Genetic Diversity and Distribution of Korean Isolates of *Ralstonia solanacearum*

Yeonhwa Jeong, Jinwoo Kim, and Yongsung Kang, Department of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Korea; **Seungdon Lee**, Plant Pathology Division, National Institute of Agricultural Science of Technology, Rural Development Administration, Suwon 441-707, Korea; and **Ingyu Hwang**, Department of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Korea

ABSTRACT

Jeong, Y., Kim, J., Kang, Y., Lee, S., and Hwang, I. 2007. Genetic diversity and distribution of Korean isolates of *Ralstonia solanacearum*. Plant Dis. 91:1277-1287.

Genetic diversity among 478 isolates of *Ralstonia solanacearum* collected from various plants in Korea between 1997 and 2005 was determined based on biovar, pathogenicity, amplified fragment length polymorphism (AFLP), 16S rRNA, endoglucanase, *hrpB*, and *mutS* gene sequence analyses. Of the isolates, 440 belonged to biovars 1, 3, or 4, and 38 belonged to biovar 2. Biovar N2 isolates were not found. The biovar 1 and 2 isolates were found mainly in southern Korea, whereas the biovar 3 and 4 isolates were widely distributed throughout all nine provinces. AFLP analysis divided the 109 representative Korean isolates into six clusters that were distinct from most of the foreign isolates. Grouping of 8 representative isolates based on their 16S rRNA gene sequences indicated that biovars 1, 3, and 4 belonged to division 1, while biovar 2 belonged to subdivision 2b. Sequence analysis of the endoglucanase, *hrpB*, and *mutS* genes from the same isolates indicated that the biovar 1, 3, and 4 isolates belonged to phylotype I, while the biovar 2 isolate belonged to phylotype IV. This study is the first comprehensive analysis of genetic diversity among Korean isolates of *R. solanacearum*.

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most important bacterial plant diseases in the world's tropical, subtropical, and warm temperate regions (10). The bacterium infects many economically important crops, including tomato (*Lycopersicon esculentum* Mill.), potato (*Solanum tuberosum* L.), pepper (*Capsicum annuum* L.), tobacco (*Nicotiana tabacum* L.), eggplant (*Solanum melongena* L.), banana (*Musa paradisiaca* L.), ginger (*Zingiber officinale* Roscoe), and peanut (*Arachis hypogaea* L.) (10).

The species *R. solanacearum* is a complex taxonomic unit with broad physiological and genetic diversity; therefore, it has been divided into seven biovars (based on biochemical properties) and five races (based on differences in host range) (10,11). Unlike other phytopathogenic bacteria, race systems of *R. solanacearum* are not based on gene-for-gene interactions (i.e., different cultivars carrying different *R* gene[s]). Instead, they are based on the pathogenicity of each isolate in different kinds of host plants (3,10). Although the biovar and race systems are widely accepted for the classification of *R. solana-*

cearum, there is no definite correlation between biovar and race: each race transects the biovars and each biovar contains various races (9,10,11). The only positive correlation between the biovar and race systems exists for biovar 2 and race 3 (10,26).

To circumvent such classical taxonomic methods and improve our understanding of the genetic relatedness among *R. solanacearum* strains, DNA-based analyses such as restriction fragment length polymorphisms (RFLP), polymerase chain reaction (PCR)-RFLP, 16S rRNA gene sequence analysis, repetitive extragenic palindromic (REP)-PCR, and amplified fragment length polymorphism (AFLP) are now used (6,8,13,21). RFLP analysis of *R. solanacearum* isolates divided the species into two major groups that reflected their geographical origins (4,5,6). The first group, called the Americanum division, contains biovar 1, 2, and N2 isolates, while the second group, the Asiaticum division, contains biovar 3, 4, and 5 isolates (4,5,6). Further analysis by 16S rRNA gene sequencing confirmed the results of the RFLP analysis and separated division 2 into subdivisions, which included the Indonesian isolates (19,21,25,27). Subgroup specific primers were designed using the sequences of the 16S-23S intergenic spacer region of the rRNA gene, the polygalacturonase gene, and the endoglucanase gene (8), but the resulting classification system was not fully consistent with the results of a PCR-RFLP analysis based on the *hrp* gene in many African biovar 1

isolates that assigned them to the Asiaticum division (22). REP-PCR analysis using different primer sets with 16S rRNA gene sequence analysis yielded the genomic fingerprints of several Japanese strains (12,13); however, the grouping obtained by this method failed to reflect the diverse hosts and geographical origins from which the isolates were obtained. Therefore, AFLP analysis has been used as an alternative DNA fingerprinting tool to classify *R. solanacearum* isolates at a subspecies level (21,32). AFLP-based analysis of *R. solanacearum* isolates collected worldwide (e.g., African, American, and Japanese) produced seven clusters reflecting the biovars and their geographical origins (21).

In 2005, a new hierarchical classification scheme based on sequence analyses of the endoglucanase, *hrpB*, and *mutS* genes was proposed (7,23). This new scheme divided the *R. solanacearum* species complex into four phylotypes. The isolates in phylotype I originate in Asia, whereas those in phylotype II are primarily from America. Phylotype III mainly comprises isolates from Africa and its surrounding islands, while those in phylotype IV are from Indonesia, Japan, and Australia (7). Phylotypes can be subgrouped into sequence variants, which are groups of isolates with highly conserved DNA sequences (7).

Bacterial wilt is a serious and chronic disease in tomato, potato, tobacco, eggplant, and pepper plants during the growing season in Korea, when the temperature and moisture levels are high (31); however, the genetic diversity among Korean *R. solanacearum* isolates is unknown. Thus, we investigated the genetic diversity of Korean isolates of *R. solanacearum* to create a race and biovar distribution map and improve the management of bacterial wilt. Between 1997 and 2005, we collected 478 Korean isolates of *R. solanacearum* and 16 foreign isolates from diverse geographical locations and from different hosts during the growing season. Then, after determining the biovars and races of all 478 isolates, we analyzed 125 representative isolates by AFLP and determined the 16S rRNA, endoglucanase, *hrpB*, and *mutS* gene sequences for 8 representative isolates. We found that the Korean isolates comprised six AFLP clusters that were distinct from the foreign isolates, and our

Corresponding author: Ingyu Hwang
E-mail: ingyuh@snu.ac.kr

Accepted for publication 9 May 2007.

doi:10.1094/PDIS-91-10-1277

© 2007 The American Phytopathological Society

16S rRNA, endoglucanase, *hrpB*, and *mutS* gene sequence analyses revealed two divisions and two phylotypes.

MATERIALS AND METHODS

Collection of isolates and culture conditions. Four hundred and seventy-eight *R.*

solanacearum isolates were collected from wilted tomato, pepper, eggplant, potato, perilla, sesame, paprika, peanut, pimiento, and sunflower plants at 90 different locations in 9 Korean provinces (Gangwon-do, Gyeonggi-do, Chungcheongbuk-do, Chungcheongnam-do, Jeollabuk-do, Jeollanam-

do, Gyeongsangbuk-do, Gyeongsangnam-do, and Jeju-do) between 1997 and 2005. The 109 representative isolates used for further analysis are listed in Table 1. All of the isolates were purified on tetracycline (TZC) agar medium, frozen in 20% glycerol and stored at

Table 1. Geographical origin, biovar, race, and amplified fragment length polymorphism (AFLP) group of *Ralstonia solanacearum* isolates used in this study

Isolate ^x	Geographical origin	Host	Biovar	Race	AFLP cluster	Source ^y
SL312	Gwangju, Gyeonggi-do	Tomato	3	1	III	This study
SL341	Gimcheon, Gyeongsangbuk-do	Tomato	3	1	III	This study
SL535	Jincheon, Chungcheongbuk-do	Tomato	4	1	III	This study
SL776	Goseong, Gyeongsangnam-do	Tomato	3	1	III	This study
SL1020	Suwon, Gyeonggi-do	Tomato	4	1	III	This study
SL1085	Cheorwon, Gangwon-do	Tomato	4	1	III	This study
SL1737	Boseong, Jeollanam-do	Tomato	4	1	III	This study
SL1743	Haman, Gyeongsangnam-do	Tomato	4	1	III	This study
SL1971	Suwon, Gyeonggi-do	Tobacco	3	1	VI	This study
SL1977	Pohang, Gyeongsangbuk-do	Tobacco	3	1	VI	This study
SL2025	Boryeong, Chungcheongnam-do	Sesame	3	1	I	This study
SL2029	Namjeju, Jeju-do	Potato	2	3	V	This study
SL2064	Namjeju, Jeju-do	Potato	2	3	V	This study
SL2078	Namjeju, Jeju-do	Potato	2	3	V	This study
SL2249	Namjeju, Jeju-do	Potato	1	1	I	This study
SL2268	Namjeju, Jeju-do	Potato	2	3	V	This study
SL2282	Namjeju, Jeju-do	Potato	2	3	V	This study
SL2308	Damyang, Jeollanam-do	Tomato	2	3	V	This study
SL2313	Namhae, Gyeongsangnam-do	Potato	2	3	V	This study
SL2316	Miryang, Gyeongsangnam-do	Potato	2	3	V	This study
SL2327	Namhae, Gyeongsangnam-do	Potato	4	1	I	This study
SL2665	Boseong, Jeollanam-do	Potato	2	3	V	This study
SL2673	Boseong, Jeollanam-do	Potato	2	3	V	This study
SL2680	Boseong, Jeollanam-do	Potato	2	3	V	This study
SL2707	Gimhae, Gyeongsangnam-do	Potato	2	3	V	This study
SL3112	Namhae, Gyeongsangnam-do	Potato	2	3	V	This study
SL3179	Bukjeju, Jeju-do	Potato	2	3	V	This study
SL3202	Namjeju, Jeju-do	Tomato	2	3	V	This study
SL3291	Namjeju, Jeju-do	Potato	2	3	V	This study
SL3505	Changnyeong, Gyeongsangnam-do	Pepper	4	1	I	This study
EW4	Hongcheon, Gangwon-do	Eggplant	3	1	II	This study
EW7	Geoje, Gyeongsangnam-do	Eggplant	4	1	II	This study
EW9	Gimhae, Gyeongsangnam-do	Eggplant	4	1	II	This study
PW5	Yanggu, Gangwon-do	Pepper	3	1	II	This study
PW16	Hampyeong, Jeollanam-do	Pepper	4	1	II	This study
PW17	Cheongdo, Gyeongsangbuk-do	Pepper	4	1	I	This study
PW22	Jeongeup, Jeollabuk-do	Pepper	4	1	II	This study
PW23	Gochang, Jeollabuk-do	Pepper	4	1	II	This study
PW33	Chilgok, Gyeongsangbuk-do	Pepper	4	1	I	This study
PW40	Yeonggwang, Jeollanam-do	Pepper	3	1	II	This study
PW44	Chuncheon, Gangwon-do	Pepper	4	1	II	This study
PAW1	Hwasun, Jeollanam-do	Paprika	4	1	VI	This study
PAW2	Tongyeong, Gyeongsangnam-do	Paprika	4	1	VI	This study
POW1	Yeonggwang, Jeollanam-do	Potato	4	1	II	This study
POW2	Yeonggwang, Jeollanam-do	Potato	4	1	II	This study
POW3	Yeonggwang, Jeollanam-do	Potato	4	1	II	This study
POW4	Yeonggwang, Jeollanam-do	Potato	4	1	II	This study
POW5	Yeonggwang, Jeollanam-do	Potato	4	1	II	This study
POW7	Muan, Jeollanam-do	Potato	4	1	II	This study
POW8	Muan, Jeollanam-do	Potato	4	1	II	This study
POW9	Muan, Jeollanam-do	Potato	4	1	II	This study
POW10	Muan, Jeollanam-do	Potato	4	1	II	This study
POW11	Boseong, Jeollanam-do	Potato	4	1	II	This study
POW12	Boseong, Jeollanam-do	Potato	4	1	II	This study
POW13	Boseong, Jeollanam-do	Potato	4	1	II	This study
POW14	Boseong, Jeollanam-do	Potato	4	1	II	This study
POW15	Boseong, Jeollanam-do	Potato	4	1	I	This study
POW16	Boseong, Jeollanam-do	Potato	4	1	I	This study
POW23	Yeongam, Jeollanam-do	Potato	4	1	II	This study
POW25	Yeongam, Jeollanam-do	Potato	4	1	II	This study
POW29	Yeongam, Jeollanam-do	Potato	4	1	II	This study

(Continued on next page)

^x Of 494 isolates, 125 representative samples were selected for AFLP analysis based on their geographical origin, biovar, race, and pathogenicity.

^y BCCM/LMG: Belgian Coordinated Collection of Microorganisms.

^z N indicates not included in the AFLP clusters.

–70°C until use (17). The isolates were identified by sole carbon source utilization analysis and fatty acid methyl ester analysis as described previously (15). Of the 16 foreign isolates, 11 were obtained from the Belgian Coordinated Collection of Microorganisms (BCCM/LMG, Ghent, Belgium), 4 were obtained from Kyushu University in Japan, and the GMI1000

isolate was provided by C. Boucher (Table 1).

Pathogenicity and host range tests. For the pathogenicity test, seedlings of tomato (cv. Kwangsoo), pepper (cv. Nokkwang), and potato (cv. Daeji) were transplanted into plastic pots (11 cm diameter) (Hungnong Seeds, Seoul, Korea) containing commercial peat mix soil

(Baroker, Seoul Agriculture Materials Co., Seoul, Korea) and grown in a greenhouse at 25 to 30°C. Each plant was inoculated at the fifth to sixth true leaf stage, and each inoculation was repeated three times. To prepare the inoculum, bacterial cells were grown on TZC agar medium for 24 h at 28°C, suspended in sterile distilled water, and adjusted to 10⁸ CFU/ml. The roots of

Table 1. (Continued from previous page)

Isolate ^a	Geographical origin	Host	Biovar	Race	AFLP cluster	Source ^b
POW30	Yeongam, Jeollanam-do	Potato	4	1	II	This study
POW33	Yeongam, Jeollanam-do	Potato	4	1	IV	This study
POW37	Miryang, Gyeongsangnam-do	Potato	4	1	II	This study
POW39	Miryang, Gyeongsangnam-do	Potato	4	1	II	This study
POW41	Miryang, Gyeongsangnam-do	Potato	4	1	II	This study
POW44	Miryang, Gyeongsangnam-do	Potato	4	1	II	This study
POW47	Miryang, Gyeongsangnam-do	Potato	4	1	II	This study
POW49	Miryang, Gyeongsangnam-do	Potato	4	1	II	This study
POW51	Gimhae, Gyeongsangnam-do	Potato	4	1	IV	This study
POW52	Yeongam, Jeollanam-do	Potato	4	1	IV	This study
POW53	Yeongam, Jeollanam-do	Potato	4	1	IV	This study
POW54	Yeongam, Jeollanam-do	Potato	4	1	IV	This study
POW55	Yeongam, Jeollanam-do	Potato	4	1	IV	This study
POW56	Miryang, Gyeongsangnam-do	Potato	4	1	IV	This study
POW57	Wando, Jeollanam-do	Potato	4	1	IV	This study
POW58	Haenam, Jeollanam-do	Potato	4	1	IV	This study
POW68	Wando, Jeollanam-do	Potato	4	1	IV	This study
POW69	Wando, Jeollanam-do	Potato	4	1	IV	This study
POW70	Wando, Jeollanam-do	Potato	4	1	IV	This study
POW71	Wando, Jeollanam-do	Potato	4	1	IV	This study
POW72	Wando, Jeollanam-do	Potato	4	1	IV	This study
POW73	Wando, Jeollanam-do	Potato	4	1	IV	This study
POW74	Wando, Jeollanam-do	Potato	4	1	IV	This study
POW75	Wando, Jeollanam-do	Potato	4	1	IV	This study
POW76	Wando, Jeollanam-do	Potato	4	1	IV	This study
TW14	Chungju, Chungcheongbuk-do	Tomato	4	1	III	This study
TW21	Nonsan, Chungcheongnam-do	Tomato	3	1	III	This study
TW23	Pyeongtaek, Gyeonggi-do	Tomato	4	1	III	This study
TW24	Paju, Gyeonggi-do	Tomato	4	1	III	This study
TW25	Paju, Gyeonggi-do	Tomato	4	1	III	This study
TW26	Paju, Gyeonggi-do	Tomato	4	1	III	This study
TW27	Paju, Gyeonggi-do	Tomato	4	1	III	This study
TW28	Paju, Gyeonggi-do	Tomato	4	1	III	This study
TW29	Yeongcheon, Gyeongsangbuk-do	Tomato	4	1	III	This study
TW30	Yeongcheon, Gyeongsangbuk-do	Tomato	4	1	III	This study
TW31	Hadong, Gyeongsangnam-do	Tomato	4	1	I	This study
TW32	Hadong, Gyeongsangnam-do	Tomato	4	1	III	This study
TW33	Hadong, Gyeongsangnam-do	Tomato	4	1	III	This study
TW38	Gimje, Jeollabuk-do	Tomato	4	1	II	This study
TW47	Goseong, Gyeongsangnam-do	Tomato	4	1	II	This study
TW56	Gimhae, Gyeongsangnam-do	Tomato	4	1	II	This study
TW60	Gimcheon, Gyeongsangbuk-do	Tomato	4	1	II	This study
TW64	Gumi, Gyeongsangbuk-do	Tomato	4	1	II	This study
TW65	Gumi, Gyeongsangbuk-do	Tomato	4	1	II	This study
TOW3	Cheongwon, Chungcheongbuk-do	Tobacco	3	1	VI	This study
PEW1	Chilgok, Gyeongsangbuk-do	Peanut	4	1	N ^z	This study
SW1	Pyeongtaek, Gyeonggi-do	Sunflower	4	1	N	This study
SEW2	Yonchon, Gyeonggi-do	Sesame	4	1	N	This study
7501	Japan	Eggplant	4	1	II	Kyushu Univ.
7502	Japan	Eggplant	4	1	II	Kyushu Univ.
7601	Japan	Eggplant	4	1	II	Kyushu Univ.
N6211	Japan	Pepper	4	1	II	Kyushu Univ.
GMI1000	Guyana	Tomato	3	1	N	C. Boucher
LMG2294	Colombia	Potato	2	3	VII	BCCM/LMG
LMG2296	Zimbabwe	Tomato	1	1	N	BCCM/LMG
LMG2299	United States	Tomato	1	1	N	BCCM/LMG
LMG2300	Israel	Potato	2	3	VII	BCCM/LMG
LMG2301	Trinidad	Banana	1	2	N	BCCM/LMG
LMG2306	Portugal	Tomato	2	3	VII	BCCM/LMG
LMG17138	Brazil	Potato	1	1	N	BCCM/LMG
LMG17139	Cyprus	Potato	2	3	VII	BCCM/LMG
LMG17140	Sweden	Potato	2	3	VII	BCCM/LMG
LMG17142	U.K.	Potato	2	3	VII	BCCM/LMG
LMG17144	Fiji	Potato	3	1	N	BCCM/LMG

the test plants were immersed in 50 ml of bacterial suspension for 2 h without artificial wounding. Control plant roots were immersed in sterile water. The inoculated plants (three tomato, three pepper, and three potato plants for each isolate) were planted in plastic pots and incubated in a greenhouse at 25 to 30°C under natural light. Symptom development was recorded daily for 15 days using the following scale: – = no symptoms; + = 25% wilt symptoms, one or two leaves wilted; ++ = 50% wilt symptoms, three or four leaves wilted; and +++ = greater than 75% wilt symptoms, five leaves to whole plant wilted.

Biovar determination. Four hundred and seventy-eight isolates were classified into biovars based on their ability to oxidize three disaccharides (lactose, maltose, and cellobiose) and three hexose alcohols (mannitol, sorbitol, and dulcitol) as well as their ability to utilize trehalose, meso-inositol, and D-ribose. The characterization of the biovars was performed following the method of Hayward (9) with modifications. The bacterial cells were grown on TZC agar medium for 24 h at 28°C and then suspended in sterile distilled water (10⁸ CFU/ml). Two ml of base medium (2 g NH₄H₂PO₄, 0.4 g KCl, 0.4 g MgSO₄·7H₂O, 2 g peptone, and 0.6 ml 1% [wt/vol] bromothymol blue per liter, pH 7.1) containing 1% filter-sterilized lactose, maltose, cellobiose, mannitol, sorbitol, dulcitol, trehalose, meso-inositol, and D-ribose were dispensed into the wells of 24-well tissue culture plates (SPL Life Sciences, Seoul, Korea). Each well was inoculated with 5 µl of the prepared suspension, and the test was repeated three times with noninoculated controls. The plates were incubated at 28°C for 14 days, and color changes in each well were recorded daily.

AFLP analysis. Genomic DNA from 125 representative isolates (Table 1) representing different hosts, biovars, and geo-

graphical origins was isolated as described by Sambrook et al. (24), followed by AFLP analysis as described by Poussier et al. (21) with modifications. Genomic DNA (100 ng/sample) was digested with 1 U *SacI* (Takara Bio Inc., Otsu, Japan), 1 U *MspI* (Takara), and 2 µl 10× OPA buffer (500 mM potassium acetate, 100 mM magnesium acetate, 100 mM Tris-acetate, pH 7.5) at 25°C for 16 h. Adapter ligation was achieved by adding 5 pmol *SacI* adapter (5'-TGTACGACGTCTAC-3' and 5'-CTCGTAGACTGCGTACAAGCT-3'), 50 pmol *MspI* adapter (5'-GACGATGAGTCTGAA-3' and 5'-CGTTCAGGATCTCATC-3'), 35 U T4 DNA ligase (Takara), 2 µl 10× T4 DNA ligase buffer (Takara), and sterilized water to make a final volume of 20 µl. The digested and ligated DNA was diluted 10-fold and stored at –20°C until use. Selective amplifications were performed using two primers complementary to the adapter sequences with additional selective nucleotides at their 3' ends (*SacI*-selective primer: 5'-CTCGTAGACTGCGTACAAGCTC-3', *MspI*-selective primer: 5'-GATGAGTCCTGAACGGCG-3'). Digested and ligated DNA (1.3 µl) was added to 3.7 µl of a mixture containing 0.5 µl 10× AFLP buffer (500 mM KCl, 100 mM Tris-Cl [pH 7.0], 15 mM MgCl₂, and 1% Triton-X 100), 3.475 ng *SacI* selective primer labeled with γ-[³²P]ATP (Amersham Biosciences, Uppsala, Sweden), 7.54 ng *MspI* selective primer, and 0.125 U *Taq* polymerase (Takara). DNA amplification by PCR was performed using a thermocycler (model PTC-200, MJ Research Inc., Waltham, MA) and the following program: 1 cycle of 30 s at 94°C, 30 s at 65°C, 1 min at 72°C, and 30 s at 94°C followed by 13 cycles of 1 cycle of 30 s at 94°C, 23 cycles of 30 s at 56°C (with a progressive decrease in annealing temperature of 0.7°C during each cycle), 1 min at 72°C, 30 s at 94°C, and 1 cycle of 30 s at 56°C followed by 5 min at

72°C. The amplified fragments were separated by electrophoresis on 6% polyacrylamide gels, and the patterns were visualized as described by Vos et al. (29). Individual DNA fragments, ranging between 150 and 1,000 bp, that were detected reproducibly were scored as present or absent. Binary matrices consisting of 0 (absence of a given band) and 1 (presence of a given band) were analyzed to obtain simple matching coefficients among the isolates using NTSYS-pc (version 2.0, Exeter Biological Software, Setauket, NY). The simple matching coefficients were then clustered to generate similarity trees with the SHAN clustering program, using the unweighted pair-group method with arithmetic average (UPGMA) algorithm in NYSYS-pc. Classification of the AFLP data was performed as described by Poussier et al. (21).

16S rRNA gene sequencing. The 16S rRNA gene sequence was determined for 8 representative isolates (SL2249, SL2029, SL341, SL2025, TOW3, POW57, TW56, and N6211) taken from each AFLP cluster (I to VI). The 16S rRNA genes of the 8 isolates were amplified by PCR in 50-µl reaction volumes containing 5 µl 10× PCR buffer (Takara), 5 µl each dNTP (2.5 mM, Takara), 1 µl each primer (100 pmol, 27mF: 5'-AGAGTTTGATCMTGGCTCAG-3', 1492mR: 5'-GGYTACCTTGTACGACTT-3'), 0.5 µl of *Taq* polymerase (250 U/µl, Takara), and 2 µl bacterial suspension (*A*_{600nm} = 0.1). The amplifications were performed in an automated thermocycler (model PTC-200, MJ Research) with an initial denaturation at 94°C for 5 min, followed by 29 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min, with a final extension at 72°C for 10 min. The amplified DNA was cloned into the *SmaI* site of pBluescript II (KS +) (Stratagene, Inc., La Jolla, CA) as described by Sambrook et al. (24). The se-

Table 2. The 16S rRNA nucleotide sequence differences among 18 isolates of *Ralstonia solanacearum*

Isolate ^x	Origin	Host	Bio-var	Division or sub-division ^y	GenBank accession no.	Nucleotide(s) at position(s) ^z																				
						458-																				
						152	165	167	169	269	375	387	397	410	416	460	474	649	669	737	923	1424	1428	1451	1456	1472
SL341	Korea	Tomato	3	1	DQ924950	A	C	A	U	C	U	U	G	G	U	ACU	U	G	A	U	A	C	A	C	A	U
SL2025	Korea	Sesame	3	1	DQ924951	A	C	A	C	C	U	U	A	G	G	ACU	U	G	A	U	A	C	A	C	A	U
SL2029	Korea	Potato	2	2b	DQ924952	A	C	G	C	C	U	U	A	G	G	UUC	A	G	G	C	A	C	A	C	A	U
SL2249	Korea	Potato	1	1	DQ924953	A	C	A	C	C	U	U	A	G	G	ACU	U	G	A	U	A	C	A	C	A	U
POW57	Korea	Potato	4	1	DQ924954	A	C	A	C	C	U	C	A	G	G	ACU	U	G	A	U	A	C	A	C	A	U
TW56	Korea	Tomato	4	1	DQ924957	G	C	A	C	C	U	U	A	A	G	ACU	U	G	A	U	A	C	A	C	A	U
TOW3	Korea	Tobacco	3	1	DQ924955	A	C	A	C	C	G	U	A	G	G	ACU	U	G	A	U	A	C	A	C	A	U
N6211	Japan	Pepper	4	1	DQ924956	A	C	A	C	C	U	U	A	G	G	ACU	U	G	A	U	G	C	A	C	A	U
ACH0158	Australia	Potato	2	2a	U28224	A	C	A	C	C	U	U	A	G	G	UUC	A	A	A	U	A	U	G	C	G	C
ACH0171	Australia	Eggplant	3	1	U27986	A	C	A	C	C	U	U	A	G	G	ACU	U	G	A	U	A	C	A	C	A	U
CFBP712	Burkina Faso	Eggplant	1	2a	AF207892	A	C	A	C	U	U	U	A	G	G	UUC	A	G	A	U	A	U	G	U	G	C
CFBP734	Madagascar	Potato	1	2c	AF207895	A	U	A	C	C	U	U	A	G	G	UCU	A	G	A	U	A	U	G	U	G	C
GMI1000	Guyana	Tomato	3	1	AL646052	A	C	A	C	C	U	U	A	G	G	ACU	U	G	A	U	A	C	A	C	A	U
MAFF211266	Japan	Tomato	4	1	AF207897	A	C	A	C	C	U	U	A	G	G	ACU	U	G	A	U	A	C	A	C	A	U
MAFF301559	Japan	Potato	N2	2b	AB024608	A	C	G	C	C	U	U	A	G	G	UUC	A	G	G	C	A	C	A	C	A	U
NCPBP1018	Angola	Potato	1	2c	AF207893	A	U	A	C	C	U	U	A	G	G	UCU	A	G	A	U	A	U	G	U	G	C
R142	Indonesia	Clove	2	2b	U28233	A	C	G	C	C	U	U	A	G	G	UUC	A	G	G	C	A	C	A	C	A	U
R791	Indonesia	Tomato	3	1	U27987	A	C	A	C	C	U	U	A	G	G	ACU	U	G	A	U	A	C	A	C	A	U

^x Except for the first 8 isolates, data shown are from Horita and Tsuchiya (12), Poussier et al. (21), and Taghavi et al. (27).

^y Based on data from Poussier et al. (21) and Taghavi et al. (27).

^z *Escherichia coli* numbering (30).

quences of both strands of the 16S rRNA gene from each isolate were determined using an ABI3730XL automated DNA sequencer (Applied Biosystems Inc., Foster City, CA), and the sequences were aligned using the Clustal X program (14) with the published (12,21,27) 16S rRNA gene sequences for the following *R. solanacearum* isolates: ACH0158, ACH0171, CFBP712, CFBP734, GMI1000, MAFF-211266, MAFF301559, NCPB1018, R142, and R791. Sequence differences and their positions (using the *Escherichia coli* numbering system [30]) are shown in Table 2. A dendrogram was constructed using the UPGMA method of the Molecular Evolutionary Genetics Analysis (MEGA 3.1) program (18). The strength of the tree branches was tested using 1,000 bootstrap trials. Classification of the sequences was performed as described by Poussier et al. (21) and Taghavi et al. (27).

Phylotype analysis. The endoglucanase, *hrpB*, and *mutS* gene sequences of the 8 representative isolates (SL2249, SL2029, SL341, SL2025, TOW3, POW57, TW56, and N6211) used for the 16S rRNA gene sequence analysis were determined for phylotype analysis. The endoglucanase and *hrpB* genes were amplified as described by Poussier et al. (20) with modifications, and the *mutS* gene was amplified as described by Prior and Fegan (23) with modifications.

The endoglucanase gene of the 8 isolates was amplified by PCR in 50- μ l reaction volumes containing 5 μ l 10 $\times$ *Pfu* polymerase buffer (Stratagene), 5 μ l of each 2.5 mM dNTP, 1 μ l of each primer (100 pmol, Endo-F: 5'-ATGCATGCCGCTGGT CGCCGC-3', Endo-R: 5'-GCGTTGCCC

GGCACGAACACC-3'), 1.25 U of *Pfu* Turbo DNA Polymerase (Stratagene), and 2 μ l of bacterial suspension (A_{600nm} = 0.1). The amplifications were performed in an automated thermocycler (model PTC-200, MJ Research) with an initial denaturation at 96°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 70°C for 1 min, and extension at 72°C for 2 min, with a final extension at 72°C for 10 min.

The *hrpB* gene of the 8 isolates was amplified by PCR in 50- μ l reaction volumes containing 5 μ l of 10 $\times$ *Pfu* polymerase buffer (Stratagene), 5 μ l of each 2.5 mM

dNTP, 1 μ l of each primer (100 pmol, RShrpBf: 5'-TGCCATGCTGGGAAACAT CT-3', RShrpBr: 5'-GGGGGCTTCGTT GAACTGC-3'), 1.25 U of *Pfu* Turbo DNA Polymerase (Stratagene), and 2 μ l bacterial suspension (A_{600nm} = 0.1). The amplifications were performed in an automated thermocycler (model PTC-200, MJ Research) with an initial denaturation at 95°C for 5 min, followed by 10 cycles of denaturation at 95°C for 30 s, annealing at 64°C for 30 s, and extension at 68°C for 2 min. The final 20 cycles were the same as the first 10 except that an additional 20 s were added to the elongation step for each new

Table 4. Pathogenicity of Korean *Ralstonia solanacearum* isolates

Race	Biovar (no. of isolates)	Host	Pathogenicity ^y			AFLP ^z cluster
			Tomato	Pepper	Potato	
1	1 (3) 4 (9)	Paprika Potato Tomato	+++	+	+++	I, VI
1	3 (48) 4 (356)	Pepper Potato Sesame Tobacco Tomato	+++	+++	+++	I, II, III, IV
1	4 (16)	Eggplant Potato Tomato	+++	–	+++	II, III
1	3 (5)	Tomato	+++	+++	–	III
1	3 (3)	Tobacco	+++	–	+	VI
3	2 (38)	Potato Tomato	+	–	+++	V

^y Development of symptoms was recorded daily for 15 days according to the following scale: – = no symptoms; + = 25% wilt symptoms, one or two leaves wilted; ++ = 50% wilt symptoms, three or four leaves wilted; and +++ = greater than 75% wilt symptoms, five leaves to whole plant wilted. The pathogenicity test was repeated three times with three replications.

^z Amplified fragment length polymorphism.

Table 3. *Ralstonia solanacearum* isolates used in endoglucanase, *hrpB*, and *mutS* gene sequence analyses

Isolate ^x	Origin	Host	Biovar	Phylotype ^y	GenBank accession no.		
					Endoglucanase gene sequence	<i>hrpB</i> gene sequence	<i>mutS</i> gene sequence
SL341	Korea	Tomato	3	I	EF523197	EF523205	EF523213
SL2025	Korea	Sesame	3	I	EF523199	EF523207	EF523215
SL2029	Korea	Potato	2	IV	EF523198	EF523206	EF523214
SL2249	Korea	Potato	1	I	EF523200	EF523208	EF523216
POW57	Korea	Potato	4	I	EF523202	EF523210	EF523218
TW56	Korea	Tomato	4	I	EF523201	EF523209	EF523217
TOW3	Korea	Tobacco	3	I	EF523203	EF523211	EF523219
N6211	Japan	Pepper	4	I	EF523204	EF523212	EF523220
CFBP712	Burkina Faso	Eggplant	1	II-bhr	AF295267	AF295620	AY756773
CFBP734	Madagascar	Potato	1	III	AF295274	AF295627	AY756746
CFBP2972	Martinique	Potato	1	II-bhr	AF295264	AF295617	AY756807
CFBP3858	Netherlands	Potato	2	II-nhr	AF295259	AF295612	AY756748
GMI1000	Guyana	Tomato	3	I	AF295251	AF295604	AY756804
JT516	Reunion Island	Potato	2	II-nhr	AF295258	AF295611	AY756783
MAFF211266	Japan	Tomato	4	I	AF295250	AF295603	AY756791
MAFF301558	Japan	Potato	N2	IV	AY465002	AY465037	AY756812
MAFF301559	Japan	Potato	N2	IV	AY465001	AY465036	N ^z
NCPB1018	Angola	Potato	1	III	AF295271	AF295624	AY756772
NCPB3190	Malaysia	Tomato	3	I	AF295253	AF295606	AY756738
R142	Indonesia	Clove	2	IV	AY465003	AY465038	N
UW9	Costa Rica	<i>Heliconia</i> sp.	1	II-nhr	AF295257	AF295610	AY756744
UW162	Peru	<i>Musa</i> sp.	1	II-nhr	AF295256	AF295609	AY756795

^x Except for the first 8 isolates, the data shown are from Poussier et al. (20), Prior and Fegan (23), and Villa et al. (28).

^y Based on data from Fegan and Prior (7) and Prior and Fegan (23). bhr and nhr denote broad host range and narrow host range, respectively.

^z N: no available sequence.

cycle, and a final extension at 68°C for 7 min was added.

The *mutS* gene of the 8 isolates was amplified by PCR in 50- μ l reaction volumes containing 5 μ l of 10 $\times$ *Pfu* polymerase buffer (Stratagene), 5 μ l of each 2.5 mM dNTP, 1 μ l of each primer (100 pmol, mutS-RsF.1570: 5'-ACAGCGCCTTGAGCCGGTACA-3', mutS-RsR.1926: 5'-GCTGATCACCGGCCCGAACAT-3'), 1.25 U of *Pfu* Turbo DNA Polymerase (Stratagene), and 2 μ l bacterial suspension (A_{600nm} = 0.1). The amplifications were performed in an automated thermocycler (model

PTC-200, MJ Research) with an initial denaturation at 96°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 66°C for 1 min, and extension at 72°C for 1.5 min, with a final extension at 72°C for 5 min.

The amplified DNA fragments were separated by 1.5% agarose gel electrophoresis in 0.5 $\times$ TBE buffer, purified using the MEGAquick-spin PCR & Agarose gel DNA extraction system (Intron Biotechnology, Seoul, Korea) according to the manufacturer's instructions, and sequenced on both strands. The primers used to se-

quence endoglucanase, *hrpB*, and *mutS* were: Endo-F/Endo-R for the endoglucanase gene, RShrpBf/RShrpBr for *hrpB*, and mutS-RsF.1570/mutS-RsR.1926 for *mutS* (20,23). The partial sequences were aligned using the Clustal X program (14) with the published (20,23,28) endoglucanase, *hrpB*, and *mutS* gene sequences for the following *R. solanacearum* isolates: CFBP712, CFBP734, CFBP2972, CFBP-3858, GMI1000, JT516, MAFF211266, MAFF301558, MAFF301559, NCPPB1018, NCPPB3190, R142, UW9, and UW162. The partial sequences obtained in this study have been deposited in the GenBank database under the accession numbers listed in Table 3. Evolutionary distances between the sequences were computed using the algorithm of Jukes and Cantor (16), and phylogenetic trees were constructed from the genetic distance data using the neighbor-joining method in the Molecular Evolutionary Genetics Analysis (MEGA 3.1) program (18). The strength of the tree branches was tested using 1,000 bootstrap trials. Classification of the sequences was performed as described by Fegan and Prior (7) and Prior and Fegan (23).

RESULTS

Host range and biovar determination.

The Korean isolates were tested for biovar determination and pathogenicity on various host plants. Of the 478 isolates tested, 3 were biovar 1, 38 were biovar 2, 56 were biovar 3, and 381 were biovar 4 (Table 4). Among the Korean biovar 2 isolates, none were identified as biovar N2. All biovar 1 isolates were pathogenic on tomato and potato and weakly pathogenic on pepper (Table 4). Among the 56 biovar 3 isolates, 48 were pathogenic on tomato, pepper, and potato, 5 were pathogenic on tomato and pepper but nonpathogenic on potato, and 3 were pathogenic on tomato and weakly pathogenic on potato but nonpathogenic on pepper (Table 4). Of the 381 biovar 4 isolates, 356 were pathogenic on tomato, pepper, and potato, 16 were pathogenic on tomato and potato and nonpathogenic on pepper, and 9 were pathogenic on tomato and potato and weakly pathogenic on pepper (Table 4). Overall, all biovar 1, 3, and 4 isolates belonged to race 1, and all biovar 2 isolates from tomato and potato were pathogenic on potato and weakly pathogenic or nonpathogenic on tomato and pepper, corresponding to race 3 (Table 4). These results indicate that 92% (440 of 478 isolates) of the Korean isolates belong to race 1, and 85% (404 of 478 isolates) of them are highly virulent on tomato, pepper, and potato.

Geographical distribution. Figure 1 shows the geographical distribution of the various Korean isolates, reflecting their genetic diversity. Biovar 1 isolates of race 1 taken from tomato and potato plants were present only in Jeju-do (Fig. 1). Bio-

Fig. 1. Locations in Korea where *Ralstonia solanacearum* isolates were collected (Gg: Gyeonggi-do; Gw: Gangwon-do; Cb: Chungcheongbuk-do; Cn: Chungcheongnam-do; Jb: Jeollabuk-do; Jn: Jeollanam-do; Gb: Gyeongsangbuk-do; Gn: Gyeongsangnam-do; Jj: Jeju-do). Symbols indicate isolates (▲: biovar 1, race 1, amplified fragment length polymorphism (AFLP) cluster I; ■: biovar 3, race 1, AFLP cluster I; □: biovar 3, race 1, AFLP cluster II; △: biovar 3, race 1, AFLP cluster III; ●: biovar 4, race 1, AFLP cluster I; ○: biovar 4, race 1, AFLP cluster II; ◐: biovar 4, race 1, AFLP cluster III; ◑: biovar 4, race 1, AFLP cluster IV; ★: biovar 2, race 3, AFLP cluster V). Each symbol indicates representative locations for the same geographical origin, host, biovar, race, and AFLP cluster.

var 2 isolates belonging to race 3 were found at 2 locations in Jeju-do, 2 locations in Jeollanam-do, and 3 locations in Gyeongsangnam-do (Fig. 1). Biovar 3 isolates belonging to race 1 were collected from wilted tomato, potato, tobacco, eggplant, pepper, sesame, and paprika plants at 2 locations in Gangwon-do, 4 locations in Gyeonggi-do, 4 locations in Chungcheongbuk-do, 6 locations in Chungcheongnam-do, 3 locations in Jeollanam-do, 4 locations in Gyeongsangbuk-do, 4 locations in Gyeongsangnam-do, and 2 locations in Jeju-do (Fig. 1). Biovar 4 isolates belonging to race 1 were the most widespread among the four biovars. These isolates were recovered from diseased tomato, potato, pepper, eggplant, sesame, paprika, pimiento, peanut, and sunflower plants at 6 locations in Gangwon-do, 10 locations in Gyeonggi-do, 7 locations in Chungcheongbuk-do, 4 locations in Chungcheongnam-do, 3 locations in Jeollabuk-do, 9 locations in Jeollanam-do, 10 locations in Gyeongsangbuk-do, 11 locations in Gyeongsangnam-do, and 2 locations in Jeju-do (Fig. 1).

AFLP analysis. A total of 110 DNA fragments ranging from 150 to 1,000 bp, which were consistently produced, were selected to analyze 109 Korean and 16 foreign isolates. Among the isolates tested, 62 unique AFLP fragments were identified, and among those 10 were present only in the Korean biovar 2 (race 3) isolates (Fig. 2). A dendrogram generated by NTSYS-pc separated the isolates into two divisions: the first contained all 109 Korean isolates, 4 Japanese isolates, and the Guyanese isolate (GMI1000), while the second contained the 11 LMG isolates (Fig. 3). Isolates belonging to the first division were further divided into six clusters, designated I through VI, at a 0.62 correlation level. Three Korean isolates (SW1, SEW2, and PEW1) and GMI1000 did not fit into any of the clusters (Fig. 3) at the 0.62 correlation level. The first cluster, with a coefficient of 0.75, contained biovar 1, 3, and 4 (race 1) isolates from tomato, pepper, potato, and sesame collected in Chungcheongnam-do, Gyeongsangnam-do, Gyeongsangbuk-do, and Jeollanam-do (Figs. 1 and 3). The second cluster, with a coefficient of 0.70, contained biovar 3 and 4 (race 1) isolates collected from tomato, pepper, potato, and eggplant in Gyeongsangbuk-do, Gyeongsangnam-do, Jeollabuk-do, and Jeollanam-do as well as biovar 4 isolates from eggplant and pepper in Japan (Figs. 1 and 3). The third cluster, with a coefficient of 0.72, included biovar 3 and 4 (race 1) isolates collected from tomato plants in Gangwon-do, Gyeonggi-do, Chungcheongbuk-do, Chungcheongnam-do, Gyeongsangbuk-do, Gyeongsangnam-do, and Jeollanam-do (Figs. 1 and 3). The fourth cluster, with a coefficient of 0.70, included biovar 4 (race 1) isolates from potato collected in Jeol-

lanam-do and Gyeongsangnam-do (Figs. 1 and 3). The fifth cluster, with a coefficient of 0.62, included biovar 2 (race 3) isolates from tomato and potato collected in Jeollanam-do, Gyeongsangnam-do, and Jeju-do (Figs. 1 and 3). The sixth cluster, with a coefficient of 0.685, included biovar 3 and 4 (race 1) isolates from paprika and tobacco collected in Gyeonggi-do, Chungcheongbuk-do, Gyeongsangbuk-do, and Gyeongsangnam-do (Figs. 1 and 3). The Korean isolates and isolate GMI1000 had a genetic similarity coefficient of 0.59. The second division, with a coefficient of 0.48, included biovar 1 and 3 isolates, and the seventh cluster contained biovar 2 isolates originating from 11 countries (Fig. 3, Table 1).

16S rRNA gene sequence analysis. We sequenced the complete 16S rRNA genes from 7 Korean isolates (biovar 1: SL2249;

biovar 2: SL2029; biovar 3: SL341, SL2025, and TOW3; and biovar 4: POW57 and TW56) and 1 Japanese isolate (biovar 4: N6211). By comparing 1,468 nucleotides, all of the isolates were grouped into four clusters (Fig. 4A). Among them, the Korean and Japanese isolates were separated into two groups. Cluster I contained isolates of biovars 1, 3, and 4 (race 1), and cluster II contained a biovar 2 (race 3) isolate (Fig. 4A). The sequences of the cluster I isolates were identical, while the cluster II isolates differed from the cluster I isolates at seven nucleotide positions. Comparative analysis of the 16S rRNA gene sequences from the 7 Korean isolates, 1 Japanese isolate, and 10 other foreign isolates was performed based on previously published work by Horita and Tsuchiya (12), Poussier et al. (21), and

Fig. 2. Amplified fragment length polymorphism (AFLP) fingerprints of *Ralstonia solanacearum* isolates. Lanes: 1, GMI1000; 2, PW17; 3, PW33; 4, POW1; 5, POW7; 6, N6211; 7, 7601; 8, SL341; 9, TW30; 10, POW55; 11, POW57; 12, SL2029; 13, SL2313; 14, SL1977; 15, TOW3; 16, LMGG2296; 17, LMG17138; 18, PEW1; 19, SEW2. Arrows indicate specific fragments in the cluster V (biovar 2) isolates.

Fig. 3. Dendrogram constructed by the unweighted pair-group method with the arithmetic average showing the correlation between amplified fragment length polymorphism (AFLP) fingerprints of the *Ralstonia solanacearum* isolates. Roman numerals indicate clusters. LMG: Laboratorium voor Microbiologie Universiteit Gent, Belgium.

Taghavi et al. (27). Taghavi et al. (27) reported that nucleotides 458, 459, 460, and 474 (*E. coli* numbering) are unique and conserved between the divisions; therefore, we compared those positions as well as others that we previously reported to be variable. The cluster I isolates (race 1), which belong to division 1 according to Taghavi et al. (27), had the same nucleotides at positions 458 to 460 (ACU) and 474 (U), as did those of the Japanese isolate (biovar 4: MAFF211266), an Australian isolate (biovar 3: ACH0171), an Indonesian isolate (biovar 3: R791), and the Guyanese isolate (biovar 3: GMI1000) (Table 2, Fig. 4A). Six of the 7 Korean isolates belonged to cluster I. The cluster II isolates, which belong to subdivision 2b, including Korean biovar 2 isolate SL2029, Indonesian biovar 2 isolate R142, and Japanese biovar N2 isolate MAFF301559, had identical sequences (Table 2, Fig. 4A). The cluster III isolates, including African biovar 1 isolate CFBP712 and Australian

biovar 2 isolate ACH0158, were distinct from the other isolates at nucleotides 458 to 460 (UUC), 474 (A), 1424 (U), 1428 (G), 1456 (G), and 1472 (C), but were identical at positions 458 to 460, 474, 1424, 1428, 1456, and 1472. Therefore, these isolates belong to subdivision 2a according to Taghavi et al. (27) (Table 2, Fig. 4A). The cluster IV isolates, including the African biovar 1 isolates CFBP734 and NCPPB1018, differed from the other isolates by having a U at position 165 and UUC at 458 to 460 (Table 2); consequently, these isolates were grouped in subdivision 2c according to the classification of Poussier et al. (21) (Table 2, Fig. 4A). The bootstrap value for the 16S rRNA gene sequences among the cluster I isolates ranged from 19 to 84%, while the bootstrap value among cluster II was 99%. The bootstrap values for clusters III and IV were 61 and 98%, respectively, and between clusters III and IV was 83% (Fig. 4A).

Phylogenetic analysis. Partial endoglucanase gene sequences (694 nucleotides) covering approximately 45% of the complete endoglucanase gene from the 8 isolates used for 16S rRNA gene sequence analysis and 14 reference isolates were compared and divided into four clusters (Table 3, Fig. 4B). Among them, the 7 Korean isolates and one Japanese isolate belonged to either cluster I or cluster II (Fig. 4B). The cluster I isolates belonged to phylotype I along with the Japanese isolate (MAFF211266), the Malaysian isolate (NCPPB3190), and the Guyanese isolate (GMI1000; Fig. 4B). The cluster II isolates, composed of Korean biovar 2 isolate SL2029, Indonesian biovar 2 isolate R142, and Japanese biovar N2 isolates MARR301558 and MAFF301559, belonged to phylotype IV (Table 3, Fig. 4B). The cluster III isolates, including the biovar 1 African isolates NCPPB1018 and CFBP734, corresponded to phylotype III (Table 3, Fig. 4B). The cluster IV isolates

Fig. 4. A, Dendrogram constructed by the unweighted pair-group method with the arithmetic average showing the phylogenetic relationships among *Ralstonia solanacearum* strains based on a comparison of their 16S rRNA sequences. Numbers at branch points are percentages of bootstrap replicates in which clusters were found. Bars represent the sequence differences among isolates. ^aBased on data from Poussier et al. (21) and Taghavi et al. (27). B, Phylogenetic tree based on a comparison of the partial endoglucanase gene sequences. C, Phylogenetic tree based on a comparison of the partial *hrpB* gene sequences. D, Phylogenetic tree based on a comparison of the partial *mutS* gene sequences. The trees (B, C, and D) were constructed by the neighbor-joining method. Values at branches indicate percent bootstrap support for 1,000 replicates. Bars indicate one nucleotide change per 100 nucleotide positions. ^bBased on data from Fegan and Prior (7) and Prior and Fegan (23).

were divided into two distinct groups, which belonged to phylotype II broad host range (bhr) and phylotype II narrow host range (nhr). The phylotype II bhr group included biovar 1 isolates from Martinique (CFBP2972) and Burkina Faso (CFBP712), and the phylotype II nhr group included biovar 1 isolates from Peru (UW162) and Costa Rica (UW9) and biovar 2 isolates from the Netherlands (CFBP3858) and Reunion Island (JT516; Table 3, Fig. 4B).

The partial *hrpB* gene sequences, consisting of 1,049 bases covering approximately 75% of the entire gene, of the 8 isolates were compared to published sequences of 14 reference isolates (Table 3). The phylogenetic tree was divided into four clusters (Fig. 4C). Cluster I, which included 6 Korean isolates (SL2249, SL341, SL2025, TOW3, POW57, and TW56), 2 Japanese isolates (N6211 and MAFF211266), a Malaysian isolate (NCPPB3190), and a Guyanese isolate (GMI1000), belonged to phylotype I (Table 3, Fig. 4C). The cluster II isolates included biovar 1 African isolates (NCPPB1018 and CFBP734) corresponding to phylotype III (Table 3, Fig. 4C). The cluster III isolates, composed of Korean biovar 2 isolate SL2029, Indonesian biovar 2 isolate R142, and the Japanese biovar N2 isolates MARR301558 and MAFF301559, belonged to phylotype IV (Table 3, Fig. 4C). The cluster IV isolates included the phylotype II bhr group, which included biovar 1 isolates from Martinique (CFBP2972) and Burkina Faso (CFBP712), and the phylotype II nhr group, which included biovar 1 isolates from Peru (UW162) and Costa Rica (UW9), as well as biovar 2 isolates from the Netherlands (CFBP3858) and Reunion Island (JT516; Table 3, Fig. 4C).

A phylogenetic tree was generated by comparing 694 nucleotides from the partial *mutS* gene sequences of the 8 isolates and compared to the published sequences of 12 isolates (Table 3). Consistent with our analysis of the *hrpB* gene, the tree was divided into four clusters (Fig. 4D). The cluster I isolates, which belonged to phylotype I, included the Korean biovar 1 isolate SL2249, the Korean biovar 3 isolates SL341, SL2025, and TOW3, the Korean biovar 4 isolates POW57 and TW56, the Japanese biovar 4 isolates N6211 and MAFF211266, a Malaysian isolate (biovar 3: NCPPB3190), and a Guyanese isolate (biovar 3: GMI1000; Table 3, Fig. 4D). The cluster II isolates, which included the African biovar 1 isolates NCPPB1018 and CFBP734, corresponded to phylotype III (Table 3, Fig. 4D). The cluster III isolates were composed of a Korean biovar 2 isolate (SL2029) and a Japanese biovar N2 isolate (MARR301558) and belonged to phylotype IV (Table 3, Fig. 4D). The cluster IV isolates were composed of the phylotype II bhr group, including biovar 1

isolates from Martinique (CFBP2972) and Burkina Faso (CFBP712), and the phylotype II nhr group, including biovar 1 isolates from Peru (UW162) and Costa Rica (UW9), as well as biovar 2 isolates from the Netherlands (CFBP3858) and Reunion Island (JT516; Table 3, Fig. 4D).

DISCUSSION

Analysis of 109 representative Korean isolates and 16 foreign isolates of *R. solanacearum* by AFLP indicated the existence of two major groups. Among the Korean isolates, six AFLP clusters were revealed, but it is not clear if the genetic diversity among the isolates depends on their geographical origin, host, or biovar. Some inconsistencies were revealed in the groupings when biovar was considered. In division I, biovar 4 was found in all of the clusters except cluster V, which indicates that biovar 4 is the most common biovar among the Korean isolates. The only consistent group in our AFLP analysis was cluster V in division I, which included all of the Korean biovar 2 (race 3) isolates. Because all of the foreign biovar 2 isolates were part of division II, it appears that the Korean biovar 2 isolates are different from those of foreign origin. This indicates that biovar is useful for analyzing genetic diversity among *R. solanacearum* isolates, but that it cannot be used to distinguish biovar 2, which is consistent with previous observations (21,22).

Our AFLP analysis was further validated by 16S rRNA, endoglucanase, *hrpB*, and *mutS* gene sequence analyses using 7 Korean and 1 Japanese isolate, and our results support the classification system proposed by Poussier et al. (21), Fegan and Prior (7), and Prior and Fegan (23). Phylogenetic analysis of 16S rRNA gene sequences revealed that the Korean biovar 1, 3, and 4 isolates belonged to division 1, while the Korean biovar 2 isolate belonged to subdivision 2b, as suggested by Poussier et al. (21). The nucleotides at positions 458 to 460 of the 16S rRNA gene may be useful for distinguishing biovar 2, although there are exceptions. Phylogenetic trees based on the three DNA regions (endoglucanase, *hrpB*, and *mutS* gene) consistently divided the isolates of *R. solanacearum* into four clusters. The cluster distribution of the isolates also corresponds to the phylotype grouping described by Fegan and Prior (7) and Prior and Fegan (23). The Korean biovar 1, 3, and 4 isolates used in this study belonged to phylotype I, while the Korean biovar 2 isolate belonged to phylotype IV. Taken together, our AFLP, 16S rRNA, endoglucanase, *hrpB*, and *mutS* gene sequence analyses indicate that biovar 2 isolates are genetically distinct from the other biovars and have a close genetic relationship with the Japanese biovar N2 isolates.

According to Cook and Sequeira (6), the Americanum division includes biovars 1,

2, and N2, while the Asiaticum division contains biovars 3, 4, and 5; however, our analysis showed that the Korean isolates included biovars 1, 2, 3, and 4, suggesting that the Asiaticum division should be revised to include other biovars. Interestingly, the Korean and Japanese isolates belonging to biovars 1, 2, 3, and 4 exhibited weak genetic relationships with the American, African, and European isolates, indicating that the genetic backgrounds of the Korean isolates are distinct from those of the foreign isolates used in this study. In addition, since a biovar N2 isolate was not observed among the Korean isolates, it is possible that biovar N2, which is believed to have originated in tropical regions, may not yet be present in Korea.

Among the Korean samples, biovar 2 isolates were found only in a cultivated tomato and potato field in the south. Because biovar 2 isolates are limited to the southern part of Korea and were grouped in a single cluster by AFLP, 16S rRNA, endoglucanase, *hrpB*, and *mutS* gene sequence analyses, it is possible that those isolates might have originated in foreign countries. Buddenhagen (2) suggested that race 3 (biovar 2) originated in the Andean region of South America, and that the occurrence of the race outside of South America is a result of the distribution of infected potato tubers by international trade. There is no record of the existence of biovar 2 isolates in Korea before the potato cultivar Daeji was imported from Japan between 1992 and 2002 (1). It is not obvious from where the Korean biovar 2 isolate originated; however, considering that Daeji is the only potato cultivar grown in southern Korea, the biovar 2 isolates might have arrived with imported potato tubers. To address this possibility, additional Japanese isolates may be needed for a more comprehensive analysis.

ACKNOWLEDGMENTS

This study was supported by the Technology Development Program for Agriculture and Forestry by the Ministry of Agriculture and Forestry, and the Crop Functional Genomics Center of the 21st Century Frontier R&D Program, which is funded by Ministry of Science and Technology of the Republic of Korea.

LITERATURE CITED

1. Anonymous. 1991-2005. Trade in the agriculture, forestry and fishery sector. In: Agricultural and Forestry Statistical Yearbook. Ministry of Agriculture and Forestry, Republic of Korea.
2. Buddenhagen, I. 1985. Bacterial wilt revisited. Pages 126-143 in: Bacterial Wilt Disease in Asia and the South Pacific. G. J. Persley, ed. Aust. Cent. Int. Agric. Res. Proc. 13. Canberra, Australia.
3. Carney, B., and Denny, T. P. 1990. A cloned avirulence gene from *Pseudomonas solanacearum* determines incompatibility on *Nicotiana tabacum* at the host species level. J. Bacteriol. 172:4836-4843.
4. Cook, D., Barlow, E., and Sequeira, L. 1989. Genetic diversity of *Pseudomonas solanacearum*: Detection of restriction fragment length polymorphism with DNA probes that specify

- virulence and the hypersensitive response. *Mol. Plant-Microbe Interact.* 2:113-121.
5. Cook, D., Barlow, E., and Sequeira, L. 1991. DNA probes as tools for the study of host-pathogen evolution: The example of *Pseudomonas solanacearum*. Pages 103-108 in: *Advances in Molecular Genetics of Plant-Microbe Interactions*. Vol. 1. H. Henneke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, Netherlands.
6. Cook, D., and Sequeira, L. 1994. Strain differentiation of *Pseudomonas solanacearum* by molecular genetic methods. Pages 77-93 in: *Bacterial Wilt: The Disease and its Causative Agent, Pseudomonas solanacearum*. A. C. Hayward and G. L. Hartman, eds. CAB International, Wallingford, UK.
7. Fegan, M., and Prior, P. 2005. How complex is the *Ralstonia solanacearum* species complex? Pages 449-461 in: *Bacterial Wilt Disease and the Ralstonia solanacearum Species Complex*. C. Allen, P. Prior, and A. C. Hayward, eds. American Phytopathological Society, St. Paul, MN.
8. Fegan, M., Taghavi, M., Sly, L. I., and Hayward, A. C. 1998. Phylogeny, diversity and molecular diagnostics of *Ralstonia solanacearum*. Pages 19-33 in: *Bacterial Wilt Disease: Molecular and Ecological Aspects*. P. Prior, C. Allen, and J. Elphinstone, eds. INRA Edition, Paris.
9. Hayward, A. C. 1964. Characteristics of *Pseudomonas solanacearum*. *J. Appl. Bacteriol.* 27:265-277.
10. Hayward, A. C. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. *Annu. Rev. Phytopathol.* 29:65-87.
11. He, L. Y., Sequeira, L., and Kelman, A. 1983. Characteristics of strains of *Pseudomonas solanacearum*. *Plant Dis.* 67:1357-1361.
12. Horita, M., and Tsuchiya, K. 2000. Comparative analysis of Japanese and foreign strains of *Ralstonia solanacearum* based on 16S ribosomal RNA gene sequences. *J. Gen. Plant Pathol.* 66:132-137.
13. Horita, M., and Tsuchiya, K. 2001. Genetic diversity of Japanese strains of *Ralstonia solanacearum*. *Phytopathology* 91:399-407.
14. Jeanmougin, F., Thompson, J. D., Gouy, M., Higgins, D. G., and Gibson, T. J. 1998. Multiple sequence alignment with Clustal X. *Trends Biochem. Sci.* 23:403-405.
15. Jeong, Y., Kim, J., Kim, S., Kang, Y., Nagamatsu, T., and Hwang, I. 2003. Toxoflavin produced by *Burkholderia glumae* causing rice grain rot is responsible for inducing bacterial wilt in many field crops. *Plant Dis.* 87:890-895.
16. Jukes, T. H., and Cantor, C. R. 1969. Evolution of protein molecules. Pages 21-132 in: *Mammalian Protein Metabolism*. H. N. Munro, ed. Academic Press, New York.
17. Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. *Phytopathology* 44:693-695.
18. Kumar, S., Tamura, K., and Nei, M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* 5:150-163.
19. Li, X., Dorsch, M., Del Dot, T., Sly, L. I., Stackebrandt, E., and Hayward, A. C. 1993. Phylogenetic studies of the rRNA group II pseudomonads based on 16S rRNA sequences. *J. Appl. Bacteriol.* 74:324-329.
20. Poussier, S., Prior, P., Luisetti, J., Hayward, A. C., and Fegan, M. 2000. Partial sequencing of the *hrpB* and endoglucanase genes confirms and expands the known diversity within the *Ralstonia solanacearum* species complex. *Syst. Appl. Microbiol.* 23:479-486.
21. Poussier, S., Trigalet-Demery, D., Vandewalle, P., Goffinet, B., Luisetti, J., and Trigalet, A. 2000. Genetic diversity of *Ralstonia solanacearum* as assessed by PCR-RFLP of the *hrp* gene region, AFLP and 16S rRNA sequence analysis, and identification of an African subdivision. *Microbiology* 146:1679-1692.
22. Poussier, S., Vandewalle, P., and Luisetti, J. 1999. Genetic diversity of African and worldwide strains of *Ralstonia solanacearum* as determined by PCR-restriction fragment length polymorphism analysis of the *hrp* gene region. *Appl. Environ. Microbiol.* 65:2184-2194.
23. Prior, P., and Fegan, M. 2005. Recent developments in the phylogeny and classification of *Ralstonia solanacearum*. *Acta Hort.* 695:127-136.
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
25. Seal, S. E., Jackson, L. A., and Daniels, M. J. 1992. Use of tRNA consensus primers to indicate subgroups of *Pseudomonas solanacearum* by polymerase chain reaction amplification. *Appl. Environ. Microbiol.* 58:3759-3761.
26. Smith, J. J., Offord, L. C., Holderness, L. C., Holderness, M., and Saddler, G. S. 1995. Genetic diversity of *Burkholderia solanacearum* race 3 in Kenya. *Appl. Environ. Microbiol.* 61:4263-4268.
27. Taghavi, M., Hayward, C., Sly, L. I., and Fegan, M. 1996. Analysis of the phylogenetic relationships of strains of *Burkholderia solanacearum*, *Pseudomonas syzygii*, and the blood disease bacterium of banana based on 16S rRNA gene sequence. *Int. J. Syst. Bacteriol.* 46:10-15.
28. Villa, J. E., Tsuchiya, K., Horita, M., Natural, M., Opina, N., and Hyakumachi, M. 2005. Phylogenetic relationships of *Ralstonia solanacearum* species complex strains from Asia and other continents based on 16S rDNA, endoglucanase, and *hrpB* gene sequences. *J. Gen. Plant Pathol.* 71:39-46.
29. Vos, P., Hogers, R., Bleeker, M., Reijmans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res.* 23:4407-4414.
30. Woese, C. R., Gutell, R., Gupta, R., and Noller, H. F. 1983. Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. *Microbiol. Rev.* 47:621-669.
31. Yun, G. S., Park, S. Y., Kang, H. J., Lee, K. Y., and Cha, J. S. 2004. Contamination level of *Ralstonia solanacearum* in soil of greenhouses cultivating tomato plants in Chungbuk province and characteristics of the isolates. *Res. Plant Dis.* 10:58-62.
32. Yu, Q., Alvarez, A. M., Moore, P. H., Zee, F., Kim, M. S., de Silva, A., Hepperly, P. R., and Ming, R. 2003. Molecular diversity of *Ralstonia solanacearum* isolate from ginger in Hawaii. *Phytopathology* 93:1124-1130.