BACTERIAL AND PHYTOPLASMA DISEASES

Molecular typing of Japanese strains of *Ralstonia solanacearum* in relation to the ability to induce a hypersensitive reaction in tobacco

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Abstract The genetic diversity of 120 *Ralstonia solanacearum* strains isolated from a variety of host plants across Japan was assessed on the basis of hypersensitive response (HR) in tobacco leaves and phylogenetic analyses of endoglucanase gene *egl*, *hrpB*, and *gyrB*. Phylogenetic analysis of *egl* revealed that only three strains belonged to phylotype IV, and 117 strains belonged to phylotype I. Partial sequences of HrpB were identical among phylotype I strains except for one strain. Analyses using the partial nucleotide sequences of the *gyrB* and *egl* gene fragments grouped phylotype I strains into 11 *gyrB* and 8 *egl* types, respectively, whereas analyses using the partial amino acid sequences of GyrB and Egl grouped phylotype I strains into 4 GyrB and 5 Egl types, respectively. Using multilocus sequence typing of GyrB and Egl, we identified 10 unique sequence types within the Japanese phylotype I strains. Strains belonging to the GyrB42 or GyrB66 type caused wilt in tobacco, and strains belonging to GyrB2 or GyrB9 type elicited HR, demonstrating that HR induction in tobacco is genetically differentiated in the Japanese strains of *R. solanacearum*.

Keywords *Ralstonia solanacearum* · HR · Endoglucanase · Phylotype · GyrB

Introduction

Ralstonia solanacearum is a soilborne phytopathogenic bacterium in the *β*-subdivision of the Proteobacteria (Yabuuchi et al. 1995). *R. solanacearum*, distributed in tropical, subtropical, and some warm temperature regions of the world, causes bacterial wilt in an unusually broad host range of plants in more than 200 species from highly diverse botanical monocot and dicot families (Hayward 1991). In addition, recent reports indicate that the range of

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plants infected by *R. solanacearum* is increasing (Wicker et al. 2007). This bacterium has a high level of phenotypic (metabolic and pathogenic) and genotypic diversity.

Four monophyletic groups of strains, termed phylotypes, have been distinguished on the basis of nucleotide sequences of multiple genes, the ITS region, the *hrpB* gene, and the endoglucanase gene *egl* (Fegan and Prior 2005). These phylotypes correlate with the geographical origins of the strains. Phylotype I is composed of strains originating primarily from Asia, phylotype II from America, phylotype III from Africa and its surrounding islands in the Indian Ocean, and phylotype IV from Indonesia, including *Pseudomonas syzygii* and the blood disease bacterium (BDB) of banana (Prior and Fegan 2005; Villa et al. 2005). Although *R. solanacearum* can be distinguished from other bacteria by phylogenetic analysis of 16S rDNA sequences (Taghavi et al. 1996), DNA–DNA hybridization analysis has revealed that the relatedness between isolates of *R. solanacearum* is often less than the expected limit of more than 70% within a species. Because of this high genetic variation between isolates, *R. solanacearum* is considered a "species complex" (Gillings and Fahy 1994).

R. solanacearum and other gram-negative plant pathogens such as *Pseudomonas syringae*, *Xanthomonas campestris*, and *X. oryzae* infect their hosts by through wounds or natural openings. Once in the intercellular spaces (the apoplast), they produce virulence proteins called type III effectors that are secreted directly into host cells using the type III secretion system (TTSS) to enhance microbial fitness (Alfano and Collmer 2004). Interaction of the effectors and cognate host factors determine the host plant response to virulent and avirulent pathogens (Dangl and Jones 2001). Avirulent strains of *R. solanacearum* elicit a hypersensitive response (HR) on host plants. *R. solanacearum* have been estimated to possess up to 60–80 TTSS effectors (Genin and Boucher 2004).

The genome sequence of *R. solanacearum* GMI1000 strain, which contains two replicons, 3.7-megabase chromosome and 2.1-megabase megaplasmid, revealed the presence of alternative codon-usage regions (ACURs), suggesting that these sequences were acquired by horizontal gene transfer (Salanoubat et al. 2002). Significant proportions of effector genes are located within ACURs. This is probably a reflection of its wide geographic distribution and unusually broad host range.

While the genetic diversity of *R. solanacearum* strains collected from diverse geographic areas has been studied, phenotypic and genotypic variation of *R. solanacearum* populations within localized geographical regions have been reported for several regions (Horita and Tsuchiya 2001; Jeong et al. 2007; Lewis-Ivey et al. 2007; Nouri et al. 2009; Villa et al. 2005; Wicker et al. 2007; Yu et al. 2003). Tobacco and tomato are two of the most studied hosts. In *R. solanacearum* AW1 strain, which belongs to phylotype II, avirulence gene *avrA* is responsible for eliciting HR in tobacco (Carney and Denny 1990). Sequence analysis of *avrA* genes in the *R. solanacearum* population in the southeastern United States revealed that HR-positive strains have the 792-bp wild-type *avrA*, which is inactivated by miniature inverted-repeat transposable elements in HR-negative strains (Robertson et al. 2004). HR-negative strains cause wilt in both tobacco and tomato. Japanese strain OE1-1 also causes wilt in both tomato and tobacco (Kanda et al. 2003). Other Japanese strains 8107 and RS1000 cause wilt in tomato, but elicit HR in tobacco (Kiba et al. 2003; Mukaihara et al. 2004). Recently we have reported that HR-eliciting Japanese strains in tobacco were divided into two types, GMI1000-type and RS1000-type, according to their *avrA* sequences (Liu et al. 2009). The RS1000-type *avrA* has 57% identity with that of GMI1000. We also reported that several Japanese strains possessing the RS1000-type *avrA*, such as OE1-1, did not induce HR in tobacco but wilted the plants, indicating that AvrA is not likely to be the only determinant of HR induction of Japanese strains. Although all the Japanese biovar 4 strains induce tobacco HR (Horita and Tsuchiya 2001), the scheme based on oxidative metabolism of sugars and alcohols is not sufficient to encompass the diversity of strains represented in the species *R. solanacearum*. The objective of this study was to investigate genetic diversity of the Japanese strains of *R. solanacearum* and classify them on the basis of HR induction in tobacco leaves.

Materials and methods

Bacterial strains, media and culture conditions

The *R. solanacearum* strains used in this study (Table 1) were streaked on BG media (Boucher et al. 1985; 1% of bacto peptone, 0.1% of yeast extract, 0.1% of casamino acids, 0.5% of glucose, and 1.5% of agar) supplemented with polymyxin B (50 μg/ml) and incubated at 28°C for 2 days. B medium (Boucher et al. 1985; 1% of bacto peptone, 0.1% of yeast extract, and 0.1% of casamino acids) was inoculated with cells of a selected single colony and incubated overnight at 28°C. *Escherichia coli* strain DH12S (Invitrogen Corp., Carlsbad, CA, USA) was grown in Luria–Bertani (LB) medium at 37°C. Concentrations of ampicillin, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal), and isopropyl-beta-D-thiogalactopyranoside (IPTG) were 100, 40 μg/ml, and 100 μM, respectively.

DNA preparation

Chromosomal DNA of *R. solanacearum* strain was prepared with AquaPure Genomic DNA Isolation Kit

Table 1 List of Japanese strains of *Ralstonia solanacearum* used in this study

Code no.	Strain	Isolated from	Japanese prefecture	HR induction[a]	HrpB[b]	GyrB[c]	Egl[d]
2	8107	*Lycopersicon esculentum*	Nara	HR	42	2	42
3	MAFF301524	*Solanum tuberosum*	Nara	HR	42	2	42
5	MAFF730103	*Lycopersicon esculentum*	Gunma	HR	42	2	42
6	MAFF730126	*Solanum melongena*	Nara	HR	42	2	42
8	MAFF211471	*Zingiber officinale*	Kochi	HR	42	2	42
9	MAFF211479	*Zingiber officinale*	Kochi	HR	42	9	9
17	A-3-4-48	*Zingiber officinale*	Kochi	HR	42	9	9
20	D-1-3-37	*Zingiber officinale*	Kochi	HR	42	9	9
21	D-1-4-43	*Zingiber officinale*	Kochi	HR	42	9	9
22	D-2-1-1	*Zingiber mioga*	Kochi	HR	42	9	9
23	D-2-1-3	*Zingiber mioga*	Kochi	HR	42	9	9
24	D-2-1-6	*Zingiber mioga*	Kochi	HR	42	9	9
25	MAFF211496	*Zingiber mioga*	Kochi	HR	42	9	9
26	MAFF211491	*Zingiber mioga*	Kochi	HR	42	9	26
27	MAFF211266	*Lycopersicon esculentum*	Hiroshima	HR	42	2	42
28	MAFF211267	*Lycopersicon esculentum*	Shimane	HR	42	2	42
29	MAFF211268	*Solanum melongena*	Kochi	HR	42	2	42
30	MAFF211269	*Solanum melongena*	Kochi	HR	42	2	42
31	MAFF211272	*Curcuma alismatifolia*	Kochi	HR	42	9	9
32	MAFF301070	*Lycopersicon esculentum*	Kochi	HR	42	2	32
33	MAFF301520	*Solanum melongena*	Saitama	HR	42	2	42
34	MAFF301521	*Solanum melongena*	Nara	HR	42	2	42
35	MAFF301523	*Solanum melongena*	Tokyo	HR	42	2	42
36	MAFF301528	*Lycopersicon esculentum*	Gunma	HR	42	2	42
37	MAFF301560	*Strelitzia reginae*	Nagasaki	HR	42	2	32
38	MAFF301841	*Solanum melongena*	Kanagawa	HR	42	2	32
39	MAFF302549	*Limonium* sp.	Kochi	HR	42	2	9
40	Kami1-1	*Capsicum annuum*	Kochi	HR	42	2	42
41	MAFF211516	*Capsicum annuum*	Kochi	Vir	42	42	42
42	OE1-1	*Solanum melongena*	Okayama	Vir	42	42	42
44	MAFF730138	*Lycopersicon esculentum*	Kochi	Vir	42	9	42
49	MAFF211282	*Solanum melongena*	Kochi	Vir	42	42	42
55	Ikeuchi-2	*Solanum melongena*	Kochi	Vir	42	42	42
56	Shiraishi-2	*Solanum melongena*	Kochi	Vir	42	42	42
57	Mijaji-2	*Solanum melongena*	Kochi	Vir	42	42	42
58	06-Tsugita	*Solanum melongena*	Kochi	Vir	42	42	42
59	04-Takeda	*Solanum melongena*	Kochi	Vir	42	42	42
60	04-Hamada	*Solanum melongena*	Kochi	Vir	42	42	42
62	Souen-2	*Solanum melongena*	Kochi	Vir	42	42	42
63	Maekawa-1	*Solanum melongena*	Kochi	Vir	42	42	42
66	MAFF301485	*Lycopersicon esculentum*	Oita	Vir	42	66	42
68	MAFF211271	*Solanum tuberosum*	Shizuoka	C	68	68	68
69	MAFF301069	*Nicotiana tabacum*	Shizuoka	Vir	42	66	42
70	MAFF301525	*Lycopersicon esculentum*	Tottori	Vir	42	42	42
71	MAFF301527	*Capsicum annuum*	Oita	VIr	42	66	42
72	MAFF301558	*Solanum tuberosum*	Nagasaki	C	68	68	68
73	MAFF301559	*Solanum tuberosum*	Nagasaki	C	68	68	68
74	MAFF211514	*Lycopersicon esculentum*	Kochi	Vir	42	9	42

Table 1 continued

Code no.	Strain	Isolated from	Japanese prefecture	HR induction[a]	HrpB[b]	GyrB[c]	Egl[d]
75	D-1-4-48	*Lycopersicon esculentum*	Kochi	Vir	42	42	42
78	MAFF211274	*Curcuma alismatifolia*	Kochi	HR	42	9	9
79	MAFF211276	*Curcuma alismatifolia*	Kochi	HR	42	9	9
80	MAFF211278	*Curcuma alismatifolia*	Kochi	HR	42	9	9
82	Aki3	*Zingiber mioga*	Kochi	HR	42	9	9
85	MAFF211492	*Zingiber mioga*	Kochi	HR	42	9	9
87	MAFF211493	*Zingiber mioga*	Kochi	HR	42	9	9
88	Suzaki6-1	*Zingiber mioga*	Kochi	HR	42	9	9
89	MAFF211497	*Zingiber mioga*	Kochi	HR	42	9	9
92	MAFF211484	*Zingiber officinale*	Kochi	HR	42	9	9
93	MAFF211486	*Zingiber officinale*	Kochi	HR	42	9	9
95	D-2-1-36	*Zingiber officinale*	Kochi	HR	42	9	32
96	D-2-1-37	*Zingiber officinale*	Kochi	HR	42	2	9
99	D-2-3-6	*Zingiber officinale*	Kochi	HR	42	9	9
101	D-2-3-14	*Zingiber officinale*	Kochi	HR	42	9	9
102	D-2-3-21	*Zingiber officinale*	Kochi	HR	42	2	32
103	D-2-3-23	*Zingiber officinale*	Kochi	HR	42	2	42
106	MAFF211499	*Capsicum annuum*	Kochi	HR	42	2	42
112	KomatsuYoshi-A	*Solanum melongena*	Kochi	Vir	42	42	42
201	SUPP100	*Solanum melongena*	Chiba	HR	42	2	42
202	SUPP117	*Lycopersicon esculentum*	Saitama	HR	42	2	42
203	SUPP203	*Strelitzia reginae*	Shizuoka	HR	42	2	32
205	SUPP1510	*Delphinium* sp.	Shizuoka	HR	205	2	205
206	SUPP1541	*Angelica keiskei*	Tokyo	HR	42	2	32
207	SUPP1725	*Campanula lactiflora*	Shizuoka	HR	42	2	32
208	SUPP1875	*Nicotiana tabacum*	Shizuoka	Vir	42	66	42
209	SUPP1876	*Fragaria* sp.	Shizuoka	Vir	42	66	42
210	SUPP2164	*Lycopersicon esculentum*	Shizuoka	Vir	42	66	42
211	SUPP2531	*Lycopersicon esculentum*	Shizuoka	Vir	42	66	42
213	Rst018	*Lycopersicon esculentum*	Kumamoto	Vir	42	42	42
215	Rst016	*Lycopersicon esculentum*	Fukushima	HR	42	2	42
216	Rst017	*Lycopersicon esculentum*	Shizuoka	HR	42	2	42
217	Rst023	*Lycopersicon esculentum*	Nagano	HR	42	2	42
218	Rst026	*Solanum melongena*	Yamanashi	Vir	42	42	42
219	Rst033	*Lycopersicon esculentum*	Hokkaido	HR	42	2	42
220	Rst043	*Lycopersicon esculentum*	Saitama	HR	42	2	42
221	Rst065	*Lycopersicon esculentum*	Saitama	HR	42	2	42
222	Rst067	*Lycopersicon esculentum*	Kumamoto	Vir	42	66	42
223	Rst069	*Lycopersicon esculentum*	Ehime	HR	42	2	42
224	Rst071	*Lycopersicon esculentum*	Kanagawa	HR	42	2	9
225	Rst087	*Lycopersicon esculentum*	Yamagata	HR	42	2	42
226	Rst095	*Lycopersicon esculentum*	Gunma	HR	42	2	42
227	HAIP001	*Lycopersicon esculentum*	Hyogo	HR	42	2	42
228	HAIP004	*Lycopersicon esculentum*	Hyogo	HR	42	2	9
229	HAIP010	*Lycopersicon esculentum*	Hyogo	HR	42	2	9
230	HAIP016	*Lycopersicon esculentum*	Hyogo	HR	42	2	32
231	HAIP026	*Lycopersicon esculentum*	Hyogo	HR	42	2	32
232	HAIP067	*Lycopersicon esculentum*	Hyogo	HR	42	2	32

Table 1 continued

Code no.	Strain	Isolated from	Japanese prefecture	HR induction[a]	HrpB[b]	GyrB[c]	Egl[d]
233	HAIP070	*Lycopersicon esculentum*	Hyogo	HR	42	2	32
234	HAIP101	*Solanum melongena*	Hyogo	HR	42	2	42
235	HAIP104	*Solanum melongena*	Hyogo	HR	42	2	9
236	HAIP107	*Lycopersicon esculentum*	Hyogo	HR	42	2	32
237	HAIP110	*Lycopersicon esculentum*	Hyogo	HR	42	2	32
238	HAIP119	*Lycopersicon esculentum*	Hyogo	HR	42	2	42
239	HAIP122	*Lycopersicon esculentum*	Hyogo	HR	42	2	42
240	HAIP140	*Solanum tuberosum*	Nagasaki	HR	42	2	42
241	HAIP141	*Solanum tuberosum*	Nagasaki	HR	42	2	42
242	HAIP142	*Capsicum annuum*	Hyogo	Hr	42	2	42
243	TBW A-1-1-1-1	*Lycopersicon esculentum*	Mie	Vir	42	42	42
244	06SM212A-2	*Solanum melongena*	Mie	HR	42	2	42
245	PBW A-3-1-1	*Capsicum annuum*	Mie	HR	42	2	42
246	PBW C-1	*Capsicum annuum*	Mie	HR	42	2	42
247	8242	*Lycopersicon esculentum*	Tottori	HR	42	2	42
248	Nasu19	*Solanum melongena*	Okayama	Vir	42	42	42
249	Nasu30-2	*Solanum melongena*	Okayama	Vir	42	42	42
250	06-E-1	*Solanum melongena*	Okayama	Vir	42	42	42
251	DTR07-1	*Solanum melongena*	Okayama	Vir	42	42	42
252	Y17-4	*Lycopersicon esculentum*	Shiga	Vir	42	42	42
253	S293-1	*Lycopersicon esculentum*	Wakayama	HR	42	2	42
254	S507-1	*Lycopersicon esculentum*	Tochigi	HR	42	2	42
255	S434-3	*Solanum melongena*	Okayama	Vir	42	42	42
256	S543-4	*Solanum melongena*	Tochigi	Vir	42	42	42

[a] Leaves of 4-week-old tobacco (*Nicotiana tabacum* BY) plants infiltrated with 50 μl of 10^8 CFU/ml and monitored daily for disease development. HR was developed within 24 h (HR) or the infiltrated leaves had necrotic lesions by 48–60 h, and the plants wilted within 2 weeks (Vir). The infiltrated area slowly collapsed after 48 h (C)

[b] Partial *hrpB* gene fragments were sequenced and translated into amino acids. Code numbers show strains with identical HrpB amino acid sequences

[c] Partial *gyrB* gene fragments were sequenced and translated into amino acids. Code numbers show strains with identical GyrB amino acid sequences

[d] Partial *egl* gene fragments were sequenced and translated into amino acids. Code numbers show strains with identical Egl amino acid sequences

(Bio-Rad, Hercules, CA, USA) according to the manufacturer's instruction. Plasmid DNA was purified using GenElute Plasmid Miniprep Kit (Sigma–Aldrich, St. Louis, MO, USA).

Sequencing of endoglucanase gene

The partial *egl* fragments were amplified with primers eglA (5′-GGAGACAUATGCATGCCGCTGGTCGCCGC-3′) and eglB (5′-GGGAAAGUGCGTTGCCCGGCACGAACACC-3′). The 20-μl PCR mixture contained 10× TurboCx buffer (Stratagene, La Jolla, CA, USA), 10× PCR enhancer (Invitrogen Corp.), dNTP, primers, and Pfu-TurboCx DNA polymerase (Stratagene). The PCR condition was an initial denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 65°C for 1 min, and 72°C for 1 min. The PCR products separated on 0.8% agarose gel electrophoresis were purified by E.Z.N.A. Gel Extraction Kit (Omega Bio-tek, Doraville, GA, USA) and cloned on pNEB206A with a USER Friendly cloning kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol. Plasmids were purified from multiple numbers of white colonies on LB supplemented with ampicillin, IPTG, and X-gal. Nucleotide sequence of *egl* on the plasmid was determined using BigDye Terminator v3.1 Cycle Sequencing kits (Applied Biosystems, Foster City, CA, USA) with either M13-47 (5′-TGTAAAACGACGGCCAGT-3′) or RV-M (5′-CAGGAAACA

GCTATGACC-3′) primer and analyzed with Applied Biosystems 3130 genetic analyzer (Applied Biosystems).

Sequencing of *gyrB*

The partial *gyrB* gene fragments were amplified with degenerate primers UP-IE (5′-CAGGAAACAGCTAT GACCAYGSNGGNGGNAARTTYRA-3′) and APrU (5′-T GTAAAACGACGGCCAGTGCNGGRTCYTTYTCYTGR CA-3′) using PrimeSTAR HS DNA Polymerase (Takara Bio, Otsu, Japan). The PCR condition was one cycle at 95°C for 2 min, followed by 35 cycles at 98°C for 10 s, 58°C for 5 s and 72°C for 1 min. The PCR products were purified and directly sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with either M13-47 or RV-M primer.

Sequencing of *hrpB*

The partial *hrpB* gene fragments were amplified with primers RShrpBf (5′-TGCCATGCTGGGAAACATCT-3′) and hrpB-SQ2 (5′-GATACTTGGCGGACAGCCG-3′) using PrimeSTAR HS DNA Polymerase (Takara Bio). The PCR condition was one cycle at 95°C for 2 min, followed by 35 cycles at 98°C for 10 s, 56°C for 5 s and 72°C for 1.5 min. The PCR products separated on 0.8% agarose gel electrophoresis were purified and cloned on pBluescript KS+ pre-digested with *Eco*RV. Plasmids were purified from multiple numbers of white colonies on LB supplemented with ampicillin, IPTG, and X-gal. The nucleotide sequence of *hrpB* on the plasmid was determined using BigDye Terminator v3.1 Cycle Sequencing kits (Applied Biosystems) with either M13-47 or RV-M primer.

Phylogenetic analysis

The determined nucleotide sequences or the translated amino acid sequences were aligned using the program CLUSTAL W (Thompson et al. 1994). A phylogenetic tree was constructed with the program CLC Sequence Viewer using the UPGMA algorithm (CLC bio, Aarhus, Denmark).

HR test

Tobacco seedlings (*Nicotiana tabacum* cv. Bright Yellow and *N. benthamiana*) were transplanted into 6-cm-diameter plastic pots containing a mixture of vermiculite/peat moss (3:1) in a growth room at 25°C under 10,000 lux for 16 h per day (Kanda et al. 2003). Eight-week-old test plants with four to five true leaves were inoculated with bacteria using leaf infiltration method. Bacterial cell suspension (10^8 CFU/ml) prepared in 10 mM $MgSO_4$ were infiltrated into tobacco leaves with a syringe without needle. Each

assay was repeated in three successive trials. Plants were inspected at 24, 48, and 72 h post inoculation (hpi) and daily for 14 days (Yoshimochi et al. 2009).

The sequences of partial *hrpB* fragments, *gyrB* fragments, and *egl* fragments have been deposited in DDBJ as accessions AB508353–AB508472, AB508473–AB508592, and AB508593–AB508712, respectively.

Results

Phylotyping of the Japanese strains of *R. solanacearum*

The endoglucanase gene *egl* has been used to classify *R. solanacearum* into four phylotypes (Wicker et al. 2007). The partial gene fragments of *egl* were amplified from 120 strains of *R. solanacearum* isolated across Japan from variety of host plants such as tomato, tobacco, eggplant, potato, pepper, and the zingiberaceous plants (Table 1). The
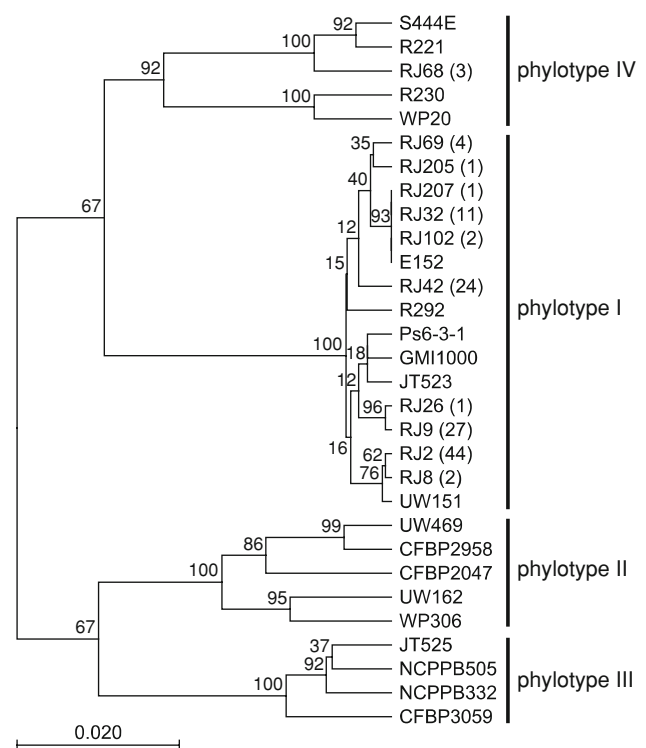
Fig. 1 Phylotype classification of the Japanese strains of *R. solanacearum* based on the partial *egl* nucleotide sequences. The 688-bp partial *egl* fragments were aligned with Clustal W. The phylogenetic tree was constructed by the UPGMA algorithm. Values at the branches indicate the bootstrap percentage. "*RJ*" is used as a prefix to indicate the Japanese strains used in this study. Numbers in parentheses indicate strain numbers having the same *egl* sequences. The *egl* sequences of S444E, R221, R230, WP20, E152, R292, Ps6-3-1, GMI1000, JT523, UW151, UW469, CFBP2958, CFBP2047, UW162, WP306, JT525, NCPPB505, NCPPB332, and CFBP3059 are from Villa et al. (2005)

nucleotide sequences of the amplified fragments were determined and compared to those of strains belonging to phylotypes I–IV. Parts of the *egl* sequences of MAFF 211471 (code no. 8), MAFF 211479 (code no. 9), MAFF 211266 (code no. 27), MAFF 211271 (code no. 68), MAFF 301558 (code no. 72), MAFF 301559 (code no. 73), and MAFF 211493 (code no. 87) were matched to the deposited *egl* sequences of these strains (AY464998, AY464997, AF295250, AY465000, AY465002, AY465001, AY465013). All but three strains were classified as phylotype I (Fig. 1). Three strains isolated from potato were classified as phylotype IV, supporting previous results (Villa et al. 2005).

HR induction in tobacco leaves by *R. solanacearum*

When leaves of *N. tabacum* were infiltrated separately with 117 strains of *R. solanacearum* belonging to phylotype I, 83 of these strains elicited HR in the infiltrated leaves within 24 h, while 34 strains caused wilt tobacco in 2 weeks (Table 1). Strains isolated from the zingiberaceous plants, ginger, mioga, or curcuma, elicited HR. HR induction by strains isolated from other plants was variable. No clear relationship between HR induction in tobacco leaves and the original host plants was observed. When a different *Nicotiana* species, *N. benthamiana*, was infiltrated with the strains, we observed the same phenomena.

Phylogenetic analyses

Five housekeeping genes, dispersed in the chromosome, and three virulence-related genes, located on the megaplasmid were used to classify *R. solanacearum* (Castillo and Greenberg 2007). Of these eight genes, we chose *hrpB*, *egl*, and *gyrB* for phylogenetic analysis in this study. The *gyrB* gene is a housekeeping gene and the *egl* and *hrpB* genes are virulence genes. The 1088-bp partial *hrpB* gene fragments were sequenced. The *hrpB* sequences of three phylotype IV strains were identical to each other, but distinct from those of other phylotype I strains. Part of the *hrpB* sequences of MAFF 211266 (code no. 27) was matched to the deposited *hrpB* sequences of this strain (AF295603). There were five alleles in phylotype I strains, represented by strain 8107 (code no. 2), strain MAFF 211479 (code no. 9), strain MAFF 301070 (code no. 32), strain OE1-1 (code no. 42), and strain SUPP1510 (code no. 205). These differed by a few nucleotides from one another and were similar to GMI1000. Almost all the allelic differences are synonymous nucleotide substitutions, and there were only two types of translated amino acid sequences (Fig. 2). HrpB sequences of OE1-1 (code no. 42), 8107 (code no. 2), MAFF 211479 (code no. 9) and MAFF 301070 (code no. 32) were identical to that of
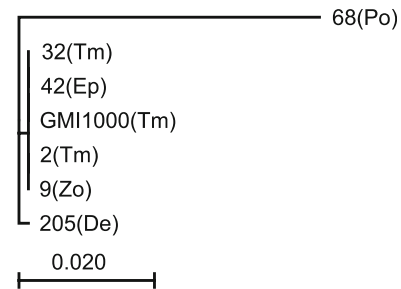
Fig. 2 Phylogenetic tree of the Japanese strains of *R. solanacearum* based on HrpB. The 362-amino acid sequences of translated partial HrpB proteins were aligned with Clustal W. The phylogenetic tree was constructed using the UPGMA algorithm. Phylotype IV strain (code no. 68) was used as the outgroup. Two letters in parentheses indicate the host plants from which each strain was initially isolated: Tm, *Lycopersicon esculentum*; Ep, *Solanum melongena*; Po, *Solanum tuberosum*; Zo, *Zinger officinale*; De, *Delphinium* sp.

GMI1000 and differed by one amino acid from that of SUPP1510 (code no. 205).

The *gyrB* sequences of three phylotype IV strains were identical and distinct from those of phylotype I strains. There were 11 *gyrB* alleles in phylotype I strains. We constructed phylogenetic trees based on both *gyrB* nucleotide sequences and GyrB amino acid sequences. When two trees were compared, the resolution was higher in the amino acid-based tree than in the nucleotide-based tree. The deduced amino acid sequences of GyrB in phylotype I strains grouped into four types (Fig. 3a), represented by strain 8107 (code no. 2), strain MAFF 211479 (code no. 9), strain OE1-1 (code no. 42) and strain MAFF 301485 (code no. 66). Although the bootstrap value was not high, GyrB of GMI1000 was grouped into another type (Fig. 3a). Strains belonging to each GyrB type were isolated from a range of host species; no clear relationship between GyrB types and the original host plants was observed.

The phylogenetic tree based on Egl amino acid sequences showed higher resolution than the phylogenetic tree based on *egl* nucleotide sequences. The deduced amino acid sequences of Egl in phylotype I grouped into five types (Fig. 3b), represented by strains MAFF 211479 (code no. 9), MAFF 211491 (code no. 26), MAFF 301070 (code no. 32), OE1-1 (code no. 42), and SUPP1510 (code no. 205). Although the bootstrap value was not high, Egl of GMI1000 was grouped into another type (Fig. 3b).

Multilocus sequence typing

We phylogenetically analyzed phylotype I strains of *R. solanacearum* using three independent genes. After combining the sequence data for the three genes, we applied multilocus sequence typing (MLST) to classify Japanese strains of *R. solanacearum*. The diversity of amino acid
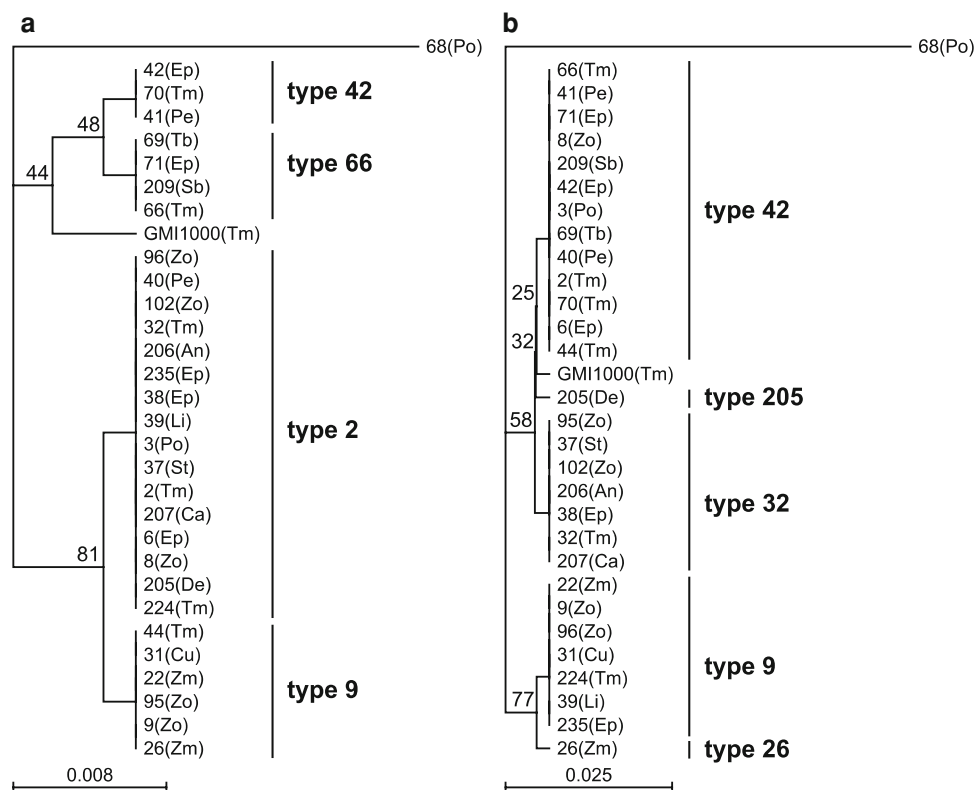
Fig. 3 Phylogenetic tree of the Japanese phylotype I strains of *R. solanacearum*. The 264-amino-acid sequences of translated partial GyrB (**a**) and the 297-amino-acid sequences of translated partial Egl (**b**) were aligned with Clustal W. The phylogenetic trees were constructed with the UPGMA algorithm. The phylotype IV strain (code no. 68) was used as the outgroup. Values at the branches indicate the bootstrap percentage. The *scales* indicate genetic distances. Two letters in parentheses indicate host plants from which each strain was initially isolated: Tm, *Lycopersicon esculentum*; Tb, *Nicotiana tabacum*; Ep, *Solanum melongena*; Po, *Solanum tuberosum*; Pe, *Capsicum annuum*; Li, *Limonium* sp.; An, *Angelia keiskei*; Ca, *Campanula lactiflora*; Sb, *Fragaria* sp.; Zo, *Zinger officinale*; Zm, *Zinger mioga*; Cu, *Curcuma alismatifolia*; St, *Strelitzia reginae*; De, *Delphinium* sp.

sequences in HrpB was very low (Fig. 2). In this study, we used amino acid sequences of GyrB and Egl for the MLST scheme and identified 10 unique sequence types within the Japanese phylotype I strains (Table 2): type A (GyrB42/Egl42), type B (GyrB2/Egl42), type C (GyrB2/Egl32), type D (GyrB2/Egl9), type E (GyrB2/Egl205), type F (GyrB66/Egl42), type G (GyrB9/Egl42), type H (GyrB9/Egl32), type I (GyrB9/Egl9), and type J (GyrB9/Egl26). We classified the phylotype I strains on the basis of MLST types and the original host plants (Table 2). Strains belonging to MLST types A and G were isolated from the family Solanaceae. Strains of MLST type F were isolated from species in the family Solanaceae and from strawberry. Strains belonging to MLST types B, C and D were isolated from a wide range of plants, including both dicots and monocots. Strains belonging to MLST types H, I, and J were isolated from species in the Zingiberaceae. MLST type E contained only one strain (code no. 205), which was isolated from *Delphinium* sp.

Discussion

One hundred and seventeen Japanese phylotype I strains of *R. solanacearum* were classified into 10 MLST types when the amino acid sequences of GyrB and Egl were combined for analysis. When HR induction in tobacco of these strains was assessed, the strains could be clearly differentiated on the basis of GyrB types (Table 3). While strains belonging to GyrB types 42 and 66, which corresponded to MLST types A and F, caused wilt of tobacco, the strains belonging to GyrB types 2 and 9 elicited HR in infiltrated tobacco leaves with two exceptions for MLST type G (Table 3). Strains GyrB42 and GyrB66 form a distinct clade from strains GyrB2 and GyrB9 (Fig. 3a). We used two proteins GyrB and Egl to conduct the MLST analysis. In comparison of two phylogenetic trees based on GyrB and Egl, the tree topologies were different (Fig. 3), suggesting that genes *gyrB* and *egl* evolved in a different manner. The housekeeping gene *gyrB* has been proposed to be a suitable

Table 2 Code number of phylotype I strains of *Ralstonia solanacearum* classified according to host of origin and the MLST types based on amino acid sequences for GyrB and Egl (sequence numbers in parentheses)

Host	MLST type (GyrB sequence no./Egl sequence no.)									
	A (42/42)	F (66/42)	G (9/42)	B (2/42)	C (2/32)	D (2/9)	E (2/205)	H (9/32)	I (9/9)	J (9/26)
Lycopersicon esculentum	70 75 213 243 252	66 210 211 222	44 74	2 5 27 28 36 202 215 216 217 219 220 221 223 225 226 227 238 239 247 253 254	32 230 231 232 233 236 237	224 228 229				
Nicotiana tabacum		69 208								
Solanum melongena	42 49 55 56 57 58 59 60 62 63 112 218 248 249 250 251 255 256			6 29 30 33 34 35 201 234 244	38	235				
Solanum tuberosum				3 240 241						
Capsicum annuum	41	71		40 106 242 245 246						
Limonium sp.						39				
Angelica keiskei					206					
Campanula lactiflora					207					
Fragaria sp.		209								
Zingiber officinale				8 103	102	96		95	9 17 20 21 92 93 99 101	
Zingiber mioga									22 23 24 25 82 85 87 88 89	26
Curcuma alismatifolia									31 78 79 80	
Strelitzia reginae					37 203					
Delphinium sp.							205			

phylogenetic marker (Yamamoto and Harayama 1998). On the other hand, the *egl* gene, encoding a plant cell wall-degrading enzyme, is directly involved in pathogenesis (Liu et al. 2005). Diversifying selection of the host may accelerate the accumulation of new divergent alleles (Castillo and Greenberg 2007). By combining two genes that evolved in a different manner, we could classify *R. solanacearum* strains at a much higher resolution. Such a case may be seen in the strains belonging to MLST type G. Type G strains cause wilt in tobacco. The GyrB type of the MLST type G strain is GyrB9. However, other GyrB9 strains elicited HR in tobacco (Table 3). When we used only GyrB for diversifying HR-inducing strains, MLST G strains became the exception. On the other hand, Egl of MLST type G is Egl42, which is the same as MLST types A and F (Table 3). Strains belonging to MLST types A and F cause wilt in tobacco. When GyrB and Egl are combined for the analysis, MLST type G strains are placed in the right group.

The strains belonging to MLST types A, F, and G, which cause wilt in tobacco, were originally isolated from the species in the Solanaceae (Table 2). The strains belonging to MLST types B, C, and D, which elicited HR in tobacco, were isolated from both Solanaceae and Zingiberaceae species. These MLST types are composed of GyrB2. When ginger plants were inoculated with strains from types B,

C, and D isolated from the family Solanaceae—8107 (code no. 2), MAFF 301524 (code no. 3), MAFF 730126 (code no. 6), Kami-1 (code no. 40), MAFF 301070 (code no. 32), MAFF 301841 (code no. 38), Rst071 (code no. 224), and HAIP104 (code no. 235)—no wilting was observed (unpublished data). The type B strain isolated from ginger, MAFF 211471 (code no. 8), was fully virulent on species from the family Solanaceae (Tsuchiya et al. 2005). We can speculate that the ancestral strains of MLST types B, C, and D were avirulent on tobacco and that some strains gained the ability to cause disease on the family Zingiberaceae.

On the other hand, HR strains belonging to MLST types H, I, and J were isolated exclusively from the Zingiberaceae species. The GyrB type is GyrB9, which is a distinct clade from GyrB2 (Fig. 3a). Strains isolated from ginger and mioga have unique sequences in both 16S rRNA and *egl* genes (Villa et al. 2005). An AFLP analysis of *R. solanacearum* strains on the island of Hawaii indicated that strains from ginger have a high degree of similarity within strains and little similarity with strains from all other hosts (Yu et al. 2003). Type I strains MAFF 211479 (code no. 9), MAFF 211496 (code no. 25), MAFF 211272 (code no. 31), and MAFF 211274 (code no. 78) and type J strain MAFF 211491 (code no. 26) did not wilt plants from the family Solanaceae (Tsuchiya et al. 2005). The ancestral strains of

Table 3 Relationship between MLST types based on amino acid sequences for GyrB and Egl for phylotype I strains of *Ralstonia solanacearum* and response elicited (lesions or HR)

MLST type (GyrB sequence no./Egl sequence no.)	Host of origin [Code no. of strain] eliciting host response	
	Lesions	HR
A (42/42)	Tm [70 75 213 243 252]	
	Ep [42 49 55 56 57 58 59 60 62 63 112 218 248 249 250 251 255 256]	
	Pe [41]	
F (66/42)	Tm [66 210 211 222]	
	Tb [69 208]	
	Pe [71]	
	Sb [209]	
G (9/42)	Tm [44 74]	
B (2/42)		Tm [2 5 27 28 36 202 215 216 217 219 220 221 223 225 226 227 238 239 247 253 254]
		Ep [6 29 30 33 34 35 201 234 244]
		Po [3 240 241]
		Pe [40 106 242 245 246]
		Zo [8 103]
C (2/32)		Tm [32 230 231 232 233 236 237]
		Ep [38]
		An [206]
		Ca [207]
		Zo [102]
		St [37 203]
D (2/9)		Tm [224 228 229]
		Ep [235]
		Li [39]
		Zo [96]
E (2/205)		De [205]
H (9/32)		Zo [95]
I (9/9)		Zo [9 17 20 21 92 93 99 101]
		Zm [22 23 24 25 82 85 87 88 89]
		Cu [31 78 79 80]
J (9/26)		Zm [26]

R. solanacearum strains were isolated from Tm, *Lycopersicon esculentum*; Tb, *Nicotiana tabacum*; Ep, *Solanum melongena*; Po, *Solanum tuberosum*; Pe, *Capsicum annuum*; Li, *Limonium* sp.; An, *Angelia keiskei*; Ca, *Campanula lactiflora*; Sb, *Fragaria* sp.; Zo, *Zinger officinale*; Zm, *Zinger mioga*; Cu, *Curcuma alismatifolia*; St, *Strelitzia reginae*; De, *Delphinium* sp. Leaves of 4-week-old tobacco (*N. tabacum* BY) plants infiltrated with 50 μl of 10^8 CFU/ml and monitored daily for disease development. Infiltrated leaves developed necrotic lesions at 48–60 h, and plants wilted within 2 weeks (virulence); or HR was developed within 24 h (avirulence)

MLST types H, I, and J might be distinct from the ginger strains of MLST types B, C, and D. A rep-PCR analysis of the ginger strains defined two fingerprint types, types I and II (Horita et al. 2004). The MLST type B strain MAFF 211471 (code no. 8) is fingerprint type II, and the MLST type I strains MAFF 211479 (code no. 9), MAFF 211272 (code no. 31), and MAFF 211274 (code no. 78), are fingerprint type I (Horita et al. 2004), which could support our speculation that the ginger strains might have two distinct origins.

In this study, while almost all strains belonged to phylotype I, three strains were classified into phylotype IV. These three strains (MAFF211271, code no. 68; MAFF301558, code no. 72; MAFF301559, code no. 73) were all race 3 and isolated from potato. They belong to phylotype IV and caused the collapse of the infiltrated area of tobacco leaves (Villa et al. 2005). They did not cause wilt in tobacco. We obtained the same results with these potato strains, indicating that tobacco plants inoculated

with phylotype IV Japanese strains reacted differently from those inoculated with phylotype I strains.

The type III effectors play an important role in determining host range (Dangl and Jones 2001). The *avrA* gene is involved in pathogenicity on tobacco in the phylotype II population of *R. solanacearum* in the southeastern United States (Robertson et al. 2004). The combination of two genes, *avrA* and *popP1*, is responsible for HR induction of phylotype I strain GMI1000 (Poueymiro et al. 2009), which was collected in South America. Wild-type GMI1000 elicited HR and an *avrA popP1* double mutant was virulent on tobacco. Recently, we reported that HR-eliciting Japanese strains in tobacco were divided into two types according to AvrA sequences: one type strain contained AvrA identical to that of GMI1000, and another type strain contained AvrA identical to that of OE1-1 (Liu et al. 2009). While the strains with GMI1000-type AvrA elicited HR in tobacco, both strains with OE1-1-type AvrA elicited HR or wilted tobacco. Although all the virulent strains lacked *popP1*, HR was elicited even by several strains without *popP1*. From these results, we speculate that *avrA* and *popP1* in Japanese strains might not be responsible for HR induction in tobacco. Draft genome sequencing of strains OE1-1 and MAFF 730135 (potato strain, race 3) has been ongoing. Strain OE1-1 belonging to MLST type A caused wilt of tobacco, and strain MAFF 730135 belonging to MLST type B elicited HR in tobacco. By comparing the two genome sequences, we have found several effector genes, whose structures are quite different between OE1-1 and MAFF 730135 (unpublished data). These effector genes could be candidate virulence determinants on tobacco. As a next step, we will investigate the distribution of the candidate effector genes in the strains belonging to each of 10 MLST types.

The *hrpB* gene has been used to analyze the phylogenetic relationship among strains of *R. solanacearum* (Poussier et al. 2000; Villa et al. 2005). In this study, we also used *hrpB* to classify the Japanese strains. Although the *hrpB* sequence of phylotype IV strain was distinct from those of phylotype I strains, the sequence variation among phylotype I strains was subtle. Most of the substitution was synonymous, and only two types of amino acid sequences of partial HrpB were detected. Although South American phylotype I strain GMI1000 had different GyrB and Egl types from Japanese phylotype I strains (Fig. 3), the full length of HrpB of GMI1000 was 100% identical to OE1-1 (Liu et al. 2009). Contrary to the *hrp* gene cluster of other plant pathogens, the *R. solanacearum hrp* locus and flanking regions containing the virulence genes is not a pathogenicity island (Salanoubat et al. 2002). Instead, the *hrp* locus is believed to be composed of a core group of ancestral pathogenicity genes (Genin and Boucher 2004), indicating that diversity of genes in the *R. solanacearum*

hrp locus including *hrpB* is low. All these data suggest that the *hrpB* gene might not be suitable for classifying *R. solanacearum* strains in the same phylotype, at least in phylotype I.

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