacearum* mutants included in the positive pool were divided into several subgroups, and the plant assay was repeated until a single positive mutant was specified. We did not choose pools that showed a small increase in the cAMP level (12.5 to 50 fmol/well) because no positive mutant was isolated from these pools in the beginning of this screening using approximately 24,000 mutants.

Cloning and sequencing of *rip* genes.

Genomic DNA from mini-Tn5^{cya} insertion mutants purified with a Genomic DNA Extraction Kit (Qiagen, Valencia, CA, U.S.A.) was digested and self-ligated for the transformation of JM109. Because the transposon contains the ColE1-type origin (Fig. 1A), when genomic DNA was digested with an appropriate restriction enzyme, the surrounding region of the transposon can be rescued as a plasmid in *E. coli*. *Apa*I, *Cla*I, *Kpn*I, or *Mlu*I was used to clone the upstream region of the transposon, and *Bam*HI, *Not*I, or *Sma*I was used for the downstream rescue. For sequencing the Tn insertion sites, the primer P1041 (5'-G AGTACGGCTGCGGGGATG-3') was used for O-end junctions and the primer P2043 (5'-AACGGATTCACTCA AGA-3') for I-end junctions.

Construction of *R. solanacearum* mutants expressing Cya fusion protein and adenylate cyclase activity assay.

For the *rip* genes newly isolated in this study, a genomic DNA (2 µg) from *R. solanacearum* Tn insertion mutants was electroporated into the strain RS1204 (Δ hrpB), RS1212 (Δ hpaB), or RS1254 (Δ hrpY) under the conditions previously described (Mukaihara et al. 2004) and selected for the Kan^r recombinants. An *hrpB*^c mutant was constructed for each Δ hrpB strain by electroporating the genomic DNA of the *hrpB*^c strain RS1239 (Tamura et al. 2005) and selecting the gentamycin-resistant recombinants. For the *rip* genes previously identified, a plasmid integration system was used to construct the Δ hpaB mutant expressing the Cya fusion protein as described previously (Mukaihara and Tamura 2009). The protocol of in vitro or in planta adenylate cyclase activity assay and the procedure of cAMP extraction from each sample have been described (Murata et al. 2006).

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