Identification of novel *Ralstonia solanacearum* type III effector proteins through translocation analysis of *hrpB*-regulated gene products

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The Hrp type III secretion system (TTSS) is essential for the pathogenicity of *Ralstonia solanacearum* on host plants. Hrp TTSS is a specialized secretion system that injects virulence proteins, the so-called type III effector proteins, into plant cells. In *R. solanacearum*, the expression of Hrp TTSS-related genes is regulated by an AraC-type transcriptional activator, HrpB. We have identified 30 *hrpB*-regulated *hpx* (*hrpB*-dependent expression) genes and three well-known *hrpB*-regulated genes, *popA*, *popB* and *popC*, as candidate effector genes in *R. solanacearum* strain RS1000. In this study, we newly cloned 11 additional candidate effector genes that share homology with known *hpx* genes from *R. solanacearum* RS1000. Using a *Cya* reporter system, we investigated the translocation of these 44 gene products into plant cells via the Hrp TTSS and identified 34 effector proteins. These include three effector families composed of more than four members, namely the Hpx4, Hpx30 and GALA families. The Hpx30 family effectors are 2200–2500 aa in size and appear to be the largest class of effector proteins among animal- and plant-pathogenic bacteria. Members of this family contain 12–18 tandem repeats of a novel 42 aa motif, designated SKWP repeats.

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INTRODUCTION

Ralstonia solanacearum is a Gram-negative soilborne pathogen that causes bacterial wilt in more than 100 plant species, including agriculturally important crops such as tomato, potato, tobacco, banana and eggplant (Hayward, 1991). This pathogen enters plant roots from natural openings or wounds, invades xylem vessels, proliferates and spreads throughout the vascular system, and secretes a large amount of extracellular polysaccharides (EPSs) that impair water transport, resulting in the lethal wilting of infected plants (Schell, 2000).

The type III secretion system (TTSS) is essential for the pathogenicity of many plant- and animal-pathogenic bacteria (Cornelis & Van Gijsegem, 2000; Galán & Collmer, 1999; He *et al.*, 2004). Some components of the TTSS are similar to those of the flagellar basal body (Gophna *et al.*, 2003). In fact, the TTSS machinery complexes isolated from several animal-pathogenic bacteria, such as *Salmonella typhimurium*, *Shigella flexneri* and

enteropathogenic *Escherichia coli*, have been shown to form a cylindrical structure, similar to a flagellar basal body, which is composed of outer and inner rings, an inner rod that traverses the two rings and a needle-like projection (Galán & Wolf-Watz, 2006). In plant pathogens, the TTSS is called the Hrp (hypersensitive response and pathogenicity) because defects in the TTSS result in the loss of both the ability to induce hypersensitive responses and pathogenicity in plants (Alfano & Collmer, 1997). A long filamentous extracellular appendage associated with the Hrp TTSS, called the Hrp pilus, has been found (Jin & He, 2001; Roine *et al.*, 1997; Van Gijsegem *et al.*, 2000; Weber, *et al.*, 2005). These TTSSs are considered to act as molecular syringes or conduits for injecting virulence factors, called effector proteins, into host cells, where they suppress or stimulate a variety of host cellular functions to promote the growth of the pathogen (Alfano & Collmer, 2004; Jones & Dangle, 2006; Mudgett, 2005; Nomura *et al.*, 2005).

Various approaches have been taken to identify type III effector proteins in plant-pathogenic bacteria. Genes belonging to the *hrp* regulon have been isolated as candidate effector genes by genetic screening (Fouts *et al.*, 2002; Noël *et al.*, 2001), by analysing specific promoter sequences in genome DNA sequences (Cunnac *et al.*, 2004; Zwiesler-Vollick *et al.*, 2002) or by whole-genomic microarray analyses (Ferreira *et al.*, 2006; Occhialini *et al.*,

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Abbreviations: EPS, extracellular polysaccharide; LRR, leucine-rich repeat; PIP, plant-inducible promoter; TTSS, type III secretion system.

A supplementary table of primers and plasmids is available with the online version of this paper.

2005) because the expression of many effector genes is coregulated with that of *hrp* genes by common regulatory proteins in many plant pathogens. The N-terminal export signals of effector proteins have also been used as a characteristic to survey candidate effector genes in genomic DNA sequences (Petnicki-Ocwieja *et al.*, 2002). True effector proteins were selected from these candidates by testing their ability to be translocated into plant cells, mainly using a *Bordetella pertussis* calmodulin-dependent adenylate cyclase (Cya) reporter system (Casper-Lindley *et al.*, 2002; Cunnac *et al.*, 2004; Schechter *et al.*, 2004; Sory & Cornelis, 1994).

In *R. solanacearum*, the components of the Hrp TTSS are encoded within a large *hrp/hrc* gene cluster and their expressions are controlled by an AraC-type transcriptional activator HrpB, which is also encoded within the *hrp* gene cluster (Genin *et al.*, 1992; Van Gijsegem *et al.*, 1995). Mutations in *hrc* genes result in the complete loss of the ability of the pathogen to proliferate in host plants (Boucher *et al.*, 1987). This observation provides clear evidence that the Hrp-delivered effector proteins function in plant cells to enable the growth of this pathogen in host plants. In a previous study, using a transposon-based genetic screen, we identified 30 *hrpB*-regulated *hpx* (*hrpB*-dependent expression) genes in *R. solanacearum* strain RS1000 (Mukaihara *et al.*, 2004). Most of the *hpx* genes were considered to encode effector proteins because they contained a PIP (plant-inducible promoter) box in the promoter region and were considered to be directly regulated by HrpB (Mukaihara *et al.*, 2004). In fact, the secretion of some Hpx proteins, such as Hpx23, Hpx24, Hpx25 and Hpx26, into culture media via the Hrp TTSS has been observed (Tamura *et al.*, 2005). In this study, adding the above 30 *hpx* genes and the well-known HrpB-regulated *popABC* genes (Arlat *et al.*, 1994; Guéneron *et al.*, 2000), we newly cloned 11 additional candidate effector genes that share homology with known *hpx* genes from *R. solanacearum* RS1000. Using a Cya reporter system, we investigated the translocation of these gene products into plant cells via the Hrp TTSS.

METHODS

Bacterial strains, media and culture conditions. The bacterial strains used in this study are listed in Table 1. All of the *R. solanacearum* strains are derivatives of RS1000 (race 1 biovar 4 phylotype I) (Mukaihara *et al.*, 2004). RS1002 is a spontaneous NaI^r derivative of RS1000 (Mukaihara *et al.*, 2004). RS1085 is an EPS^- derivative of RS1002. In RS1085, the high production of EPSs and the formation of fluid colonies are eliminated. We used derivatives of the strain in this study because we can easily apply any genetic manipulations to the strain for comparison with the wild-type. *E. coli* S17-1 (Simon *et al.*, 1983) was used as a host for *oriT/mob* plasmids in conjugation experiments. BG medium (Boucher *et al.*, 1985) was used for the growth of *R. solanacearum* strains at 28 °C and was also used as the *hrp*-repressing medium. An *hrp*-inducing medium was prepared with the following composition: 50 mM potassium phosphate buffer, 7.6 mM $(\text{NH}_4)_2\text{SO}_4$, 1.7 mM MgCl_2 and 1.7 mM NaCl supplemented with 10 mM glutamate and 10 mM sucrose (Tamura *et al.* 2005). The *E. coli* strains were grown at 37 °C in LB medium (Sambrook *et al.*, 1989). Antibiotics were added to the media as described previously (Mukaihara *et al.*, 2004).

Construction of *R. solanacearum* mutants expressing Cya fusion protein and adenylate cyclase activity assay. For the construction of *R. solanacearum* mutants expressing the Cya fusion protein, a plasmid-based system was used as described previously (Murata *et al.*, 2006). Briefly, a DNA fragment encoding a part (first 127–371 aa) or the full length of the protein was amplified from each candidate effector gene and inserted into pARO-HA-'Cya (Murata *et al.*, 2006). Primer sets used for the study are listed in Supplementary Table S1 (available with the online version of this paper). The resulting plasmid was integrated into the genome of *R. solanacearum* strain RS1085 and its ΔhrpY derivative, RS1254, by a single homologous recombination event at the native locus after conjugation with *E. coli* S17-1 as previously described (Mukaihara *et al.*, 2004). The resulting strains expressing the *cya* fusion genes from their original promoters were used for *in planta* adenylate cyclase activity assay. For the *in vitro* adenylate cyclase activity assay, an *hrpB*^c (*hrpB* constitutive) mutant was constructed for each *R. solanacearum* strain by the electroporation of RS1239 chromosomal DNA and the selection of Gm^r recombinants as described previously (Mukaihara *et al.*, 2004). The resulting *hrpB*^c mutants were used in the assay. The primers and plasmids used to construct the Cya fusions are listed in Supplementary Table S1. The adenylate cyclase activity assay and cAMP extraction protocols have been described (Murata *et al.*, 2006).

Table 1. Bacterial strains

Strain	Description	Reference
<i>R. solanacearum</i>		
RS1002	RS1000 NaI^r	Mukaihara <i>et al.</i> (2004)
RS1085	RS1002 ΔepsP	Mukaihara <i>et al.</i> (2004)
RS1204	RS1085 $\Delta(\text{hrpB-hrcT})$	Murata <i>et al.</i> (2006)
RS1239	RS1085 RSc2836::mini-Tn5 <i>hrpB</i> [constitutively expressing <i>hrpB</i> (<i>hrpB</i> ^c)]	Tamura <i>et al.</i> (2005)
RS1254	RS1085 ΔhrpY	Mukaihara <i>et al.</i> (2004)
RS1312	RS1254 <i>hrpB</i> ^c	This study
<i>E. coli</i>		
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1</i> $\Delta(\text{lac-proAB})$ F' [<i>traD36 proAB</i> ⁺ <i>lacI</i> ^q <i>lacZ</i> ΔM15]	Sambrook <i>et al.</i> (1989)
S17-1	<i>thi pro hsdR</i> [−] <i>hsdM</i> ⁺ <i>recA</i> [chr::RP4-2-Tc::Mu-Km::Tn7]	Simon <i>et al.</i> (1983)

RESULTS

Cloning of candidate effector genes from *R. solanacearum* RS1000

Based on the complete genome sequence of strain GMI1000 (Salanoubat *et al.*, 2002), 11 additional candidate effector genes were newly cloned from strain RS1000 (Table 2). Ten of these genes are homologues of *hpx* genes that were identified in a previous study: RSp0099, RSp0846 and RSc2139 (similar to *hpx4* and *hpx10*); RSp0296, RSp0930, RSp1374, RSc3401 and RSc2130 (similar to *hpx30*) and RSp0028 and RSp0914 [type I/GALA leucine-rich repeat (LRR) protein genes] (Mukaihara *et al.*, 2004). During sequencing analysis, we found a gene RSp0845 encoded downstream of RSp0846. Although RSp0845 did not show similarity to known genes found in databases, this was added to the list because a PIP box motif is present in its putative promoter region, and therefore this gene was considered to be a candidate effector gene (Table 2). Sequence analysis revealed that, in eight of the 11 candidate effector genes, a PIP box motif (consensus 5'-TTCGB-N₁₅-TTCGB-3') and a probable -10 sequence (consensus 5'-YANNRT-3') are present in the putative promoter region (Table 2), suggesting that these genes are regulated by *hrpB*. No PIP box motif was observed upstream of RSc2139, RSc2130 and RSp0914.

Construction and expression of Cya fusion of candidate effector proteins in *R. solanacearum*

Our previous genetic screening resulted in the identification of 30 *hrpB*-regulated genes, designated *hpx* (*hrpB*-

dependent expression), and three well-known *hrpB*-regulated genes, *popA*, *popB* and *popC* (DDBJ accession no. AB302224), as candidate effector genes in *R. solanacearum* RS1000 (Mukaihara *et al.*, 2004). By adding the 11 candidates newly isolated in this study (Table 2) to the above-mentioned 33 candidates, 44 genes in total were examined in this study in terms of whether the encoded protein product can be translocated into plant cells as an effector protein. Using a plasmid integration system, we constructed a series of *R. solanacearum* *hrp*⁺ and *Hrp*-deficient Δ *hrpY* strains expressing a candidate effector protein in which an adenylate cyclase domain (Cya) of *B. pertussis* adenylate cyclase toxin CyaA was C-terminally fused with a part (first 127–371 aa) or the full length of the protein. It is important to note that, in these strains, *cya* fusion genes are expressed from their original promoters on the chromosome of RS1000 (see Methods for the construction of Cya fusions). Then, to check the activity of the Cya fusion protein, the *hrpB*^c mutation was introduced into each strain. When the Cya fusion protein was expressed in the *hrpB*^c background in an *hrp*-inducing medium, calmodulin-dependent adenylate cyclase activities were detected in cell lysates; these were comparable in the *hrp*⁺ and Δ *hrpY* strains even though the expression levels varied among the fusion proteins (Table 3). This indicates that functional fusion proteins were expressed at similar levels in both series of strains.

Before the *in planta* Cya activity assay, to determine whether the 11 candidate effector genes newly cloned in this study are regulated by *hrpB*, we compared the expression level of the Cya fusion protein between the

Table 2. Newly cloned candidate effector genes from *R. solanacearum* RS1000

Gene	DDBJ* accession no.	Probable PIP box and -10 sequence†		Protein size (aa)	Similar Hpx protein
		Position	Nucleotide sequence		
RSp0099	AB302225	-176	TTCGC -N ₁₅ - TTCGC -N ₃₁ -TAGCAT	1127	Hpx4 (19.0 % identity, 64.4 % similarity) and Hpx10 (21.6 % identity, 65.3 % similarity)
RSp0846	AB302226	-98	TTCGG -N ₁₅ - TTCGG -N ₂₈ -CACATT	1238	Hpx4 (53.7 % identity, 80.7 % similarity) and Hpx10 (22.2 % identity, 63.8 % similarity)
RSp0845	AB302226	-319	TTCGC -N ₁₅ - TTCGT -N ₃₁ -TAGGCT	1375	No homology, encoded downstream of RSp0846
RSc2139	AB302227	-	Not found	1067	Hpx4 (20.4 % identity, 63.1 % similarity) and Hpx10 (22.0 % identity, 65.7 % similarity)
RSp0296	AB302230	-421	TTCGC -N ₁₅ - TTCGC -N ₃₃ -TACACT	2338	Hpx30 (38.0 % identity, 73.5 % similarity)
RSp0930	AB302231	-470	TGCGC -N ₁₅ - TTCGC -N ₃₃ -TATCGT	2208	Hpx30 (24.8 % identity, 66.0 % similarity)
RSp1374	AB302232	-303	TTCGG -N ₁₅ - TTCGT -N ₃₁ -CATTCT	2504	Hpx30 (48.4 % identity, 82.3 % similarity)
RSc3401	AB302233	-683	TTCGT -N ₁₅ - TTCGC -N ₃₁ -TACACT	2353	Hpx30 (22.2 % identity, 65.4 % similarity)
RSc2130	AB302234	-	Not found	817	N-terminal portion of Hpx30 (45.7 % identity, 82.7 % similarity)
RSp0028	AB302228	-90	TTCGC -N ₁₅ - TTCGT -N ₃₁ -TACGCT	607	Type I/GALA LRR protein
RSp0914	AB302229	-	Not found	493	Type I/GALA LRR protein

*DDBJ, DNA Data Bank of Japan.

†Position indicates position of the first nucleotide (T) of the first TTCGB motif of a PIP box (consensus 5'-TTCGB-N₁₅-TTCGB-3') relative to the start codon of an ORF. 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').

Table 3. *In vitro* adenylate cyclase activity of cell lysates from *R. solanacearum* *hrp*⁺ and Δ *hrpY* strains expressing the Cya fusion protein in the *hrpB*^c background

Fusion protein*	cAMP level† (fold increase‡)			
	+ Calmodulin		- Calmodulin	
	<i>hrpB</i> ^c	<i>hrpB</i> ^c Δ <i>hrpY</i>	<i>hrpB</i> ^c	<i>hrpB</i> ^c Δ <i>hrpY</i>
None	0.01 ± 0.002 (1.0)	0.01 ± 0.002 (1.0)	0.01 ± 0.002 (1.0)	0.01 ± 0.002 (1.0)
Hpx1'-Cya	8.53 ± 0.67 (853)	9.22 ± 0.45 (920)	0.04 ± 0.01 (4.0)	0.06 ± 0.01 (7.0)
Hpx2'-Cya	46.2 ± 3.55 (4620)	45.3 ± 5.29 (4530)	0.15 ± 0.03 (15.0)	0.40 ± 0.03 (40.0)
Hpx3'-Cya	2.26 ± 0.37 (226)	2.92 ± 0.08 (292)	0.01 ± 0.002 (1.0)	0.01 ± 0.001 (1.0)
Hpx4 ₁₋₂₃₀ '-Cya	3.66 ± 0.49 (366)	11.6 ± 0.72 (1160)	0.02 ± 0.004 (2.0)	0.03 ± 0.01 (3.0)
Hpx5 (LrpE) ₁₋₂₃₀ '-Cya	30.0 ± 2.52 (3000)	28.9 ± 2.40 (2890)	0.12 ± 0.02 (12.0)	0.07 ± 0.01 (7.0)
Hpx6'-Cya	1427 ± 154 (142700)	1474 ± 140 (147400)	1.15 ± 0.11 (115)	1.09 ± 0.11 (109)
Hpx7'-Cya	228 ± 12.5 (22800)	223 ± 17.1 (22300)	0.28 ± 0.02 (28.0)	0.58 ± 0.06 (58.0)
Hpx8'-Cya	26.1 ± 2.38 (2610)	16.4 ± 1.17 (1640)	0.04 ± 0.01 (4.0)	0.03 ± 0.003 (3.0)
Hpx9 ₁₋₂₃₀ '-Cya	4.47 ± 0.50 (447)	14.8 ± 0.25 (1480)	0.02 ± 0.003 (2.0)	0.07 ± 0.01 (7.0)
Hpx10 ₁₋₂₃₀ '-Cya	139 ± 23.0 (13900)	306 ± 31.6 (30600)	0.23 ± 0.04 (23.0)	0.38 ± 0.01 (38.0)
Hpx11 ₁₋₂₃₀ '-Cya	9.34 ± 0.93 (934)	10.4 ± 1.24 (1040)	0.03 ± 0.01 (3.0)	0.02 ± 0.003 (2.0)
Hpx12'-Cya	300 ± 14.4 (30000)	385 ± 13.7 (38500)	0.27 ± 0.01 (27.0)	0.51 ± 0.03 (51.0)
Hpx13 (GALA6) ₁₋₁₉₀ '-Cya	18.1 ± 4.33 (1810)	36.2 ± 3.42 (3620)	0.10 ± 0.03 (10.0)	0.13 ± 0.02 (13.0)
Hpx14 (GALA7) ₁₋₂₂₉ '-Cya	194 ± 21.6 (19400)	176 ± 31.0 (17600)	0.54 ± 0.08 (54.0)	0.52 ± 0.05 (52.0)
Hpx15 (GALA4) ₁₋₁₆₈ '-Cya	31.3 ± 3.13 (3130)	43.1 ± 1.70 (4310)	0.12 ± 0.02 (12.0)	0.12 ± 0.02 (12.0)
Hpx16 (GALA5) ₁₋₁₇₀ '-Cya	3.54 ± 0.65 (354)	4.80 ± 0.51 (480)	0.02 ± 0.002 (2.0)	0.02 ± 0.004 (2.0)
Hpx17 ₁₋₂₃₀ '-Cya	26.3 ± 2.08 (2630)	35.7 ± 1.53 (3570)	0.12 ± 0.01 (12.0)	0.40 ± 0.06 (40.0)
Hpx18'-Cya	449 ± 54.9 (44900)	645 ± 32.6 (64500)	0.58 ± 0.07 (58.0)	0.64 ± 0.05 (64.0)
Hpx19'-Cya	3351 ± 317 (335100)	3620 ± 251 (362000)	0.62 ± 0.04 (62.0)	0.71 ± 0.09 (71.0)
Hpx20 (GALA2) ₁₋₁₂₇ '-Cya	14.1 ± 1.09 (1410)	17.6 ± 2.53 (1760)	0.07 ± 0.01 (7.0)	0.15 ± 0.02 (15.0)
Hpx21 ₁₋₂₃₀ '-Cya	16.3 ± 2.75 (1630)	27.1 ± 1.92 (2710)	0.06 ± 0.01 (6.0)	0.12 ± 0.01 (12.0)
Hpx22 ₁₋₂₃₀ '-Cya	0.23 ± 0.02 (23.0)	0.25 ± 0.02 (25.0)	0.01 ± 0.001 (1.0)	0.01 ± 0.002 (1.0)
Hpx23'-Cya	19.2 ± 2.40 (1920)	22.3 ± 1.62 (2230)	0.09 ± 0.01 (9.0)	0.11 ± 0.02 (11.0)
Hpx24 ₁₋₂₃₀ '-Cya	87.9 ± 4.91 (8790)	159 ± 17.3 (15900)	0.14 ± 0.01 (14.0)	0.20 ± 0.02 (20.0)
Hpx25 ₁₋₂₃₀ '-Cya	260 ± 18.3 (26000)	312 ± 33.2 (31200)	0.45 ± 0.03 (45.0)	0.81 ± 0.05 (81.0)
Hpx26 ₁₋₂₃₀ '-Cya	134 ± 12.7 (13400)	229 ± 16.3 (22900)	0.06 ± 0.01 (6.0)	0.16 ± 0.01 (16.0)
Hpx27 ₁₋₂₃₀ '-Cya	5.48 ± 0.91 (548)	9.31 ± 0.35 (931)	0.04 ± 0.01 (4.0)	0.03 ± 0.01 (3.0)
Hpx28 ₁₋₂₃₀ '-Cya	10.5 ± 0.92 (1410)	17.6 ± 1.73 (1760)	0.08 ± 0.01 (8.0)	0.11 ± 0.01 (11.0)
Hpx29 ₁₋₂₃₀ '-Cya	0.67 ± 0.03 (67.0)	0.82 ± 0.12 (82.0)	0.01 ± 0.002 (1.0)	0.01 ± 0.001 (1.0)
Hpx30 ₁₋₂₃₀ '-Cya	1.03 ± 0.09 (103)	1.26 ± 0.08 (126)	0.01 ± 0.001 (1.0)	0.01 ± 0.001 (1.0)
PopA'-Cya	4119 ± 62.9 (411900)	3815 ± 231 (381500)	3.79 ± 0.28 (379)	4.25 ± 0.22 (425)
PopB'-Cya	1870 ± 178 (187000)	2586 ± 173 (258600)	1.10 ± 0.08 (110)	1.45 ± 0.12 (145)
PopC ₁₋₂₃₀ '-Cya	8.01 ± 1.62 (801)	9.50 ± 1.19 (950)	0.02 ± 0.002 (2.0)	0.03 ± 0.002 (3.0)
RSp0099 (Hpx31) ₁₋₂₃₀ '-Cya	12.1 ± 0.62 (1210)	21.7 ± 0.95 (2170)	0.12 ± 0.01 (12.0)	0.07 ± 0.001 (7.0)
RSp0846 (Hpx32) ₁₋₂₃₀ '-Cya	0.33 ± 0.03 (33.0)	0.36 ± 0.02 (36.0)	0.01 ± 0.001 (1.0)	0.01 ± 0.001 (1.0)
RSp0845 (Hpx33) ₁₋₂₃₀ '-Cya	633 ± 20.5 (63300)	807 ± 30.6 (80700)	0.11 ± 0.01 (11.0)	0.32 ± 0.03 (32.0)
RSc2139 ₁₋₂₃₀ '-Cya	0.16 ± 0.01 (16.0)	0.18 ± 0.01 (18.0)	0.01 ± 0.001 (1.0)	0.01 ± 0.002 (1.0)
RSp0296 (Hpx34) ₁₋₂₃₃ '-Cya	8.96 ± 1.46 (896)	12.1 ± 0.40 (1210)	0.04 ± 0.01 (4.0)	0.05 ± 0.01 (5.0)
RSp0930 (Hpx35) ₁₋₂₅₅ '-Cya	0.32 ± 0.06 (32.0)	0.25 ± 0.02 (25.0)	0.01 ± 0.001 (1.0)	0.01 ± 0.001 (1.0)
RSp1374 (Hpx36) ₁₋₃₇₁ '-Cya	0.67 ± 0.05 (67.0)	0.76 ± 0.04 (76.0)	0.01 ± 0.001 (1.0)	0.01 ± 0.001 (1.0)
RSc3401 (Hpx37) ₁₋₂₃₆ '-Cya	1.00 ± 0.07 (100)	0.86 ± 0.24 (86.0)	0.01 ± 0.001 (1.0)	0.01 ± 0.001 (1.0)
RSc2130 ₁₋₂₂₂ '-Cya	0.15 ± 0.01 (15.0)	0.08 ± 0.01 (8.0)	0.01 ± 0.001 (1.0)	0.01 ± 0.001 (1.0)
RSp0028 (GALA3) ₁₋₂₂₀ '-Cya	0.98 ± 0.04 (98.0)	1.05 ± 0.13 (105)	0.01 ± 0.004 (1.0)	0.01 ± 0.002 (1.0)
RSp0914 (GALA1) ₁₋₁₆₅ '-Cya	0.20 ± 0.03 (20.0)	0.25 ± 0.06 (25.0)	0.01 ± 0.003 (1.0)	0.01 ± 0.005 (1.0)

*Subscript number indicates the position of amino acids of proteins that are fused with the Cya reporter protein. Number is not shown in candidate effector proteins of which the full length of the protein is fused with the Cya reporter.

†Adenylate cyclase activity (nmol cAMP per mg total soluble protein) was measured as described in Methods; the mean ± SD of values from three independent experiments is shown.

‡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.

hrpB^c and Δ *hrpB* strains. The Cya activities of eight genes, which have a PIP box motif in the putative promoter region (Table 2), increased 8–1200-fold in the *hrpB^c* background compared with the Δ *hrpB* background (Table 4). This indicates that the expression of the PIP-box-containing genes is indeed regulated by *hrpB*. Hence, we renamed these genes *hpx* (*hrpB*-regulated expression) as follows: *hpx31* (RSp0099), *hpx32* (RSp0846), *hpx33* (RSp0845), *hpx34* (RSp0296), *hpx35* (RSp0930), *hpx36* (RSp1374), *hpx37* (RSc3401). In the case of the remaining three genes, which have no PIP box motif (RSc2139, RSc2130 and RSp0914) (Table 2), the Cya activities were similar in both *hrpB^c* and Δ *hrpB* backgrounds but their expression levels were quite low (Table 4). It was therefore considered that these genes are not efficiently expressed in the artificial *hrp*-inducing medium used in this study.

Identification of novel *R. solanacearum* type III effector proteins

R. solanacearum *hrp⁺* and Δ *hrpY* strains expressing the Cya-fused candidate effector protein in the *hrpB⁺* background were inoculated into host eggplant leaves, and the cAMP levels of the inoculated leaves were measured 12 h after inoculation (Table 5). When the *hrp⁺* strains were used, in 34 of the 44 Cya fusions the cAMP level of inoculated leaves increased 65–9300-fold compared with the background level (Table 4). In all the positive fusions, such an increase in the cAMP level was not observed when the Δ *hrpY* strains were used (Table 5), whereas the fusion proteins were expressed at similar levels in both strains (Table 3). This clearly indicates that these 34 proteins are effector proteins translocated into plant cells via the Hrp TTSS.

DISCUSSION

In this study, we identified 34 type III effector proteins from *R. solanacearum* RS1000 using a Cya reporter system (Table 5). We identified three effector families composed of more than four members, namely, the Hpx4, Hpx30 and GALA families. The Hpx4 family in *R. solanacearum* RS1000 consists of the Hpx4, Hpx10, Hpx31 and Hpx32 effectors. The RSc2139 gene also encoded a protein belonging to the Hpx4 family (Table 2) but was considered to be non-functional because we could not detect the translocation of its product into plant cells by *in planta* Cya activity assay (Table 5). One of the Hpx4 family proteins, Hpx31, has been reported to be translocated into plant cells via the Hrp TTSS and is designated RipA (*Ralstonia* effector injected into plant cells) in *R. solanacearum* GMI1000 (Cunnac *et al.*, 2004). We herein demonstrated that Hpx31 and the other three members of this family are translocated into plant cells in RS1000 (Table 5). The Hpx4 family proteins do not contain any known protein motifs found in databases (Table 2), and therefore it is difficult to predict their functions in plant cells. However, considering both their amplified copy number in *R. solanacearum* and the fact that similar proteins are distributed in other phytopathogenic bacteria, such as *Xanthomonas* spp. and *Acidovorax avenae* subsp. *citriculi* (data not shown), the Hpx4 effector family might have important functions in pathogenicity.

The Hpx30 family is a novel *R. solanacearum* effector family. The five genes encoding this type of protein, namely RSc1839 (Hpx30), RSp0296 (Hpx34), RSp0930 (Hpx35), RSp1374 (Hpx36) and RSc3401 (Hpx37), have not been detected by the genome-wide screens for *hrpB*-regulated candidate effector genes in strain GMI1000 (Cunnac *et al.*,

Table 4. *In vitro* adenylate cyclase activity of cell lysates from *R. solanacearum* *hrpB^c* and Δ *hrpB* strains expressing Cya fusions of newly cloned candidate effectors

Fusion protein*	cAMP level†		Ratio of <i>hrpB^c</i> / Δ <i>hrpB</i>	HrpB-dependent expression
	<i>hrpB^c</i>	Δ <i>hrpB</i>		
None	0.01 ± 0.002	0.01 ± 0.002	1.0	–
RSp0099 (Hpx31) _{1–230} '-Cya	12.1 ± 0.62	0.17 ± 0.01	71.2	Yes
RSp0846 (Hpx32) _{1–230} '-Cya	0.33 ± 0.03	0.01 ± 0.004	33.0	Yes
RSp0845 (Hpx33) _{1–230} '-Cya	633 ± 20.5	0.52 ± 0.02	1217	Yes
RSc2139 _{1–230} '-Cya	0.16 ± 0.01	0.16 ± 0.02	1.0	No
RSp0296 (Hpx34) _{1–233} '-Cya	8.96 ± 1.46	0.08 ± 0.01	112	Yes
RSp0930 (Hpx35) _{1–255} '-Cya	0.32 ± 0.06	0.01 ± 0.001	32.0	Yes
RSp1374 (Hpx36) _{1–371} '-Cya	0.67 ± 0.05	0.01 ± 0.004	67.0	Yes
RSc3401 (Hpx37) _{1–236} '-Cya	1.00 ± 0.07	0.09 ± 0.01	11.1	Yes
RSc2130 _{1–222} '-Cya	0.15 ± 0.01	0.08 ± 0.01	1.8	No
RSp0028 (GALA3) _{1–220} '-Cya	0.98 ± 0.05	0.12 ± 0.02	8.2	Yes
RSp0914 (GALA1) _{1–165} '-Cya	0.20 ± 0.01	0.18 ± 0.03	1.1	No

*Subscript number indicates the position of amino acids of proteins that are fused with the Cya reporter protein. Number is not shown in candidate effector proteins in which the full length of the protein is fused with the Cya reporter.

†Adenylate cyclase activity (nmol cAMP per mg total soluble protein) was measured as described in Methods; the mean ± SD of values from three independent experiments is shown.

Table 5. cAMP level of plant leaves inoculated with *R. solanacearum* *hrp*⁺ and Δ *hrpY* strains expressing the Cya fusion protein in the wild-type *hrpB* background

Fusion protein*	cAMP level† (fold increase‡)		Ratio of <i>hrp</i> ⁺ / Δ <i>hrpY</i>	Hrp-dependent translocation into plant cells
	<i>hrp</i> ⁺	Δ <i>hrpY</i>		
None	0.02 ± 0.004 (1.0)	0.02 ± 0.004 (1.0)	1.0	—
Hpx1'-Cya	0.06 ± 0.01 (3.0)	0.07 ± 0.01 (3.5)	0.9	No
Hpx2'-Cya	75.3 ± 3.81 (3765)	0.02 ± 0.002 (1.0)	3765	Yes
Hpx3'-Cya	0.02 ± 0.002 (1.0)	0.01 ± 0.002 (0.5)	2.0	No
Hpx4 ₁₋₂₃₀ '-Cya	25.7 ± 2.98 (1285)	0.01 ± 0.002 (0.5)	2570	Yes
Hpx5 (LrpE) ₁₋₂₃₀ '-Cya	0.04 ± 0.01 (2.0)	0.04 ± 0.004 (2.0)	1.0	No
Hpx6'-Cya	0.04 ± 0.01 (2.0)	0.02 ± 0.002 (1.0)	2.0	No
Hpx7'-Cya	0.10 ± 0.01 (5.0)	0.12 ± 0.01 (6.0)	0.8	No
Hpx8'-Cya	18.3 ± 1.87 (915)	0.02 ± 0.003 (1.0)	915	Yes
Hpx9 ₁₋₂₃₀ '-Cya	59.5 ± 2.40 (2975)	0.01 ± 0.005 (0.5)	5950	Yes
Hpx10 ₁₋₂₃₀ '-Cya	57.6 ± 4.29 (2880)	0.04 ± 0.001 (2.0)	1440	Yes
Hpx11 ₁₋₂₃₀ '-Cya	83.0 ± 14.9 (4150)	0.06 ± 0.01 (3.0)	1383	Yes
Hpx12'-Cya	0.09 ± 0.01 (4.5)	0.03 ± 0.01 (1.5)	3.0	No
Hpx13 (GALA6) ₁₋₁₉₀ '-Cya	54.0 ± 7.43 (2700)	0.03 ± 0.01 (1.5)	1800	Yes
Hpx14 (GALA7) ₁₋₂₂₉ '-Cya	187 ± 15.1 (9350)	0.02 ± 0.002 (1.0)	9350	Yes
Hpx15 (GALA4) ₁₋₁₆₈ '-Cya	27.5 ± 5.44 (1375)	0.02 ± 0.002 (1.0)	1375	Yes
Hpx16 (GALA5) ₁₋₁₇₀ '-Cya	4.53 ± 0.76 (227)	0.03 ± 0.003 (1.5)	151	Yes
Hpx17 ₁₋₂₃₀ '-Cya	11.3 ± 3.61 (565)	0.07 ± 0.02 (3.5)	161	Yes
Hpx18'-Cya	75.7 ± 18.2 (3785)	0.03 ± 0.01 (1.5)	2523	Yes
Hpx19'-Cya	0.05 ± 0.01 (2.5)	0.05 ± 0.01 (2.5)	1.0	No
Hpx20 (GALA2) ₁₋₁₂₇ '-Cya	89.5 ± 8.32 (4475)	0.02 ± 0.002 (1.0)	4475	Yes
Hpx21 ₁₋₂₃₀ '-Cya	14.6 ± 2.78 (730)	0.02 ± 0.01 (1.0)	730	Yes
Hpx22 ₁₋₂₃₀ '-Cya	0.04 ± 0.01 (2.0)	0.04 ± 0.01 (2.0)	1.0	No
Hpx23'-Cya	97.2 ± 19.2 (4860)	0.02 ± 0.003 (1.0)	4860	Yes
Hpx24 ₁₋₂₃₀ '-Cya	4.24 ± 1.11 (212)	0.03 ± 0.01 (1.5)	141	Yes
Hpx25 ₁₋₂₃₀ '-Cya	144 ± 21.3 (7200)	0.04 ± 0.01 (2.0)	3600	Yes
Hpx26 ₁₋₂₃₀ '-Cya	79.2 ± 18.9 (3960)	0.03 ± 0.01 (1.5)	2640	Yes
Hpx27 ₁₋₂₃₀ '-Cya	75.2 ± 5.54 (3760)	0.02 ± 0.01 (1.0)	3760	Yes
Hpx28 ₁₋₂₃₀ '-Cya	71.5 ± 4.90 (3575)	0.02 ± 0.003 (1.0)	3575	Yes
Hpx29 ₁₋₂₃₀ '-Cya	98.1 ± 8.37 (4905)	0.02 ± 0.004 (1.0)	4905	Yes
Hpx30 ₁₋₂₃₀ '-Cya	40.7 ± 5.72 (2035)	0.01 ± 0.002 (0.5)	4070	Yes
PopA'-Cya	49.4 ± 5.20 (2470)	0.25 ± 0.03 (12.5)	198	Yes
PopB'-Cya	46.9 ± 7.08 (2345)	0.16 ± 0.04 (8.0)	293	Yes
PopC ₁₋₂₃₀ '-Cya	122 ± 13.7 (6100)	0.06 ± 0.01 (3.0)	2033	Yes
Hpx31 (RSp0099) ₁₋₂₃₀ '-Cya	24.5 ± 10.2 (1225)	0.03 ± 0.002 (1.5)	817	Yes
Hpx32 (RSp0846) ₁₋₂₃₀ '-Cya	13.6 ± 2.30 (680)	0.04 ± 0.01 (2.0)	340	Yes
Hpx33 (RSp0845) ₁₋₂₃₀ '-Cya	21.9 ± 3.79 (1095)	0.05 ± 0.02 (2.5)	438	Yes
RSc2139 ₁₋₂₃₀ '-Cya	0.08 ± 0.01 (4.0)	0.04 ± 0.01 (2.0)	2.0	No
Hpx34 (RSp0296) ₁₋₂₃₃ '-Cya	13.7 ± 2.67 (685)	0.04 ± 0.01 (2.0)	343	Yes
Hpx35 (RSp0930) ₁₋₂₅₅ '-Cya	16.2 ± 1.42 (810)	0.03 ± 0.01 (1.5)	540	Yes
Hpx36 (RSp1374) ₁₋₃₇₁ '-Cya	10.8 ± 1.18 (540)	0.05 ± 0.01 (2.5)	216	Yes
Hpx37 (RSc3401) ₁₋₂₃₆ '-Cya	3.27 ± 1.20 (164)	0.05 ± 0.01 (2.5)	65.4	Yes
RSc2130 ₁₋₂₂₂ '-Cya	0.06 ± 0.01 (3.0)	0.05 ± 0.01 (2.5)	1.2	No
RSp0028 (GALA3) ₁₋₂₂₀ '-Cya	71.5 ± 8.70 (3575)	0.03 ± 0.002 (1.5)	2383	Yes
RSp0914 (GALA1) ₁₋₁₆₅ '-Cya	4.23 ± 1.50 (212)	0.02 ± 0.01 (1.0)	212	Yes

*Subscript number indicates the position of amino acids of proteins that are fused with the Cya reporter protein. Number is not shown in candidate effector proteins in which the full length of the protein is fused with the Cya reporter.

†Adenylate cyclase activity (nmol cAMP per mg total soluble protein) was measured as described in Methods; the mean ± SD of values from three independent experiments is shown.

‡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.

2004; Occhialini *et al.*, 2005). This might be due to the low expression level of the *hpx30* family genes in an *hrp*-inducing medium (Mukaihara *et al.*, 2004). The Hpx30 family effectors are 2200–2500 aa in size and appear to be the largest class of effector proteins among those identified before from animal and plant-pathogenic bacteria (Fig. 1a). The RSc2130 gene also encoded the Hpx30 family protein (Table 2) but was considered to be non-functional from the *in planta* Cya activity assay (Table 5). Members of this family contain a novel amino acid repeat domain, designated here as SKWP repeats, from its conserved motif (Fig. 1a). Hpx30, for instance, contains 18 tandem SKWP repeats of a 42 aa motif (Fig. 1b). Other Hpx30 family members contain 12–15 SKWP repeats (Fig. 1a). The size and the consensus amino acid residues of the motif are well conserved among the family members (Fig. 1c). As an exception, Hpx36 contains SKWP repeats of a 45 aa motif (Fig. 1c). Long and repetitive amino acid sequences have been reported to form an interaction domain for a certain ligand. The Hpx30 family proteins might interact with plant factor(s) via the SKWP repeat domain to exert virulence functions.

The third family consists of the Hpx13/GALA6, Hpx14/GALA7, Hpx15/GALA4, Hpx16/GALA5, Hpx20/GALA2,

RSp0028/GALA3 and RSp0914/GALA1 effectors (Fig. 2a), some of which were previously called GALA or type I LRR proteins (Cunnac *et al.*, 2004; Mukaihara *et al.*, 2004). Although two of the GALA proteins, namely GALA6/RipG (Hpx13 in RS1000) and GALA7 (Hpx14 in RS1000) have been reported in terms of their translocation into plant cells via the Hrp TTSS (Angot *et al.*, 2006; Cunnac *et al.*, 2004), in this study we demonstrated that the other members from RS1000 are also translocated into plant cells (Table 5). Members of the GALA family contain large LRRs that share almost two-thirds of the entire amino acids of the protein (Fig. 2a), which comprise a new kind of bacterial LRR protein (Cunnac *et al.*, 2004; Kajava *et al.*, 2008; Mukaihara *et al.*, 2004). Hpx13/GALA6 of RS1000, for instance, contains 17 tandem repeats of the LRR motif with 16 perfect LRRs of 24 aa and one imperfect LRR of 23 aa (Fig. 2b). On the other hand, the N-terminal sequence of the GALA effectors contains F-box motif-like sequences (Fig. 2a and b). Hence, these proteins resemble the plant F-box protein, a subunit of the SCF (for Skp1-Cullin-F-box) complex that functions as an E3 ubiquitin ligase (Bai *et al.*, 1996). Indeed, Angot *et al.* (2006) revealed that several GALA proteins specifically interact with subsets of *Arabidopsis* Skp1-like (ASK)

Fig. 1. Structures of the Hpx30 effector family. (a) Structures of the Hpx30 effector family of *R. solanacearum* RS1000. Amino acid residue numbers are shown below the bars. Grey-shaded boxes indicate SKWP repeats and the number is shown above. (b) Alignment of SKWP repeats of Hpx30. Numbers on the left indicate the number of SKWP repeats. Conserved amino acids are indicated in bold capital letters (more than 70% identity) and bold small letters (50–70% identity). The consensus sequence is shown below. x=any residue. (c) Comparison of consensus sequences from SKWP repeats of Hpx30 effector family. The consensus sequence of the SKWP motif of the Hpx30 effector family is shown below.

Fig. 2. Structures of the GALA effector family. (a) Structures of the GALA effector family of *R. solanacearum* RS1000. Amino acid residue numbers are shown below the bars. Grey-shaded boxes indicate LRRs and the number is shown above. The black boxes indicate the F-box motif-like sequences. (b) Amino acid sequence of Hpx13/GALA6. The F-box motif-like sequences are underlined. LRR repeats are aligned, with numbers on the left. Conserved amino acids (more than 70 % identity) in LRRs are indicated in blue bold letters. Amino acid residues that are substituted in Hpx13 compared with the corresponding protein (GALA6/RSc1356) in GMI1000 are shown in red.

proteins in the F-box motif-like sequences. Sequence comparison between the GALA effectors of our strain RS1000 and those of the genome-sequenced strain GMI1000 revealed that some members accumulate mutations in their LRR domain. Hyper-amino acid substitution was observed in LRRs of Hpx13 and its corresponding GALA6/RSc1356 of GMI1000; the percentage of amino acid substitution in the N-terminal non-LRR region was 2.6 % (5/190 amino acids), but in LRRs it was 27.5 % (112/407 amino acids). Most of the amino acid substitutions in LRRs occurred in the variable residues but not in the conserved residues in the LRR motif (Fig. 2b). On the other hand, two independent deletion events, I (Δ 3LRRs) and II (Δ 4LRRs), occurred in the LRR domain of RSp0914/GALA1 from RS1000 compared with that from GMI1000 (data not shown). These mutational changes in GALA effectors might contribute to the pathoadaptation of *R. solanacearum* strains to their host plants.

Eight of the remaining 18 effector proteins from *R. solanacearum* share homology with known type III effectors and enzymes (Mukaihara *et al.*, 2004). The Hpx11, Hpx23, Hpx24, Hpx25 and Hpx27 effectors share homology with the HopQ1-1, HopAA1-1, HopR1, HopD1 and HopAV1 effectors from *Pseudomonas syringae*. Hpx17 is a member of the AvrBs3 effector family from xanthomonads. Hpx29 shares homology with the IpaH effector family from *Shigella* spp. It has been revealed how some of the above-mentioned effectors function in eukaryotic host cells (Kay *et al.*, 2007; Li *et al.*, 2005; Munkvold, *et al.*, 2008; Rohde

et al., 2007; Wei *et al.*, 2007). It would be interesting to determine whether *R. solanacearum* effectors function in a similar way. Hpx11 and Hpx26 have been found to possess known enzyme motifs, respectively called the inocine-uridine-preferring nucleoside hydrolase motif and the Nudix (nucleoside diphosphates linked to some other moiety $\bar{X}$) hydrolase motif (Mukaihara *et al.*, 2004). Recently, it has been shown that an *Arabidopsis* Nudix family protein, AtNUDT7, negatively regulates the basal defence response through its nucleoside hydrolysis activity (Ge *et al.*, 2007). Although such catalytic activities have not yet been determined in Hpx11 and Hpx26, these effectors might change the amount of certain nucleoside derivatives and modulate the defence response in plant cells.

It is noteworthy that, from a database search, many proteins similar to *R. solanacearum* effector proteins are distributed among plant-pathogenic bacteria, symbiotic bacteria and even in animal-pathogenic bacteria (data not shown). Although their abilities for translocation into host cells have not yet been clarified in most cases, they may also act as effector proteins during the infection of these bacteria. On the other hand, we could not find similar proteins for Hpx2, Hpx9, Hpx18, Hpx26, Hpx33, PopA and PopB effectors, indicating that they are specific to *R. solanacearum*.

Recent studies that focused on the entire type III secretome have revealed a large repertoire of effector proteins in plant- and animal-pathogenic bacteria. A nearly complete inventory analysis using multiple functional and bioinfor-

matics-based approaches identified 41 functional type III effector proteins possessed by the phytopathogenic bacterium *P. syringae* pv. *tomato* DC3000 (Schechter *et al.*, 2006). In animal pathogens, a similar approach revealed 39 functional effector proteins in enterohaemorrhagic *E. coli* O157 Sakai (Tobe *et al.*, 2006). Considering these findings and the results obtained in our study, both plant- and animal-pathogenic bacteria seem to inject more than 40 effector proteins into host cells during infection. In this study, under the speculation that most effector genes of *R. solanacearum* are regulated by *hrpB*, we analysed 44 candidate effector genes. However, our search for *hrpB*-regulated genes is still ongoing (Mukaihara *et al.*, 2004) and a genome-wide microarray analysis has resulted in the identification of 143 *hrpB*-upregulated genes in *R. solanacearum* GMI1000 (Occhialini *et al.*, 2005). Therefore, a large number of untested genes potentially encoding effector proteins exist in *R. solanacearum*. It needs to be determined whether the remaining *hrpB*-upregulated genes that were not examined in this study encode effector proteins. As another approach in the search for effector proteins, several functional screening systems that can directly detect the *in planta* translocation activity of effector proteins have been constructed and used for some plant-pathogenic bacteria, such as *P. syringae* and *Xanthomonas campestris* (Chang *et al.*, 2005; Guttman *et al.*, 2002; Roden *et al.*, 2004). It will also be important to construct such a functional screening system for the complete inventory of type III effector proteins in *R. solanacearum*.

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