

Analysis of Genetic and Pathogenic Diversity of *Ralstonia solanacearum* Causing Potato Bacterial Wilt in Korea

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The *Ralstonia solanacearum* species complex (RSSC) can be divided into four phylotypes, and includes phenotypically diverse bacterial strains that cause bacterial wilt on various host plants. This study used 93 RSSC isolates responsible for potato bacterial wilt in Korea, and investigated their phylogenetic relatedness based on the analysis of phylotype, biovar, and host range. Of the 93 isolates, twenty-two were identified as biovar 2, eight as biovar 3, and sixty-three as biovar 4. Applied to the phylotype scheme, biovar 3 and 4 isolates belonged to phylotype I, and biovar 2 isolates belonged to phylotype IV. This classification was consistent with phylogenetic trees based on 16S rRNA and *egl* gene sequences, in which biovar 3 and 4 isolates clustered to phylotype I, and biovar 2 isolates clustered to phylotype IV. Korean biovar 2 isolates were distinct from biovar 3 and 4 isolates pathologically as well as genetically – all biovar 2 isolates were nonpathogenic to peppers. Additionally, in host-determining assays, we found uncommon strains among biovar 2 of phylotype IV, which were the tomato-nonpathogenic strains. Since tomatoes are known to be highly susceptible to RSSC, to the best of our knowledge this is the first report of tomato-nonpathogenic potato strains. These results imply the potential prevalence of greater RSSC diversity in terms

of host range than would be predicted based on phylogenetic analysis.

Keywords : diversity, potato bacterial wilt, *Ralstonia solanacearum*

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Ralstonia solanacearum is a causal agent of bacterial wilt disease, and is one of the most destructive phytopathogenic bacteria worldwide (Hayward, 1991). A soil-borne pathogen, *R. solanacearum* infects host plants through wounds and natural openings, colonizes and blocks water in the xylem, and finally causes wilting and death of the host plant (Denny, 2006). When this bacterium infects potatoes, it causes brown rot on the tubers and aboveground symptoms including wilting, stunting, and yellowing of leaves (Kelman, 1954; Martin and French, 1985).

The bacteria have an unusually broad host range of over 450 plant species, encompassing monocots and dicots (Hayward, 1991; Wicker et al., 2007). Phylogenetic analysis of *R. solanacearum* has revealed great diversity, and this group is known as the *R. solanacearum* species complex (RSSC) (Elphinstone, 2005; Genin and Denny, 2012). Previously, *R. solanacearum* has been classified into “races” based on host range (Buddenhagen et al., 1962; Hayward, 1964; He et al., 1983; Pegg and Moffett, 1971) and “biovars” based on carbohydrate utilization (Hayward, 1964, 1991). However, it has been difficult to define the correlation between races and biovars, with the exception of race 3/biovar 2. Recently, the RSSC has been divided into four phylogenetic groups (“phylotypes”) based on sequence analysis of the internal transcribed spacer (ITS) region of the 16S-23S rRNA gene (Poussier et al., 2000; Prior and

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Fegan, 2005). This scheme corresponds to geographic origin: phylotype I (Asia), phylotype II (America), phylotype III (Africa), and phylotype IV (Indonesia) (Fegan and Prior, 2005; Prior and Fegan, 2005). The scheme is also broadly consistent with various genetic typing analyses. The RSSC has recently undergone reclassification: *R. solanacearum* of phylotype II was reclassified as true *R. solanacearum*, phylotype I and III as *R. pseudosolanacearum*, *R. syzygii* of phylotype IV as *R. syzygii* supsp. *syzygii*, *R. solanacearum* of phylotype IV as *R. syzygii* supsp. *indonesiensis*, and the blood disease bacterium (BDB) of phylotype IV as *R. syzygii* supsp. *celebesensis* (Safni et al., 2014).

Korean agriculture has been severely affected by bacterial wilt. This disease has been observed not only in many economically important solanaceous crops, such as potato, tomato, and pepper plants, but also in paprika, sesame, peanut, sunflower, etc (Jeong et al., 2007; Lee and Kang, 2013; Lim et al., 2008; Seo et al., 2007; Yun et al., 2004). Therefore, there have been great efforts to overcome this disease by breeding resistant varieties or detecting pathogenic bacteria using PCR-based methods (Cho et al., 2011; Han et al., 2009; Jung et al., 2014; Kang et al., 2007; Kim et al., 2016; Lee et al., 2011).

In the present study, we collected bacteria from plants affected by potato bacterial wilt in Korea, conducted various genetic analyses, and determined host range. Our results demonstrate a relationship between genetic and pathogenic traits, and form the basis for comparative genomic analyses of the RSSC.

Materials and Methods

Collection of isolates and culture conditions. *Ralstonia solanacearum* isolates were collected by Dr. Young Kee Lee (T-numbered strains) and Dr. Seungdon Lee (SL-numbered strains) from 1998 to 2003 in Korea. Among the twenty-five locations surveyed, potato bacterial wilt was observed in twelve cities in six Korean provinces (Fig. 1). We analyzed ninety-three isolates, which are listed in Table 1. All isolates were identified to *R. solanacearum* by 16S rRNA sequence analysis. These bacteria were cultured on tetrazolium chloride (TZC) agar medium (peptone 10 g, glucose 2.5 g, casamino acid 1 g, agar 18 g, TZC 50 mg in 1 L distilled water) at 28°C for 48 h and frozen in 40% glycerol stock at -70°C (Kelman, 1954).

Isolation of genomic DNA. To prepare genomic DNA, bacterial cells grown on TZC agar medium were subcultured in LB broth (peptone 10 g, yeast extract 5 g, sodium chloride 5 g in 1 l distilled water) in a 28°C shaking incu-



Fig. 1. Potato-growing areas and geographic locations infected with potato bacterial wilt in Korea from 1998-2003. Potato-growing areas are colored gray, and symbols indicate isolates: ○, phylotype I-biovar 3; ⊙, phylotype I-biovar 4; ●, phylotype IV-biovar 2. Each symbol indicates representative locations for the same geographical origin, phylotype, and biovar.

bator for 16 h. Genomic DNA of all isolates was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Phylotype identification. The ninety-three isolates were classified into phylotypes as previously described (Prior and Fegan, 2005; Sagar et al., 2014). We determined phylotype using the method of Sagar (2014) based on phylotype-specific multiplex PCR (Pmx-PCR) using the following primers: Nmult:21:1F, 5'-CGTTGATGAGGC-GCGCAATTT-3' (specific for phylotype I, amplicon size is 144 bp when paired with Nmult22:RR); Nmult:21:2F, 5'-AAGTTATGGACGGTGAAGTC-3' (phylotype II,

Table 1. Host, year, geographical origin, phylotype, biovar, a *rsaI* gene containing, pathogenicity, pathotype, and GenBank accession numbers of 16S rRNA and endoglucanase of the ninety-three *R. solanacearum* isolates used in this study

Isolate ^a	Host	Year	Geographical origin	Phylotype	Biovar	<i>rsaI</i> ^b	Pathogenicity ^b			Pathotype ^c	16S rRNA ^c	Endo-glucanase ^d	Source
							Potato	Tomato	Eggplant				
SL1870	Potato	1998	Namjeju, Jeju-do	I	4	-	+++	+++	+++	PTEP	KY119263	KY313644	This study
SL2029	Potato	1998	Namjeju, Jeju-do	IV	2	+	+++	+	-	PTE	KY119264	KY313645	Jeong 2007
SL2064	Potato	1998	Namjeju, Jeju-do	IV	2	+	+++	+++	-	PTE	KY119265	KY313646	Jeong 2007
SL2230	Potato	1998	Namjeju, Jeju-do	I	4	-	+++	+++	+++	PTEP	KY119266	KY313647	This study
SL2268	Potato	1998	Namjeju, Jeju-do	IV	2	+	+++	++	-	PTE	KY119268	KY313649	Jeong 2007
SL2313	Potato	1998	Namhae, Gyeongsangnam-do	IV	2	+	+++	-	-	P	KY119270	KY313651	Jeong 2007
SL2312	Potato	1998	Miryang, Gyeongsangnam-do	IV	2	+	+++	-	-	P	KY119269	KY313650	This study
SL2264	Potato	1998	Muan, Jeollanam-do	I	4	-	+	++	-	PTE	KY119267	KY313648	This study
SL2543	Potato	1999	Namjeju, Jeju-do	I	4	-	+++	+++	+++	PTEP	KY119272	KY313653	This study
SL3175	Potato	1999	Namjeju, Jeju-do	IV	2	+	+++	++	-	PT	KY119285	KY313666	This study
SL3150	Potato	1999	Namjeju, Jeju-do	I	4	-	+++	+++	+++	PTEP	KY119283	KY313664	This study
SL3166	Potato	1999	Bukjeju, Jeju-do	I	4	-	+++	+++	+++	PTEP	KY119284	KY313665	This study
SL3177	Potato	1999	Bukjeju, Jeju-do	I	4	-	+++	+++	+++	PTEP	KY119286	KY313667	This study
SL2330	Potato	1999	Namhae, Gyeongsangnam-do	I	3	-	+	+++	+	PTEP	KY119271	KY313652	This study
SL3112	Potato	1999	Namhae, Gyeongsangnam-do	I	3	-	++	+++	+	PTEP	KY119282	KY313663	This study
SL3022	Potato	1999	Gimhae, Gyeongsangnam-do	IV	2	+	+++	+++	-	PT	KY119276	KY313657	This study
T11	Potato	1999	Gimhae, Gyeongsangnam-do	IV	2	+	++	++	-	PTE	KY119311	KY313692	This study
T17	Potato	1999	Gimhae, Gyeongsangnam-do	IV	2	+	+++	-	-	P	KY119316	KY313697	This study
SL2729	Potato	1999	Miryang, Gyeongsangnam-do	I	4	-	++	+++	+++	PTEP	KY119275	KY313656	This study
SL2664	Potato	1999	Boseong, Jeollanam-do	I	3	-	++	+++	+	PTEP	KY119274	KY313655	This study
SL3085	Potato	1999	Boseong, Jeollanam-do	I	4	-	+++	+++	+++	PTEP	KY119279	KY313660	This study
SL3100	Potato	1999	Boseong, Jeollanam-do	I	4	-	+++	+++	+++	PTEP	KY119280	KY313661	This study
SL2610	Potato	1999	Yeonggwang, Jeollanam-do	I	4	-	++	+++	+++	PTEP	KY119273	KY313654	This study
SL3055	Potato	1999	Yeonggwang, Jeollanam-do	I	4	-	+++	+++	+++	PTEP	KY119277	KY313658	This study
SL3079	Potato	1999	Muan, Jeollanam-do	I	4	-	+++	+++	+++	PTEP	KY119278	KY313659	This study
SL3103	Potato	1999	Haenam, Jeollanam-do	I	4	-	+	+++	-	PTE	KY119281	KY313662	This study
SL3300	Potato	2000	Namjeju, Jeju-do	I	4	-	+++	+++	+++	PTEP	KY119289	KY313670	This study
SL3303	Potato	2000	Namjeju, Jeju-do	IV	2	+	+++	-	-	P	KY119290	KY313671	This study
SL3827	Potato	2000	Namjeju, Jeju-do	IV	2	+	+++	+++	-	PT	KY119302	KY313683	This study
SL3809	Potato	2000	Namjeju, Jeju-do	I	4	-	++	+++	+++	PTEP	KY119300	KY313681	This study
SL3835	Potato	2000	Namjeju, Jeju-do	I	4	-	++	+++	+++	PTEP	KY119303	KY313684	This study
T92	Potato	2000	Namjeju, Jeju-do	I	4	-	++	+++	+++	PTEP	KY119340	KY313721	This study
T93	Potato	2000	Namjeju, Jeju-do	IV	2	+	++	++	-	PTE	KY119341	KY313722	This study

Table 1. Continued

Isolate ^a	Host	Year	Geographical origin	Phylo type	Biovar <i>rsal</i> ^b	Pathogenicity ^c			Pathotype ^e	16S rRNA ^g	Endo-glucanase ^h	Source
						Potato	Tomato	Eggplant				
SL3796	Potato	2000	Bukjeju, Jeju-do	I	4	+++	+++	+++	PTEP	KY119299	KY313680	This study
SL3822	Potato	2000	Bukjeju, Jeju-do	I	4	+++	+++	+++	PTEP	KY119301	KY313682	This study
SL3760	Potato	2000	Namhae, Gyeongsangnam-do	I	4	++	+++	++	PTEP	KY119295	KY313676	This study
T14	Potato	2000	Namhae, Gyeongsangnam-do	I	4	+++	+++	+++	PTEP	KY119313	KY313694	This study
T15	Potato	2000	Namhae, Gyeongsangnam-do	I	4	++	+++	+	PTEP	KY119314	KY313695	This study
SL3762	Potato	2000	Gimhae, Gyeongsangnam-do	I	4	++	+++	+++	PTEP	KY119296	KY313677	This study
T2	Potato	2000	Gimhae, Gyeongsangnam-do	I	4	++	+++	+	PTEP	KY119309	KY313690	This study
SL3774	Potato	2000	Miryang, Gyeongsangnam-do	I	4	+++	+++	+++	PTEP	KY119297	KY313678	This study
SL3781	Potato	2000	Miryang, Gyeongsangnam-do	I	4	+++	+++	+++	PTEP	KY119298	KY313679	This study
SL3747	Potato	2000	Boseong, Jeollanam-do	I	4	++	+++	+++	PTEP	KY119293	KY313674	This study
SL3755	Potato	2000	Boseong, Jeollanam-do	I	3	+	++	+	PTEP	KY119294	KY313675	This study
SL3283	Potato	2000	Yeonggwang, Jeollanam-do	I	4	+++	+++	+++	PTEP	KY119288	KY313669	This study
SL3705	Potato	2000	Yeonggwang, Jeollanam-do	I	4	+++	+++	+++	PTEP	KY119291	KY313672	This study
SL3730	Potato	2000	Muan, Jeollanam-do	I	4	+++	+++	+++	PTEP	KY119292	KY313673	This study
SL3282	Potato	2000	Goryeong, Gyeongsangbuk-do	I	4	+	+++	++	PTEP	KY119287	KY313668	This study
SL3873	Potato	2001	Namjeju, Jeju-do	I	4	+++	+++	+++	PTEP	KY119306	KY313687	This study
T85	Potato	2001	Namjeju, Jeju-do	I	4	+	+++	+	PTEP	KY119338	KY313719	This study
T87	Potato	2001	Namjeju, Jeju-do	I	4	++	+++	+	PTEP	KY119339	KY313720	This study
T111	Potato	2001	Namjeju, Jeju-do	I	4	++	+++	+++	PTEP	KY119352	KY313733	This study
T112	Potato	2001	Namjeju, Jeju-do	I	4	+	+++	+++	PTEP	KY119353	KY313734	This study
SL3867	Potato	2001	Bukjeju, Jeju-do	IV	2	+++	-	-	P	KY119304	KY313685	This study
SL3869	Potato	2001	Bukjeju, Jeju-do	I	4	++	+++	+++	PTEP	KY119305	KY313686	This study
T104	Potato	2001	Bukjeju, Jeju-do	IV	2	+++	-	-	P	KY119348	KY313729	This study
T105	Potato	2001	Bukjeju, Jeju-do	I	4	+	+++	+++	PTEP	KY119349	KY313730	This study
T5	Potato	2001	Gimhae, Gyeongsangnam-do	I	4	++	+++	+++	PTEP	KY119310	KY313691	This study
T18	Potato	2001	Miryang, Gyeongsangnam-do	I	4	++	+++	+++	PTEP	KY119317	KY313698	This study
T77	Potato	2001	Gimje, Jeollabuk-do	I	3	+	+++	+	PTE	KY119334	KY313715	This study
T78	Potato	2001	Gimje, Jeollabuk-do	I	4	+++	+++	+++	PTEP	KY119335	KY313716	This study
T80	Potato	2001	Gimje, Jeollabuk-do	I	4	+++	+++	+++	PTEP	KY119336	KY313717	This study
SL3879	Potato	2001	Boseong, Jeollanam-do	I	4	+++	+++	+++	PTEP	KY119307	KY313688	This study
T56	Potato	2001	Boseong, Jeollanam-do	I	4	+	+++	-	PTE	KY119325	KY313706	This study
T57	Potato	2001	Boseong, Jeollanam-do	I	4	+	+++	++	PTEP	KY119326	KY313707	This study
T58	Potato	2001	Boseong, Jeollanam-do	I	4	+	+++	-	PTE	KY119327	KY313708	This study
T59	Potato	2001	Boseong, Jeollanam-do	I	4	+++	+++	++	PTEP	KY119328	KY313709	This study
T67	Potato	2001	Yeonggwang, Jeollanam-do	I	4	+++	+++	+++	PTEP	KY119331	KY313712	This study

Table 1. Continued

Isolate ^a	Host	Year	Geographical origin	Phylogroup	Biovar	Pathogenicity ^b			Pathotype ^c	16S rRNA ⁵	Endo-glucanase ¹	Source	
						Potato	Tomato	Pepper					
T46	Potato	2001	Muan, Jeollanam-do	I	4	-	++	+++	+++	PTEP	KY119322	KY313703	This study
SL3882	Potato	2001	Haenam, Jeollanam-do	I	4	-	+++	+++	+++	PTEP	KY119308	KY313689	This study
T73	Potato	2001	Haenam, Jeollanam-do	I	4	-	++	+++	+++	PTEP	KY119333	KY313714	This study
T34	Potato	2001	Goryeong, Gyeongsangbuk-do	I	4	-	++	+++	+++	PTEP	KY119320	KY313701	This study
T115	Potato	2001	Goesan, Chungcheongbuk-do	I	4	-	+++	+++	+++	PTEP	KY119354	KY313735	This study
T117	Potato	2001	Goesan, Chungcheongbuk-do	I	4	-	+++	+++	+++	PTEP	KY119355	KY313736	This study
T95	Potato	2003	Namjeju, Jeju-do	IV	2	+	+++	+	+++	PTE	KY119342	KY313723	This study
T96	Potato	2003	Namjeju, Jeju-do	IV	2	+	+++	-	+++	P	KY119343	KY313724	This study
T98	Potato	2003	Namjeju, Jeju-do	IV	2	+	+++	-	+++	PT	KY119344	KY313725	This study
T99	Potato	2003	Namjeju, Jeju-do	I	3	-	+	+++	+++	PTEP	KY119345	KY313726	This study
T100	Potato	2003	Namjeju, Jeju-do	I	4	-	++	+++	+++	PTEP	KY119346	KY313727	This study
T101	Potato	2003	Namjeju, Jeju-do	IV	2	+	+++	-	+++	P	KY119347	KY313728	This study
T109	Potato	2003	Bukjeju, Jeju-do	I	4	-	+	+++	+++	PTEP	KY119350	KY313731	This study
T110	Potato	2003	Bukjeju, Jeju-do	I	3	-	+	+++	+	PTEP	KY119351	KY313732	This study
T12	Potato	2003	Namhae, Gyeongsangnam-do	IV	2	+	+++	-	+++	P	KY119312	KY313693	This study
T16	Potato	2003	Namhae, Gyeongsangnam-do	I	4	-	+++	+++	+++	PTEP	KY119315	KY313696	This study
T24	Potato	2003	Miryang, Gyeongsangnam-do	IV	2	+	++	-	+++	P	KY119318	KY313699	This study
T25	Potato	2003	Miryang, Gyeongsangnam-do	I	3	-	++	+++	+	PTEP	KY119319	KY313700	This study
T82	Potato	2003	Gimje, Jeollabuk-do	IV	2	+	+++	-	+++	P	KY119337	KY313718	This study
T50	Potato	2003	Boseong, Jeollanam-do	I	4	-	+++	+++	+++	PTEP	KY119323	KY313704	This study
T51	Potato	2003	Boseong, Jeollanam-do	IV	2	+	+++	+	+++	PTE	KY119324	KY313705	This study
T60	Potato	2003	Yeonggwang, Jeollanam-do	I	4	-	+++	+++	+++	PTEP	KY119329	KY313710	This study
T61	Potato	2003	Yeonggwang, Jeollanam-do	I	4	-	+++	+++	+++	PTEP	KY119330	KY313711	This study
T42	Potato	2003	Muan, Jeollanam-do	I	4	-	+++	+++	+	PTEP	KY119321	KY313702	This study
T70	Potato	2003	Haenam, Jeollanam-do	I	4	-	+	+++	+++	PTEP	KY119332	KY313713	This study

^aIsolate: SL-numbering isolates were obtained from Dr. Seungdon Lee and T-numbering isolates were from Dr. Young Kee Lee.

^b*rsal*: PCR was carried out using two primer pairs; one pair produced a 720-bp fragment containing full *rsal* gene including promoter (720-rsa1F/720-rsa1R) at 70°C annealing temperature, and another produced a 315-bp fragment containing partial *rsal* ORF region (315-rsa1F/315-rsa1R) at 59°C annealing temperature.

^cPathogenicity: Symptoms were recorded at 28 days post inoculation using the following scale: -, no symptoms; +, one to three leaves wilted; ++, four to six leaves wilted; and +++, seven more leaves or whole plant wilted.

^dPathotype: (P) only pathogenic on potato and nonpathogenic on tomato, eggplant and pepper, (PT) pathogenic on potato and tomato, and nonpathogenic on eggplant and pepper, (PTE) pathogenic on potato, tomato, and eggplant, and nonpathogenic on pepper, and (PTEP) pathogenic on all tested crops – potato, tomato, eggplant, and pepper.

⁵16S rRNA: GenBank accession number of 16S rRNA.

¹endoglucanase: GenBank accession number of endoglucanase gene.

372 bp); Nmolt:23:AF, 5'-ATTACGAGAGCAATC-GAAAGATT-3' (phylogroup III, 91 bp); Nmolt:22:InF, 5'-ATTGCCAAGACGAGAGAAGTA-3' (phylogroup IV, 213 bp), and Nmolt:22:RR, 5'-TCGCTTGACCCTATA-ACGAGTA-3' (reverse primer for all phylogroups). PCR reactions were carried out in a total volume of 20 µl with Profi-Premix (Bioneer) with primer sets and genomic DNA in an automated thermocycler (model PTC-200, MJ Research Inc., Waltham, MA, USA) as follows: initial denaturation at 96°C for 5 min, followed by 30 cycles of denaturation at 95°C for 15 sec, annealing at 59°C for 15 sec, and extension at 72°C for 30 sec, with a final extension at 72°C for 10 min. Seven microliters of each PCR product was examined by electrophoresis through 1% agarose gel, stained with ethidium bromide, and visualized on a UV-trans-illuminator.

Biovar determination. The ability of each isolate to oxidize three disaccharides (maltose, lactose, and cellobiose) and three hexose alcohols (mannitol, sorbitol, and dulcitol) was evaluated by inoculating the isolates on biovar plates following a modified Hayward method (Hayward, 1964). Along with the isolates, 1 ml of the basal medium (NH₄H₂PO₄ 1 g, KCl 0.2 g, MgSO₄·7H₂O 0.2 g, peptone 1 g, bromothymol blue 8 mg, agar 1.5 g in distilled water 1 l, pH 7.1) containing 1% sterilized carbon sources (maltose, lactose, cellobiose, mannitol, sorbitol, and dulcitol) was dispensed into the wells of 24-well plates (SPL Life Sciences, Seoul, Korea). All isolates were inoculated into individual wells with 5 µl of the 10⁸ cfu/ml bacterial suspensions, with two replicates per isolate. The plates were incubated at 28°C for 14 days. The plates were observed every day and the color change of the medium was recorded two weeks after inoculation. The test was repeated twice.

Detection of *rsaI* gene. We carried out PCR to validate the presence of *rsaI*, which has been reported to be a gene for avirulence of *R. solanacearum* on pepper hosts (Jeong et al., 2011). To eliminate errors, we prepared two *rsaI* gene primer pairs. One pair produced a 720-bp fragment containing the full *rsaI* gene including the promoter region (720-*rsaI*F; 5'-GCCGCTCGCCGCAATGCTGCC-3', 720-*rsaI*R; 5'-TGGGCTGGGTGGGACTTAACC-3'), and the other produced a 315-bp fragment containing a partial *rsaI* open reading frame (ORF) region (315-*rsaI*F; 5'-ATCACCAAGATTACCGGAAAG-3', 315-*rsaI*R 5'-TGGGCTGGGTGGGACTTAACC-3'). The reactions were carried out as described previously for phylogroup determination with a primer set of 720-*rsaI*F/720-*rsaI*R at an annealing temperature of 70°C, and with a primer set of

315-*rsaI*F/315-*rsaI*R at an annealing temperature of 59°C.

Phylogenetic analysis of 16S rRNA and partial endoglucanase (*egl*) gene sequences. The 16S rRNA genes of the 93 isolates were amplified by PCR in 25 µl reaction volumes containing 1.25 U of Pfu Turbo DNA Polymerase (Stratagene), 2.5 µl of 10× Pfu polymerase buffer, 0.25 mM of each dNTP, 1 µl of 10 pmoles of each primer (9F, 5'-GAGTTTGATCCTGGCTCAG-3'; 1512R, 5'-ACG-GCTACCTTGTTACGACTT-3'), and 50 ng of genomic DNA. The reaction was performed in an automated thermocycler (model PTC-200, MJ Research Inc.) with initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 45 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The 750-bp partial endoglucanase gene was amplified by PCR in the same reaction as the 16S rRNA gene with primer pairs of Endo-F (5'-ATGCATGCGCTGGTCCCGC-3') and Endo-R (5'-GCGTTGCCCGCACGAACACC-3') (Poussier et al., 2000). After the PCR amplicons of 16S rRNA and endoglucanase genes were confirmed by electrophoresis, the sequences of the two genes were determined using an ABI BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI3730XL automated DNA sequencer (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's instructions. The 16S rRNA and endoglucanase gene sequences were confirmed and edited with BioEdit 7.2.5 software and trimmed with EditSeq software (DNASTAR Lasergene8, DNASTAR Inc., Madison, WI, USA). The analyzed 16S rRNA and partial endoglucanase gene sequences were deposited in the GenBank database, and the accession numbers are listed in Table 1. Reference sequences for the 16S rRNA gene and endoglucanase gene were obtained from the NCBI website with the following GenBank accession numbers: AL646052.1 (GMI1000), FP885891.2 (PSI07), FP885896.1 (CMR15), FP885897.1 (CFBP2957), and CP002820.1 (Po82). The ClustalW method was used for sequence alignment and the construction of phylogenetic trees using 1,000 bootstrap replicates in the MegAlign program (DNASTAR Lasergene8, DNASTAR Inc.).

Host range determination. For the pathogenicity test, tomato (*Lycopersicon esculentum* cv. Seokwang) (Lee and Kang, 2013), eggplant (*Solanum melongena* cv. Heukmajang) (Lee, 1999), and pepper (*Capsicum annuum* cv. Nokkwang) (Jeong et al., 2007) were grown in a greenhouse at 25-35°C under natural light conditions. For the positive control, potatoes (*Solanum tuberosum* cv. Sumi) (the original cultivar name: Superior) (Park et al., 2016) were

grown in a greenhouse at 20–25°C. Two-week-old seedlings of eggplant and pepper, and 10-day-old seedlings of tomato were transplanted into plastic pots 7 cm in diameter containing commercial soil (Baroker, Seoul Agriculture Materials Co., Seoul, Korea) and grown in a greenhouse for 2–3 weeks. To prepare the inoculum, all isolates were grown on TZC plates for 48 h at 28°C. Bacterial cells were suspended in sterile distilled water and the concentration was adjusted to OD_{600} 0.1. After wounding the root of each plant by stabbing with a 3-cm-wide scoop, 50 ml of bacterial suspension of the 93 isolates was poured into each pot. Three plants from each crop were inoculated with each isolate, and pathogenicity assays were repeated two or three times in a greenhouse under natural light conditions. Symptom development was observed every 3 days and recorded at 28 days post-inoculation using the following

scale: –, no symptoms; +, one to three leaves wilted; ++, four to six leaves wilted; and +++, seven or more leaves or whole plant wilted.

The tomato plants were photographed at 12 days post-inoculation, and the eggplants and pepper plants were photographed at 19 days post-inoculation.

Results

Determination of biovar, phylotype, and *rsaI* gene. The Korean potato bacterial wilt isolates were analyzed for biovar, phylotype, and presence of the *rsaI* gene. Of the 93 isolates analyzed, twenty-two were of biovar 2 (about 24%), eight were of biovar 3 (less than 8%), and sixty-three were of biovar 4 (68%) (Table 1). The isolates of biovar 3 and biovar 4 were classified as phylotype I, and biovar 2

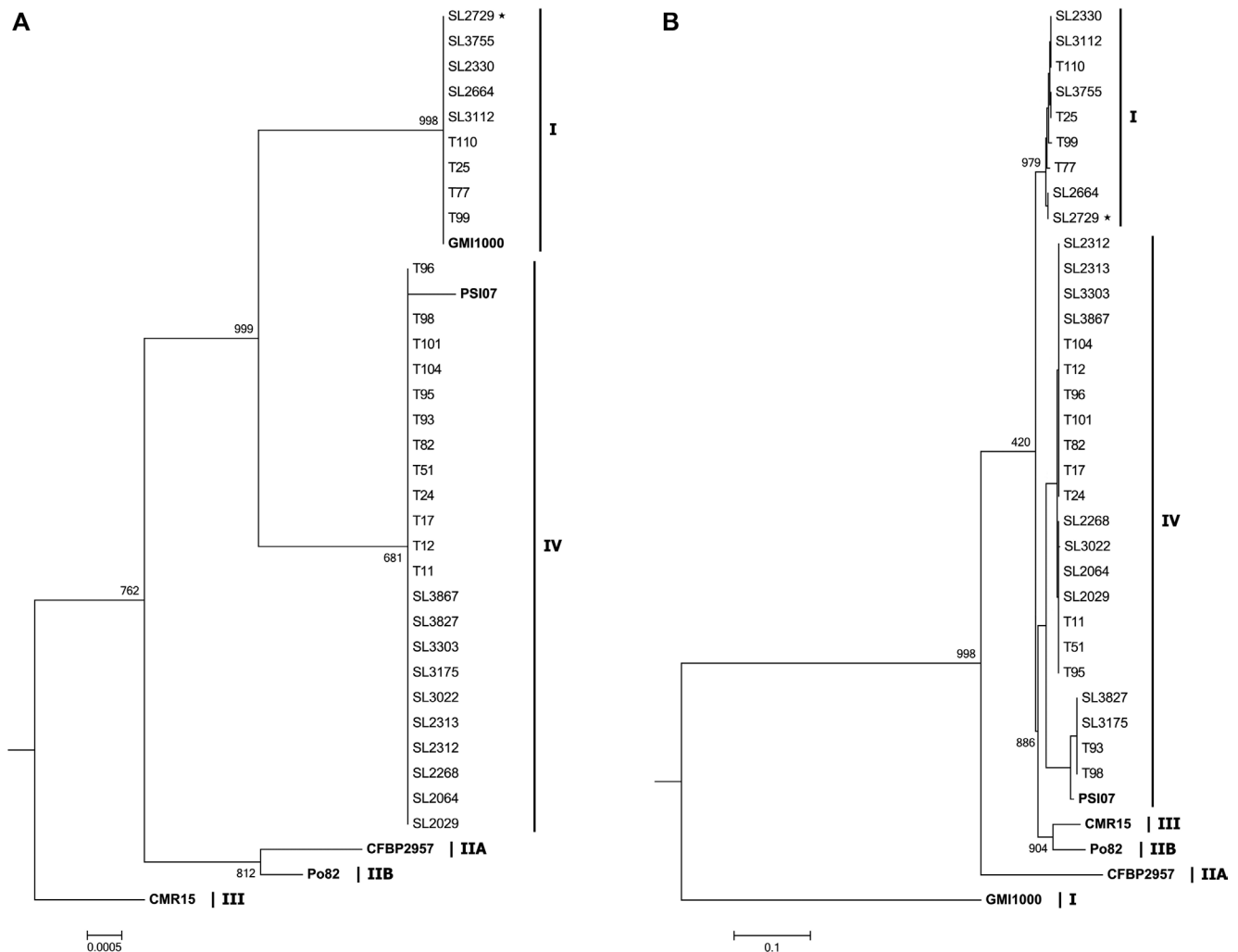


Fig. 2. Phylogenetic trees of 16S rRNA (A) and partial endoglucanase (*egl*) gene sequences (B) analyzed by ClustalW with 1,000 bootstrap replicates using MegAlign of DNASTAR Lasergene 8 (DNASTAR Inc.). Roman numerals indicate phylotypes and symbols (★) indicate the representative biovar 4 strain.

was classified as phylotype IV.

It has been reported that the Rsa1 protein, which is an aspartic protease secreted through the type II secretion systems (T2SSs), is responsible for the loss of bacterial virulence to pepper (Jeong et al., 2011). To validate the presence of this gene, we carried out *rsa1* detection PCR. For greater certainty, we used two sets of *rsa1* gene primers. One pair was for the full *rsa1* gene including the promoter (720-*rsa1*F/720-*rsa1*R), while the other was for a partial *rsa1* ORF (315-*rsa1*F/315-*rsa1*R). From the ninety-three isolates of potato bacterial wilt, all phylotype IV-biovar 2 isolates produced a full 720-bp *rsa1* gene fragment at an annealing temperature of 70°C. On the other hand, biovar 3 and biovar 4 isolates of phylotype I did not produce this fragment under these annealing conditions. Partial *rsa1* gene PCR was carried out with a 315-*rsa1*F/315-*rsa1*R primer set at an annealing temperature of 59°C, and the results were the same as for the PCR of the full *rsa1* gene, which was detected in all isolates of phylotype IV and not detected in any isolates of phylotype I.

Geographical distribution. Fig. 1 shows the geographical distribution of the potato bacterial wilt isolates in Korea from 1998 to 2003. *Ralstonia solanacearum* was first isolated in southern provinces (Gyeongsangnam-do, Jeollanam-do, and Jeju-do, Korea) with biovar 2 and biovar 4 isolates in 1998. The biovar 3 bacterium, which infects potato, was first identified in Gyeongsangnam-do in 1999,

and was subsequently found in Jeollanam-do, Jeollabuk-do, and Jeju-do. Biovar 4 bacteria that infect potatoes have been isolated in all locations where potato bacterial wilt has been observed. *R. solanacearum* of biovar 2 has been isolated in seven locations, which are the same as the regions where biovar 3 bacteria have been isolated.

Phylogenetic analysis of 16S rRNA and partial endoglucanase (*egl*) gene sequences. To evaluate the genetic relationship of Korean potato isolates, we sequenced the 16S rRNA gene (about 1,453 nucleotides) and partial endoglucanase gene (760 and 766 nucleotides) of 93 isolates (Table 1) and compared the sequences using the ClustalW program (Fig. 2).

The 16S rRNA sequences of biovar 2 isolates were identical with each other, and the sequences of biovar 3 and biovar 4 isolates were also identical. To downsize the phylogenetic tree, SL2729 was chosen to represent the biovar 4 strains (Fig. 2-A). In the 16S rRNA phylogenetic tree, Korean *R. solanacearum* isolates were separated into two groups following GMI1000 (phylotype I) and PSI07 (phylotype IV). Korean biovar 2 isolates, which were identified as phylotype IV, were clustered with PSI07 of a representative phylotype IV strain. The isolates of biovar 3 and 4, which were identified as phylotype I, were not only clustered with GMI1000, but also identical with GMI1000 in the sequenced 1,453 nucleotides.

Since the partial endoglucanase gene (*egl*) sequences

Table 2. Host range of Korean *R. solanacearum* isolates

Pathotype	Pathogenicity				Phylotype-Biovar	No. of isolates	List of isolates
	Potato	Tomato	Eggplant	Pepper			
P	+	-	-	-	IV-2	11	SL2312, SL2313, SL3303, SL3867, T12, T17, T24, T82, T96, T101, T104
PT	+	+	-	-	IV-2	4	SL3022, SL3175, SL3827, T98
					IV-2	7	SL2029, SL2064, SL2268, T11, T51, T93, T95
PTE	+	+	+	-	I-3	1	T77
					I-4	4	SL2264, SL3103, T56, T58
					I-3	7	SL2330, SL2664, SL3112, SL3755, T25, T99, T110
PTEP	+	+	+	+	I-4	59	SL1870, SL2230, SL2543, SL2610, SL2729, SL3055, SL3079, SL3085, SL3100, SL3150, SL3166, SL3177, SL3282, SL3283, SL3300, SL3705, SL3730, SL3747, SL3760, SL3762, SL3774, SL3781, SL3796, SL3809, SL3822, SL3835, SL3869, SL3873, SL3879, SL3882, T2, T5, T14, T15, T16, T18, T34, T42, T46, T50, T57, T59, T60, T61, T67, T70, T73, T78, T80, T85, T87, T92, T100, T105, T109, T111, T112, T115, T117
Total						93	

were identical among biovar 4 isolates, we analyzed the *egl* sequence of SL2729 as the representative strain of biovar 4 (Fig. 2B). While the sequences of biovar 4 isolates were identical, the *egl* sequences of biovar 3 isolates showed subtle differences in nucleotides. In a phylogenetic tree based on the *egl* gene, biovar 3 and biovar 4 isolates were grouped to phylotype I without GMI1000, which was placed as the outgroup to Korean phylotype I.

Host range determination. To determine the host range of the bacteria that cause potato bacterial wilt, we assessed the pathogenicity of isolates on several solanaceous plants—potato, tomato, eggplant, and pepper. The patterns of pathogenicity were divided into four types: first, pathogenic only to potato, but nonpathogenic to tomato, eggplant, and pepper (P); second, pathogenic to potato and tomato, but nonpathogenic to eggplant and pepper (PT); third, pathogenic to potato, tomato, and eggplant, but nonpathogenic to pepper (PTE); and finally, pathogenic on all four tested crops (potato, tomato, eggplant, and pepper, PTEP) (Table 2).

The isolates of phylotype IV—biovar 2 showed various patterns of host pathogenicity, including P, PT, and PTE. Of the twenty-two biovar 2 isolates, eleven infected only potato (P), four infected only potato and tomato (PT), and seven infected potato, tomato, and eggplant (PTE). These results showed that none of the biovar 2 isolates could in-

fect pepper. The isolates of phylotype I (including biovar 3 and 4) could be divided into two groups: pathogenic to potato, tomato, and eggplant, but nonpathogenic on pepper (PTE); and pathogenic to all test plants (PTEP). For phylotype I, one biovar 3 isolate and four biovar 4 isolates were classified as PTE (7% of phylotype I), and seven biovar 3 and fifty-nine biovar 4 isolates were classified as PTEP (93% of phylotype I). Fig. 3 shows the host range of representative pathotype strains.

Discussion

When potato bacterial wilt was first reported in Korea, we surveyed potato-growing regions from 1998 to 2003, isolated bacteria, and characterized them using various genetic and pathogenic tests. During this period, among the twenty-five potato cultivation areas, potato bacterial wilt was observed in twelve locations in southern region of Korea. By 16S rRNA sequence analysis and BLAST search, ninety-three bacteria were identified as *R. solanacearum*. These isolates were analyzed to determine their phylotype, biovar, phylogenetic relationship of 16S rRNA and *egl* gene, presence of an *rsaI* gene, and host range on major solanaceous crops—potato, tomato, eggplant, and pepper.

In Korea, the potato bacterial wilt isolates were classified into biovars 2, 3, and 4. When applied to the phylotype

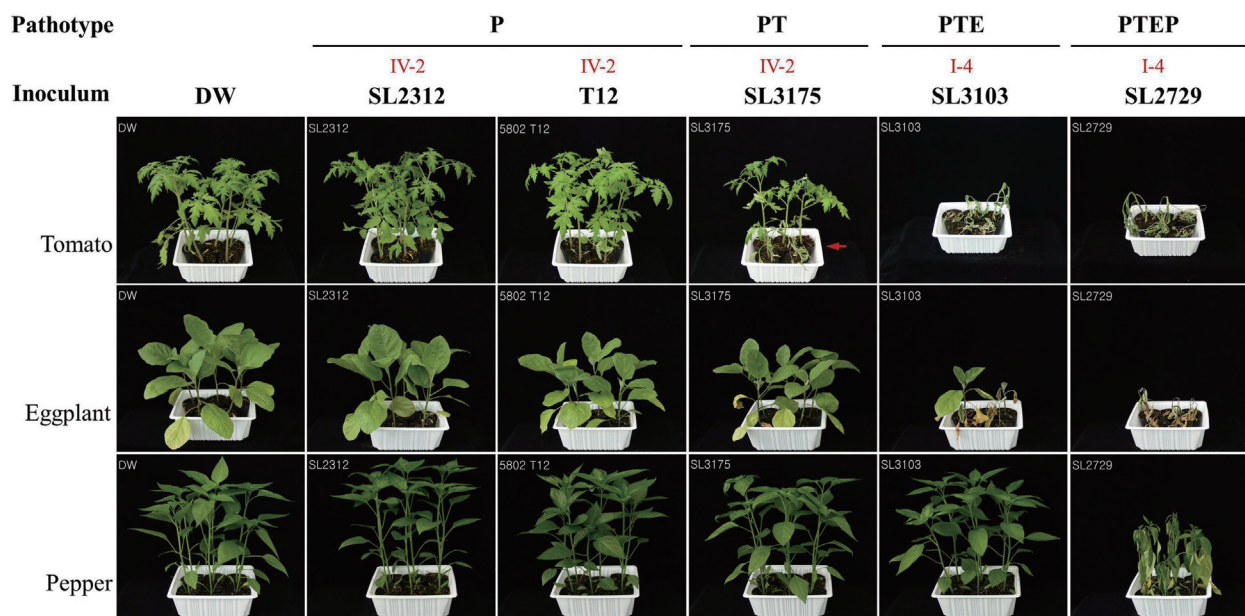


Fig. 3. Host range determination of *Ralstonia solanacearum* on tomato, eggplant, and pepper. SL2312 and T12 were not pathogenic to tomato, eggplant, and pepper (pathotype P); SL3175 was pathogenic to tomato, and not pathogenic to eggplant or pepper (PT); SL3103 was pathogenic to tomato and eggplant, and not pathogenic to pepper (PTE); and SL2729 was pathogenic to all tested plants (PTEP). IV-2, phylotype IV—biovar 2; I-4, phylotype I—biovar 4. Pictures of tomato were taken at 12 DPI, and pictures of eggplant and pepper were taken at 19 DPI.

scheme, biovar 3 and 4 isolates belonged to phylotype I (Asian origin). All biovar 2 isolates belonged to phylotype IV (Indonesian origin), and none belonged to phylotype II (American origin).

The sequence variation of isolates from biovars 3 and 4 differed between the 16S rRNA and *egl* gene sequences. For 16S rRNA gene sequences, biovar 3 and 4 isolates were identical and built a phylotype I cluster with the representative strain GMI1000. For *egl* gene sequences, while all biovar 4 isolates were identical, biovar 3 isolates had variations in some nucleotides. Surprisingly, the representative phylotype I strain GMI1000 was distinguished from Korean biovar 3 and 4 isolates and was placed outside of the Korean phylotype I clade. On the other hand, Korean biovar 2 isolates were consistently located in the phylotype IV clade with the representative strain PSI07, either in the 16S rRNA tree or in the *egl* tree. These results may suggest that Korean biovar 2 strains were introduced from a foreign country relatively recently, and that Korean biovar 3 and 4 strains represent the Asian-origin phylotype I, which evolved differentially from GMI1000.

The *egl* gene was used for “sequevar” determination of the phylotype-sequevar classification, as discussed by Fegan and Prior (2005). The evolutionary dynamics of RSSC have previously been revealed by phylogenetic and statistical analysis of housekeeping and virulence-related genes by Castillo and Greenberg (2007). Among virulence-related genes, *hrpB* and *fliC*, which are essential for species survival, have undergone purifying selection, like essential housekeeping genes. On the other hand, *egl*, which is directly related to pathogenicity, has undergone diversifying selection with a high level of recombination. The divergence of *egl* between Korean phylotype I and GMI1000 was attributed by Castillo and Greenberg (2007) to geographic isolation.

Regarding the recent introduction of Korean phylotype IV, Jeong et al. (2007) discussed the possibility of the import of phylotype IV strains from Japan. In phylogenetic analysis of 16S rDNA, *egl*, and *hrpB*, Korean phylotype IV isolate (SL2029) clustered with Japanese (MAFF301558, MAFF301559) and Indonesian (R142) phylotype IV strains. However, Korean SL2029 was closer to Japanese MAFF301558 and MAFF301559 than to Indonesian R142, which suggests that the Korean and Japanese lineages have diverged more recently than the Japanese and Indonesian lineages. Korean phylotype IV strains appeared after import of the potato cultivar Daeji (Japanese variety name: Dejima) from Japan in the 1990s. It is reasonable to infer that phylotype IV ingressed with the import of Daeji, since the timing of Daeji cultivation in southern region of Korea

is consistent with the emergence of *R. solanacearum* phylotype IV.

Among biovars 2, 3, and 4, biovar 4 is the most common in Korea and was found in all regions of potato bacterial wilt. Most biovar 4 isolates (93.6%) were pathogenic to all of the tested solanaceous plants (potato, tomato, eggplant, and pepper). The distribution and pathogenicity results are consistent with a previous report that biovar 4 was predominant in other crops in Korea (Jeong et al., 2007). It seems that the high humidity and temperatures of the Korean summer are suitable for biovar 4 outbreaks, and the cultivation season of susceptible crops permits the spread of the pathogens. In light of these results, biovar 4 is considered the main and most destructive pathovar; hence, we should monitor biovar 4 to predict and prevent the spread of bacterial wilt from potato to other crops, or vice versa.

Korean biovar 2 isolates were genetically and pathologically distinct from biovar 3 and 4. All biovar 2 isolates were classified as phylotype IV, and clustered with phylotype IV reference strain PSI07 in 16S rRNA and in *egl* phylogenetic trees. Furthermore, only biovar 2 isolates contained the *rsal* gene, which confers avirulence to pepper-infecting strains (Jeong et al., 2011). This result is consistent with our host-determining pathogenicity assays, which showed that all biovar 2 isolates were nonpathogenic to pepper. We also identified tomato-nonpathogenic biovar 2 isolates. In the course of pathogenicity assays, we observed that tomato plants were the most susceptible among all hosts, which is consistent with previous reports (Ramesh et al., 2014; Sakthivel et al., 2016). However, some biovar 2 isolates could not infect all tested plants (tomato, eggplant, and pepper), but only potato, which was the original host plant. The nonpathogenic strains on tomato, eggplant, and pepper were reported previously: R288 (phylotype I) isolated from *Morus alba* in China and MAFF211266 (phylotype I) and MAFF301558 (phylotype IV) isolated from *Solanum lycopersicum* in Japan (Lebeau et al., 2011). However, the tested cultivars (tomato L390, eggplant Florida Market, and pepper Yolo Wonder) in these earlier reports differed from those in the present study (tomato Seokwang, eggplant Heukmajang, and pepper Nokkwang), and did not include potato. Therefore, the tomato-nonpathogenic isolates used in the present study are important as a genetic resource.

From this study, we also obtained the groups of eggplant-pathogenic and nonpathogenic isolates and pepper-pathogenic and nonpathogenic isolates. These groups could be the materials for investigating of eggplant-specific (or pepper-specific) infection factors. The genomic difference between the tomato-pathogenic and tomato-nonpathogenic biovar 2 groups may provide a clue to host specificity on

tomato. Therefore we aim to conduct further comparative analyses of the tomato pathogenic and nonpathogenic genomes.

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