농학박사학위논문

한국의 세균성 풋마름병 감염 감자에서 분리한 *Ralstonia solanacearum*의 유전체 해독 및 비교분석

Comparative genome analysis of *Ralstonia solanacearum* causing potato bacterial wilt in Korea

2018년 2월

서울대학교 대학원

농생명공학부 식물미생물학전공

조 희 정

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Comparative genome analysis of *Ralstonia solanacearum* causing potato bacterial wilt in Korea

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ABSTRACT

Ralstonia solanacearum, causal agent of bacterial wilt, is one of the most destructive phytopathogen in the world. Soil-borne this bacterium invades plants mainly through the roots, colonizes and proliferates in the xylem, as the results plants wilt by blocking water. *R. solanacearum* has unusual broad host range over 450 plant species of 50 botanical families. This bacterium distributed worldwide encompassing tropical, subtropical, and temperate region. With these features, this species are very diverse and complex and call as pathogenic *Ralstonia solanacearum* species complex (RSSC). This study used 93 RSSC isolates collected from potato bacterial wilt (or brown rot) from 1998 to 2003 in Korea. To investigate the host specific factors by comparative genomic analyses, first, their properties were determined by analyzing of phylotype, biovar, and host range. Of the 93 isolates, 71 isolates determined to phylotype I and these divided into

eight isolates of biovar 3 and sixty-three isolates of biovar 4. Twenty-two isolates determined to phylotype IV and these were all biovar 2. This phylotype-biovar classification was consistent with phylogenetic trees based on 16S rRNA and *egl* gene sequences, in which all biovar 3 and 4 isolates clustered to phylotype I, and all biovar 2 isolates clustered to phylotype IV. Korean phylotype IV isolates were distinct from phylotype I isolates pathologically as well as genetically, which was all phylotype IV isolates were nonpathogenic to peppers. In pathogenicity assays for host range determining, there were four pathotypes: (P) only pathogenic on potato, (PT) pathogenic on potato and tomato, (PTE) pathogenic on potato, tomato, and eggplant, and (PTEPe) pathogenic on all tested crops – potato, tomato, eggplant, and pepper. Based on this host range, twenty-five strains were selected and sequenced whole genome. The newly sequenced 25 Korean genomes were structurally compared with previously published nine genome data using ANI values, pair-wise and multiple genome alignment. As the results, genome sequences were usually conserved among the same phylotypes, but more divergent between phylotype I and IV. After that, to investigate candidate genes responsible for host specificity, functional genome comparisons were performed based on the host range by analyzing of pan-genome and type III secretion system effectors (T3Es). The same pathotype strains exhibited considerable gene repertoires for infection of

tomato, eggplant, or pepper. In pan-genome analysis, total 127 genes present only in tomato nonpathogenic strains, 8 genes in tomato pathogenic strains, 5 genes from eggplant nonpathogenic strains, 7 genes from eggplant pathogenic strains, one gene from pepper nonpathogenic strains, and 34 genes from pepper pathogenic strains. In T3Es analysis, RipH3 and RipS3 were found only in the tomato pathogenic strains and RipAC were only in the eggplant pathogenic strains. This study showed that the host range of *R. solanacearum* required comprehensive actions of various virulence factors involving effectors, secretion systems, attachment, and enzymes, etc.

KEY WORDS: *Ralstonia solanacearum*, potato bacterial wilt, pathogen, diversity, phylotype, comparative genome, type III effector

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CONTENTS

LIST OF TABLES

Page

CHAPTER I

CHAPTER II

LIST OF FIGURES

Page

CHAPTER I

CHAPTER II

LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
ANI	Average nucleotide identity
BDB	Blood disease bacterium
BLAST	Basic local alignment search tool
bp	Base pair
DNA	Deoxyribonucleic acid
EPS	Exopolysaccharides
FAO	Food and agricultural organization
HRP	Hypersensitive response and pathogenicity
ITS	16S-23S rRNA internal transcribed spacer sequence
LRR	Leucine rich repeat domains
NCBI	National center for biotechnology information
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
Rip	*Ralstonia* injected protein
RSSC	*Ralstonia solanacearum* species complex
T3E	Type III effector
T2SS	Type II secretion systems
T3SS	Type III secretion system
TZC	Tetrazolium chloride

GENERAL INTRODUCTION

Bacterial wilt disease occurring in economically important solanaceous crops (tomato, potato, tabacco, pepper, and eggplant) is caused by *Ralstonia solanacearum*, which is one of the most destructive phytopathogenic bacteria worldwide (Hayward, 1991). *R. solanacearum* is rod shaped gram-negative bacteria having a flagellum, and belonged to burkholderiaceae family of betaproteobacteria class. This bacterium produces massive fluidal white exopolysaccharides (EPS) on glucose or sucrose containing agar medium, and forms white with pink centered colonies on tetrazolium chloride (TZC) agar (Kelman, 1954). *R. solanacearum* is a soil-borne pathogen and can live several years without host. This bacterium invades host vascular tissues through wounded roots or natural openings. The colonization and production of EPS in the stem block water in the xylem and results in wilting and death of the host plant (Denny, 2006).

R. solanacearum have an unusually broad host range over 450 plant species belonging to more than 50 families, encompassing monocots and dicots, and herbaceous and woody plants (Hayward, 1991; Jiang et al., 2017; Wicker et al., 2007). *R. solanacearum* exists in distinct geographical regions,

which include tropical, subtropical, warm and some cool temperate areas across the six continents - Asia, Africa, Europe, North and South America, and Oceania (Denny, 2006; Hayward, 1991). These features were well shown in the review paper of China (Jiang et al., 2017). *R. solanacearum* was prevalent in tropical and subtropical regions and observed various host plants not only herbaceous plants but also woody plants including mulberry, olive, and Eucalyptus spp. (Jiang et al., 2017).

R. solanacearum has revealed great diversity in the genetic and phenotypic properties as the host range, and this species is called as the *R. solanacearum* species complex (RSSC) (Fegan and Prior, 2005; Genin and Denny, 2012). The first applying term "species complex" to *R. solanacearum* was by Gillings and Fahy in 1994 to reflect the genetic variations. The concept of *R. solanacearum* species complex expended to include *R. syzygii* and the blood disease bacterium (BDB), which were closely related organisms, by Tahgavi et al. in 1996.

Traditionally, *R. solanacearum* has been classified with "races" and "biovars" system, which were based on host range and carbohydrate utilization (Buddenhagen et al., 1962; Hayward, 1964; He et al., 1983; Pegg and Moffett, 1971). Races system, which was suggested by Buddenhagen in 1962 with 3 races, classified this bacterium into 5 races according to host

range. Race 1 was strains of pathogenic to tomato, tobacco, many solanaceous and diploid bananas, race 2 was to triploid bananas and Heliconia, race 3 was to potato and tomato, race 4 was to mulberry in China, and race 5 was to ginger in Australia. “Biovars” was proposed by Hayward in 1964. It was divided into five biovars by the ability to oxidize 3 disaccharides (lactose, maltose, and cellobiose) and 3 hexose alcohols (mannitol, sorbitol, and dulcitol) (Hayward, 1964). Biovar 1 oxidized none of six carbohydrates, biovar 2 oxidized 3 disaccharides but not 3 hexose alcohols, biovar 3 oxidized all six carbohyrates, biovar 4 oxidized 3 hexose alcohols but not 3 disaccharides, and biovar 5 oxidized 3 disaccharides and mannitol. Because biovar 2 strains obtained solely from potato and tomato, it was correlated to race 3. But, there were no more correlations between races and biovars.

In 2005, based on the 16S-23S rRNA internal transcribed spacer (ITS) sequence, “phylotypes” scheme was used to classifying of *R. solanacearum*. It was determined by amplified PCR fragments using phylotype specific multiplex primer mixture (Fegan and Prior, 2005). This scheme corresponded to geographic origin; phylotype I included strains isolated primarily from Asia region, phylotype II from the Americas, phylotype III from Africa and nearby islands, and phylotype IV from Indonesia, Australia, and Japan (Fegan and Prior, 2005; Prior and Fegan,

2005). Phylotype is also broadly consistent with various genotyping analyses of AFLP, PCR-RFLP on the HRP gene cluster and sequencing analysis of 16S rRNA, *hrpB* gene, and partial endoglucanase gene.

Recently, the RSSC had reclassified using a polyphasic taxonomic analysis - classical phenotypic tests (physiological and biochemical like oxidase, catalase, hydrolysis of starch, etc), whole-cell fatty acid composition analysis, DNA-DNA hybridization, and phylogenetic analysis of 16S-23S rRNA intergenic spacer (ITS) region and partial endoglucanase (*egl*) gene sequences. By combining these results, the RSSC was renamed as following: *R. solanacearum* of phylotype II was reclassified as true *R. solanacearum*, phylotype I and III as *R. pseudosolanacearum* sp. nov., *R. syzygii* of phylotype IV as *R. syzygii* supsp. *syzygii* supsp. nov., *R. solanacearum* of phylotype IV as *R. syzygii* supsp. *indonesiensis* supsp. nov., and the blood disease bacterium (BDB) of phylotype IV as *R. syzygii* supsp. *celebesensis* supsp. nov. (Safni et al., 2014).

The genome of *R. solanacearum* was first reported in 2002 with complete sequences of GMI1000 (Salanoubat et al., 2002). This strain had two replicons containing a 3.7-Mb and a 2.1-Mb. The large replicon encoding essential housekeeping gene sets (DNA replication, repair, cell division, transcription, and translation) was referred to as the ‘chromosome’.

While having characteristics of plasmid-borne *ori* loci, the small replicon appeared to megaplasmid. The megaplasmid contained the genes associated with virulence; type III secretion system, flagellum, and exopolysaccharide synthesis. This bipartite genome structure was characteristic of *R. solanacearum*. The genome analysis of GMI1000 marked a significant advance in the pathogenicity of this bacterium by characterizing the molecular complexity. After that, many *R. solanacearum* species, which were isolated from various hosts including solanaceae plants, banana, clove, and ginger (Cao et al., 2013; Guarischi-Sousa et al., 2016; Hayes et al., 2017; Li et al., 2016; Liu et al., 2017; Patil et al., 2017; Remenant et al., 2011; Shan et al., 2013; She et al., 2015; Sun et al., 2017; Xu et al., 2011). Up to now, 78 genomes of *Ralstonia solanacearum* species have been sequenced (NCBI database in Dec 2017). The genomes of GMI1000 and FQY_4 strains were used for the phylotype I reference genomes, CFBP2957, IPO1609, and Po82 for phylotype II, CMR15 for phylotype III, and PSI07 for phylotype IV. Comparative analysis of these genomes represented considerable diversity and validated the RSSC phylotype classification scheme by clustered to one of the phylotype I, II, III, and IV groups.

A lot of factors are responsible for pathogenesis of *R. solanacearum*; type II, III, IV, and VI secretion systems, global regulatory transcription factors, exopolysaccharides, hormones, host cell wall degrading enzymes,

adhesion/surface proteins, toxins, oxidative stress resistance, etc. Among them, type III effector proteins (T3E) is translocated directly into host cells through the type III secretion system (T3SS). These proteins have various functions on host plants to invade: the GALA family has F-box and Leucine rich repeat domains (LRR) required for full virulence (Angot et al., 2006; Kajava et al., 2008; Remigi et al., 2011), the PopP family works as avirulence proteins with acetyl transferase activity (PopP2) (Deslandes et al., 2003), and some AWR family effector induces necrotic cell death on host plants (Sole et al., 2012). Likewise, T3Es destruct homeostasis of host plants by disturbing signal transduction. Due to a helpless defense system, host plants allowed bacterial infection and died by wilting (Poueymiro and Genin, 2009). To date, 94 effectors were predicted from 11 RSSC genomes including phylotype I (GMI1000 and RS1000), II (CFBP2957, Molk2, IPO1609, Po82, and UW551), III (CMR15), and IV (PSI07, R24, and BDBR229) (Peeters et al., 2013). These T3E repertoires were highly variable among strains, but it was almost identical in the same phylotypes.

Bacterial wilt in Korea has severely affected many solanaceous crops, such as tomato, potato, eggplant, and pepper, which were economically important plant. This disease also has been observed from 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). To overcome this

disease, there have been great efforts to forecast by PCR-based detection of causal agent (Cho et al., 2011; Kang et al., 2007) and to prevent by breeding resistant varieties of tomato (Han et al., 2009; Jung et al., 2014; Kim et al., 2016; Lee et al., 2011). According to classifying of Korean *R. solanacearum* using host range and carbohydrates utilization, all isolates from tomato, eggplant, pepper, paprika, sesame, peanut, and sunflower were determined to race 1 biovar 3 (R1bv3) and race 1 biovar 4 (R1bv4), both which belonged to phylotype I of Asia origin. The isolates from potato were determined to race 1 biovar 4, and race 3 biovar 2 (R3bv2), and R3bv2 belonged to phylotype IV of Indonesia origin.

Potato (*Solanum tubersoum* L.) is important crop, which is in the top four in the world with maize (*Zea mays* L.), rice (*Oryza sativa* L.), and wheat (*Triticum aestivum* L.) (FAO Crops statistics database: http://faostat.fao.org). This plant originated in the Andes region and first introduced to Europe in the 16th century. Bacterial wilt (or brown rot) is destructive bacterial diseases on potato worldwide and caused by *R. solanacearum*. 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). Estimated damage was over $950 million per year across 80 countries (Elphinstone, 2005). In the United States, because it was considered a serious threat to

potato industry, *R. solanacearum* R3bv2 has been registered as a Select Agent plant pathogen in 2002.

In Korea, potato bacterial wilt was first reported in 1998 in Jeju-do (Lee, 1999). After that, the studies about this disease were started with collecting isolates from rotten lesions, pathogenic assays on various hosts, analyze biochemical properties, genotyping using AFLP, and sequencing of 16S and partial endoglucanase gene. In 2011, it was identified the *rsa1* gene from SL2029 (R3bv2), which was avirulent for pepper infection, and this gene made pepper pathogenic SL341 (R1bv4) to pepper nonpathogen strain (Jeong et al., 2011).

To find host specific factors of *R. solanacearum*, first, the isolates from potato bacterial wilt were analyzed about genetic/biochemical properties and pathogenicity on major solanaceae crops - potato, tomato, eggplant, and pepper. These isolates were determined to phylotype I - biovar 3, phylotype I - biovar 4, and phylotype IV - biovar 2. Pathogenicity assays demonstrated that genetic traits of this bacterium was related to host range, and offered the basement for comparative genomic analyses of the RSSC. Based on sequencing and comparative genome analyses, it was presented the candidate genes responsible for host specificity including type III effectors and pathogenesis related genes.

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CHAPTER I

Analysis of genetic and pathogenic diversity of *Ralstonia solanacearum* causing potato bacterial wilt in Korea

ABSTRACT

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, I 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 my 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.

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INTRODUCTION

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

MATERIALS AND METHODS

1. 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 (Figure 1). I 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℃ for 48 hr and frozen in 40% glycerol stock at -70℃ (Kelman, 1954).

2. 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℃ shaking incubator for 16 h. Genomic DNA of all isolates was extracted using the Wizard Genomic

DNA Purification Kit (Promega) according to the manufacturer's instructions.

3. Phylotype identification

The ninety-three isolates were classified into phylotypes as previously described (Prior and Fegan, 2005; Sagar et al., 2014). I determined phylotype using the method of Sagar (2014) based on phylotype-specific multiplex PCR (Pmx-PCR) using the following primers: Nmult:21:1F, 5'-CGTTGATGAGGCGCGCAATTT-3' (specific for phylotype I, amplicon size is 144 bp when paired with Nmult22:RR); Nmult:21:2F, 5'-AAGTTATGGACGGTGGAAGTC-3' (phylotype II, 372 bp); Nmult:23:AF, 5'-ATTACGAGAGCAATCGAAAGATT-3' (phylotype III, 91 bp); Nmult:22:InF, 5'-ATTGCCAAGACGAGAGAAGTA-3' (phylotype IV, 213 bp), and Nmult:22:RR, 5'-TCGCTTGACCCTATAACGAGTA-3' (reverse primer for all phylotypes). 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) as follows: initial denaturation at 96℃ for 5 min, followed by 30 cycles of denaturation at 95℃ for 15 sec, annealing at 59℃ for 15 sec, and extension at 72℃ for 30 sec, with a final extension at

72℃ 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.

4. 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 ($NH_4H_2PO_4$ 1 g, KCl 0.2 g, $MgSO_47H_2O$ 0.2 g, peptone 1 g, bromothymol blue 8 mg, agar 1.5 g in distilled water 1L, 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). All isolates were inoculated into individual wells with 5 μL of the 10^8 CFU/mL bacterial suspensions, with two replicates per isolate. The plates were incubated at 28℃ 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.

5. Detection of *rsa1* gene

I carried out PCR to validate the presence of *rsa1*, which has been reported to be a gene for avirulence of *R. solanacearum* on pepper hosts (Jeong et al., 2011). To eliminate errors, I prepared two *rsa1* gene primer pairs. One pair produced a 720-bp fragment containing the full *rsa1* gene including the promotor region (720-rsa1F; 5'-GCCGCTCGCCGCAATGCTGCC-3', 720-rsa1R; 5'-TGGGCTGGGTGGGACTTAACC-3'), and the other produced a 315-bp fragment containing a partial *rsa1* open reading frame (ORF) region (315-rsa1F; 5'-ATCACCAAGATTACCGGAAAG-3', 315-rsa1R 5'-TGGGCTGGGTGGGACTTAACC-3'). The reactions were carried out as described previously for phylotype determination with a primer set of 720-rsa1F/720-rsa1R at an annealing temperature of 70℃, and with a primer set of 315-rsa1F/315-rsa1R at an annealing temperature of 59℃.

6. 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′-ACGGCTACCTTGTTACGACTT-3′), and 50 ng of genomic DNA. The reaction was performed in an automated thermocycler (model PTC-200, MJ Research) with initial denaturation at 95℃ for 5 min, followed by 30 cycles of denaturation at 95℃ for 45 sec, annealing at 55℃ for 45 sec, and extension at 72℃ for 1 min, with a final extension at 72℃ 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′-ATGCATGCCGCTGGTCGCCGC-3′) and Endo-R (5′-GCGTTGCCCGGCACGAACACC-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) 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 Lasergene 8). 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 Lasergene 8).

7. 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℃ 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℃. 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.) and grown in a greenhouse for 2–3 weeks. To prepare the inoculum, all isolates were grown on TZC plates for 48 hr at 28℃. 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

1. Determination of biovar, phylotype, and *rsa1* gene

The Korean potato bacterial wilt isolates were analyzed for biovar, phylotype, and presence of the *rsa1* 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 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, I carried out *rsa1* detection PCR. For greater certainty, I used two sets of *rsa1* gene primers. One pair was for the full *rsa1* gene including the promotor (720-rsa1F/720-rsa1R), while the other was for a partial *rsa1* ORF (315-rsa1F/315-rsa1R). 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℃. 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-rsa1F/315-rsa1R primer set at an annealing temperature of 59℃, 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.

Table 1. Host, year, geographical origin, phylotype, biovar, a *rsa1* 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[α]	Host	Year	Geographical origin	Phylotype	Biovar	*rsa1*[β]	Pathogenicity[δ]				Pathotyp[ε]	16S rRNA[ζ]	*egl*[η]	Source
							Potato	Tomato	Eggplant	Pepper				
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

Table 1. (Continued)

Isolate	Host	Year	Geographical origin	Phylotype	Biovar	*rsa1*	Pathogenicity				Pathotype	16S rRNA	*egl*	Source
							Potato	Tomato	Eggplant	Pepper				
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
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

Table 1. (Continued)

Isolate	Host	Year	Geographical origin	Phylotype	Biovar	*rsa1*	Pathogenicity				Pathotype	16S rRNA	*egl*	Source
							Potato	Tomato	Eggplant	Pepper				
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
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

Table 1. (Continued)

Isolate	Host	Year	Geographical origin	Phylotype	Biovar	*rsa1*	Pathogenicity				Pathotype	16S rRNA	*egl*	Source
							Potato	Tomato	Eggplant	Pepper				
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

[α]Isolate: SL-numbering isolates were obtained from Dr. Seungdon Lee and T-numbering isolates were from Dr. Young Kee Lee.

[β]*rsa1*: PCR was carried out using two primer pairs; one pair produced a 720-bp fragment containing full *rsa1* gene including promotor (720-rsa1F/720-rsa1R) at 70℃ annealing temperature, and another produced a 315-bp fragment containing partial *rsa1* ORF region (315-rsa1F/315-rsa1R) at 59℃ annealing temperature.

[δ]Pathogenicity: 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.

[ε]Pathotype: (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.

[ζ]16S rRNA: GenBank accession number of 16S rRNA.

[η]endoglucanase: GenBank accession number of endoglucanase gene.

2. Geographical distribution

Figure 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) 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.

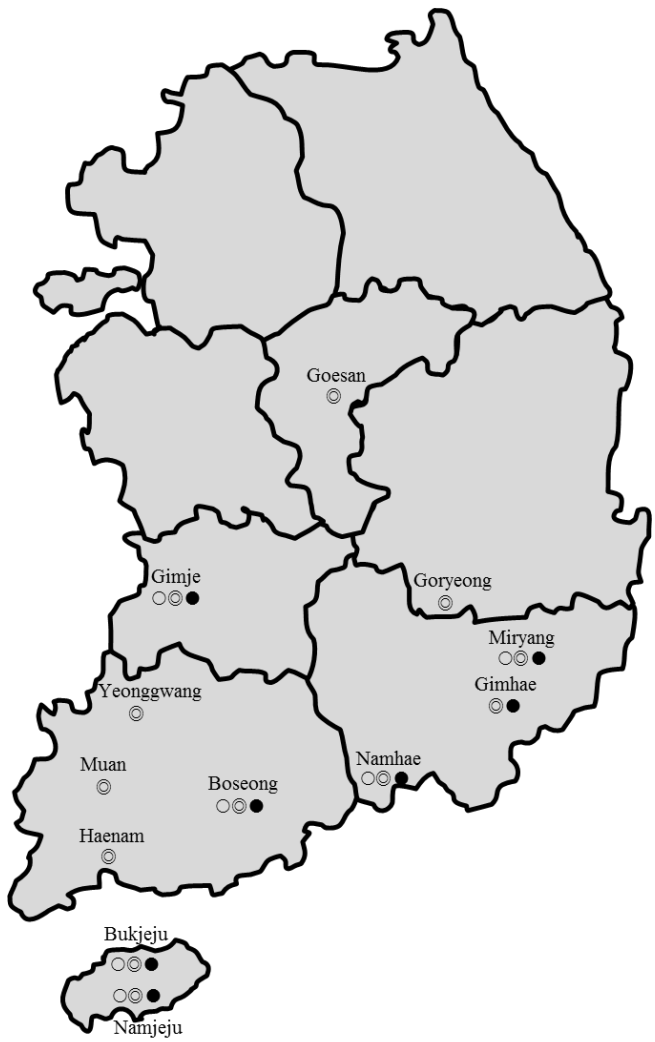

Figure 1. Potato-growing areas and geographic locations infected with potato bacterial wilt in Korea from 1998–2003. Potato-growing areas are colored gray, and symbols indicate isolates: ○, phylotype I-biovar 3; ◎, phylotype I-biovar 4; ●, phylotype IV- biovar 2. Each symbol indicates representative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

3. Phylogenetic analysis of 16S rRNA and partial endoglucanase (*egl*) gene sequences

To evaluate the genetic relationship of Korean potato isolates, I 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 (Figure 2).

The 16S rRNA sequences of biovar 2 isolates were identical to one 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. 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 were identical among biovar 4 isolates, I analyzed the *egl* sequence of SL2729 as the representative strain of biovar 4. 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.

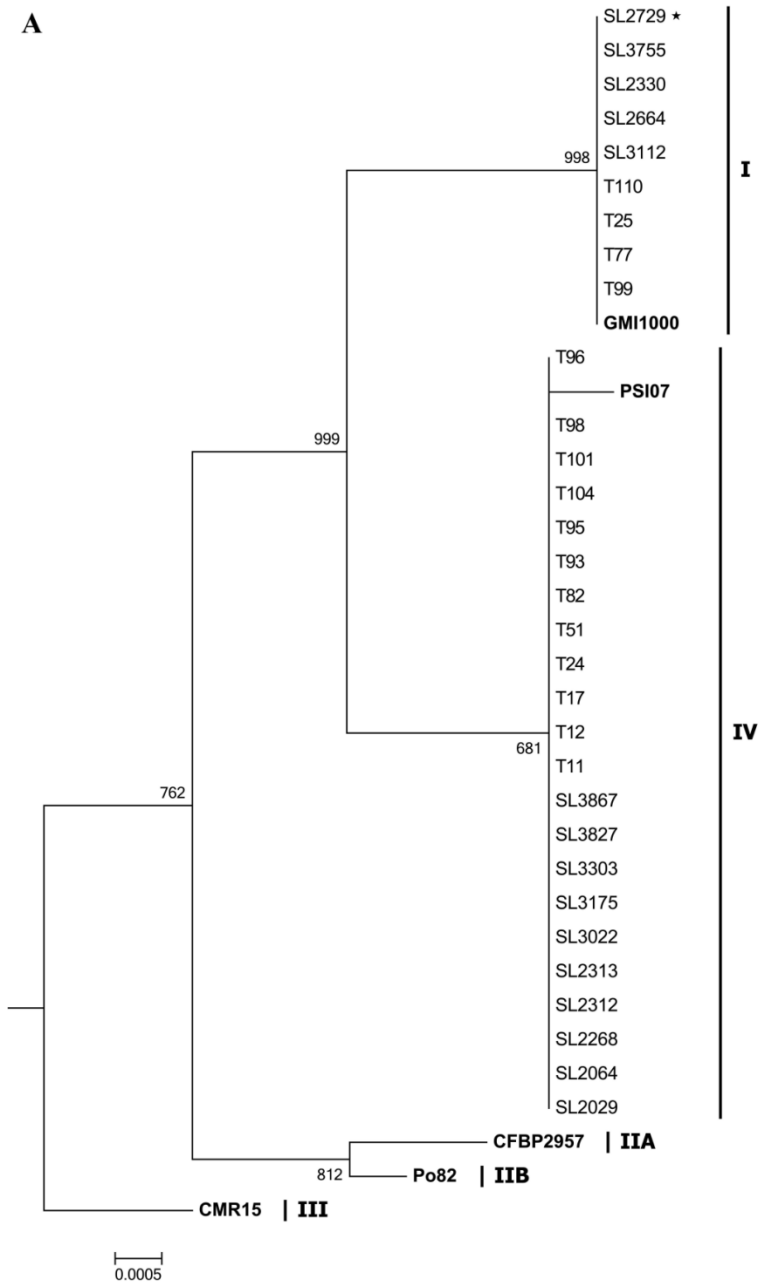

Figure 2. Phylogenetic trees of 16S rRNA (A) and partial endoglucanase (*egl*) gene sequences (B) analyzed by ClustalW with 1,000 bootstrap replicates using MegAlign of DNASTAR Lasergene 8. Roman numerals indicate phylotypes and symbols (★) indicate the representative biovar 4 strain.

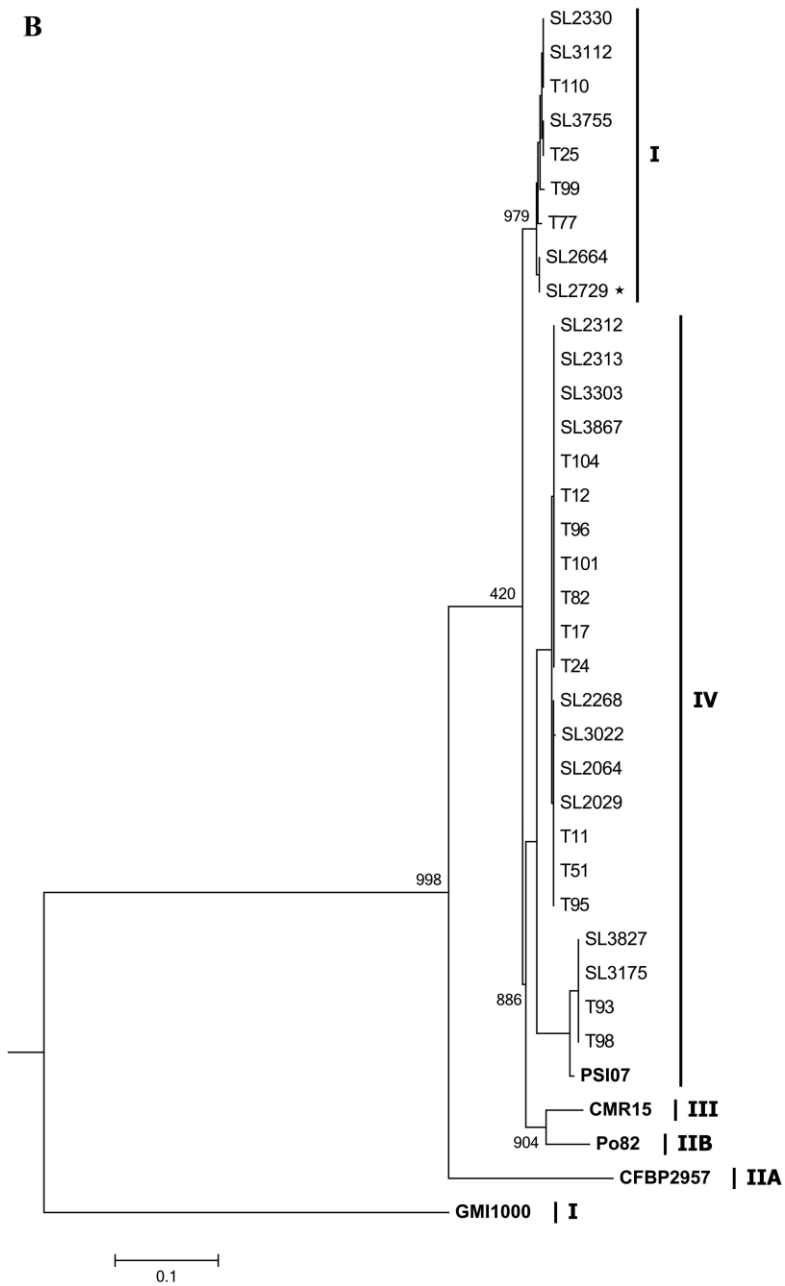

Figure 2. (continued)

4. Host range determination

To determine the host range of the bacteria that cause potato bacterial wilt, I 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 infect 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). Figure 3 shows the host range of representative pathotype strains.

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
PTE	+	+	+	–	IV-2	7	SL2029, SL2064, SL2268, T11, T51, T93, T95
					I-3	1	T77
					I-4	4	SL2264, SL 3103, T56, T58
PTEP	+	+	+	+	I-3	7	SL2330, SL2664, SL3112, 3755, T25, T99, T110
					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	

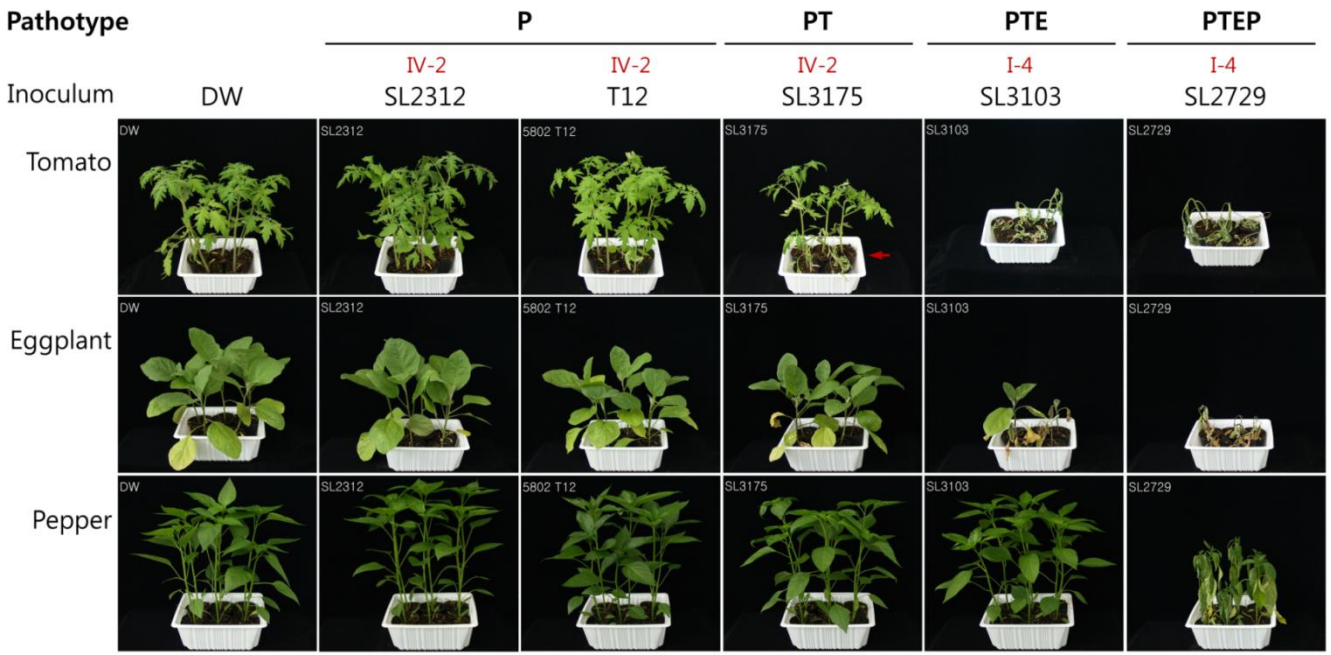

Figure 3. Host range determination of *Ralstonia solanacearum* on tomato, eggplant, and pepper. SL2312 and T12 were not pathogenic to tomato, eggplant, and pepper (pathotype P); SL3175 was pathogenic to tomato, and not pathogenic to eggplant or pepper (PT); SL3103 was pathogenic to tomato and eggplant, 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.

DISCUSSION

When potato bacterial wilt was first reported in Korea, I 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. solancearum*. These isolates were analyzed to determine their phylotype, biovar, phylogenetic relationship of 16S rRNA and *egl* gene, presence of an *rsa1* 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 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 in 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 in 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 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, I 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 *rsa1* gene, which confers avirulence to pepper-infecting strains (Jeong et al., 2011). This result is consistent with my host-determining pathogenicity assays, which showed that all biovar 2 isolates were nonpathogenic to pepper. I also identified tomato-nonpathogenic biovar 2 isolates. In the course of pathogenicity assays, I 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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CHAPTER II

Comparative genome analysis of *Ralstonia solanacearum* causing potato bacterial wilt in Korea

ABSTRACT

Soil-borne pathogenic *Ralstonia solanacearum* species complex (RSSC) is economically destructive phytopathogens in worldwide. This bacterium species causes bacterial wilt on various solanaceae plants and individually has different scope of host range. Here, after selecting of strains possessing distinctive host range (potato, tomato, eggplant, and pepper), it was sequenced the genomes of twenty-five strains isolated from potato bacterial wilts, which belonged to phylotype I and IV. With the newly sequenced genomes, structural genome comparison were analyzed with previously published genome data of nine *R. solanacearum* species including phylotype I, IIA, IIB, III, and IV to show the consistency among them. Also, it was performed phylogenetic relationship by calculating ANI values, pair-wise genome alignments using YASS tool, and multiple genome alignment with Mauve software. Genome sequences were relatively conserved among the phylotype I strains and among the phylotype IV strains, but more divergent between strains of phylotype I and IV. Subsequently, functional genome comparisons were analyzed to investigate candidate genes related to the host adaptation. The strains of same pathotype group exhibited considerable repertoires for infection of tomato, eggplant,

or pepper. By analyzing the type III secretion system effectors (T3Es), it was found three host-specific effectors; RipS3 (Skwp3) and RipH3 having only the tomato-pathogenic strains and RipAC (PopC) having only the eggplant-pathogenic strains. This study suggests that host range of *R. solanacearum* species is conferred by combination of host-specific effectors and other supportable virulence factors involving on regulatory mechanisms, secretion systems, and hydrolytic enzymes, etc.

INTRODUCTION

The genome study of *Ralstonia solanacearum* was started with complete genome sequencing of GMI1000, which marked a significant advance in the pathogenicity by characterizing the molecular complexity (Salanoubat et al., 2002). This strain contained two replicons: a 3.7-Mb chromosome, which encodes essential housekeeping genes like DNA replication and repair, and a 2.1 Mb megaplasmid, which encodes virulence genes like type III secretion systems and flagellum. After sequencing of GMI1000, many *R. solanacearum* species of various host range were sequenced completely or draftly (Cao et al., 2013; Guarischi-Sousa et al., 2016; Hayes et al., 2017; Li et al., 2016; Liu et al., 2017; Patil et al., 2017; Remenant et al., 2010; Shan et al., 2013; She et al., 2015; Sun et al., 2017; Xu et al., 2011). To date, the genomes of *R. solanacearum* species have been sequenced 78 genomes in the National Center for Biotechnology Information (NCBI database in Dec 2017). Most *R. solanacearum* strains had one chromosome and one megaplasmid but several strains carried additional plasmids. *R. solanaceraum* CMR15 (phylotype III) and PSI07 (phylotype IV) strain had an additional plasmid and each sizes were 35 kbp and 12.8 kbp (Remenant et al., 2010). In the plasmid of CMR15, it was

existed the genes encoding for type IV secretion system, from *virB1* to *virB11*, except *virB7*.

Whole-genome comparisons of these genomes represented considerable diversity and confirmed the phylotype scheme of the RSSC classification - phyloytpe I (GMI1000, FQY_4, EP1, and Y45), II (CFBP2957, IPO1609, K60, MolK2, Po82, and UW551), III (CMR15), and IV (PSI07, *R. syzygii* R24 and Blood Disease Bacterium (BDB) R229). By analyses of various biochemical properties and genomic comparison, RSSC were reclassified as followings; phylotype II srains as *Ralstonia solanacearum*, phylotype I and III strains as *Ralstonia pseudosolanacearum*, and phylotype IV strains including *R. syzygii* R24 and BDB R229 as *Ralstonia syzygii* (Safni et al., 2014).

Among the factors for pathogenicity determining, the type III secretion system (T3SS) is very important by constructing the secretion machinery. The gene organization of encoding type III secretion systems (T3SS) were usually conserved among the strains of phylotype I, II, III, and IV (Li et al., 2016). This T3SS exports and translocates virulence factors directly, specially called type III effector proteins (T3E) (Cunnac et al., 2004; Mukaihara et al., 2010). By these translocated T3Es, host plants destructed homeostasis by disturbing signal transduction or defense systems, allowed

bacterial infection, at the end, died (Poueymiro and Genin, 2009). The effector of *R. solanacearum* had been designated Rip (Ralstonia injected proteins), which included former Pop/AWR/Gala families (Peeters et al., 2013). The mining of the RSSC pan-genome identified around 110 Rips among 11 representative phylotypes strains. Individual *R. solanacearum* strains possess around 60-75 effectors and effector repertoire comparison revealed 32 core effector presented in 10 strains. Most *rip* genes had a feature in their promoter region, which was responsive to the T3SS transcriptional regulator HrpB (Cunnac et al., 2004).

Comparisons of the genes encoding type II secretion systems were analyzed with 7 reference genomes including phylotype I, II, III, and IV (Li et al., 2016). These systems were differences among phylotypes. GMI1000 and other phylotype I strains (FQY_4, YC45, and EP1) and Po82 (phylotype II) possessed three type II secretion systems, one orthodox system and two unorthodox systems. CMR15 (phylotype III) and PSI07 (phylotype IV) strains had one orthodox system and one unorthodox systems.

To find responsible genes for host specificity of *R. solanacearum*, whole genome of 25 Korean strains were sequenced completely and performed comparative genome analyses based on the host range. Although many studies had been conducted to identify the genes associated with host

specificity, by unclear host range specificity, they did not find any genes or rarely described in detail (Ailloud et al., 2015; Cellier et al., 2012; Guidot et al., 2007; Peeters et al., 2013). Compared to previous studies, this comparative genome study based on the host range determination in chapter I is valuable. The results presented the candidate genes for specific pathogenicity traits about infection of RSSC to tomato, eggplant, and pepper.

MATERIALS AND METHODS

1. Strain selection

In previous chapter, the genetic and pathogenic diversity of Korean *Ralstonia solanacearum* had been analyzed using 93 strains isolated from potato bacterial wilt in all over the country. In order to analyze the genetic relationships with host range in depth, 25 isolates had been selected based on the diverse isolation region in Korea with typical representative characters for individual phylotypes (Table 1). Strains selected based on the areas where potato bacterial wilt disease have occurred frequently; Namjeju, Bukjeju, Miryang, Namhae, Gimhae, Boseong, Haenam, Muan, Yeonggwang, Gimje, and Goesan. Among them, strains having various host range were finally selected for comparative genome study.

Table 1. List of *Ralstonia solanacearum* strains selected for genome analysis.

Strain	Host	Geographical origin	Phylotype	Biovar	Pathogenicity				Pathotype
					Potato	Tomato	Eggplant	Pepper	
SL2312	Potato	Miryang, Gyeongsangnam-do	IV	2	+++	-	-	-	P
T12	Potato	Namhae, Gyeongsangnam-do	IV	2	+++	-	-	-	P
T82	Potato	Gimje, Jeollabuk-do	IV	2	+++	-	-	-	P
T101	Potato	Namjeju, Jeju-do	IV	2	+++	-	-	-	P
SL3022	Potato	Gimhae, Gyeongsangnam-do	IV	2	+++	+++	-	-	PT
SL3175	Potato	Namjeju, Jeju-do	IV	2	+++	++	-	-	PT
T98	Potato	Namjeju, Jeju-do	IV	2	+++	+++	-	-	PT
SL2064	Potato	Namjeju, Jeju-do	IV	2	+++	+++	+++	-	PTE
T11	Potato	Gimhae, Gyeongsangnam-do	IV	2	++	++	++	-	PTE
T51	Potato	Boseong, Jeollanam-do	IV	2	+++	+	+	-	PTE
T95	Potato	Namjeju, Jeju-do	IV	2	+++	+	+++	-	PTE
SL3103	Potato	Haenam, Jeollanam-do	I	4	+	+++	+++	-	PTE
SL2330	Potato	Namhae, Gyeongsangnam-do	I	3	+	+++	+++	+	PTEPe
SL3755	Potato	Boseong, Jeollanam-do	I	3	+	+++	+	+	PTEPe
T25	Potato	Miryang, Gyeongsangnam-do	I	3	++	+++	++	+	PTEPe
T110	Potato	Bukjeju, Jeju-do	I	3	+	+++	++	+	PTEPe
SL2729	Potato	Miryang, Gyeongsangnam-do	I	4	++	+++	+++	+++	PTEPe
SL3300	Potato	Namjeju, Jeju-do	I	4	+++	+++	+++	+++	PTEPe
SL3730	Potato	Muan, Jeollanam-do	I	4	+++	+++	+++	+++	PTEPe

Table 1. (continued)

Strain	Host	Geographical origin	Phylotype	Biovar	Pathogenicity				Pathotype
					Potato	Tomato	Eggplant	Pepper	
SL3822	Potato	Bukjeju, Jeju-do	I	4	+++	+++	+++	+++	PTEPe
SL3882	Potato	Haenam, Jeollanam-do	I	4	+++	+++	+++	+++	PTEPe
T42	Potato	Muan, Jeollanam-do	I	4	+++	+++	+++	+	PTEPe
T60	Potato	Yeonggwang, Jeollanam-do	I	4	+++	+++	+++	+++	PTEPe
T78	Potato	Gimje, Jeollabuk-do	I	4	+++	+++	+++	++	PTEPe
T117	Potato	Goesan, Chungcheongbuk-do	I	4	+++	+++	+++	+++	PTEPe

[a]Pathotype: (P) only pathogenic on potato, (PT) pathogenic on potato and tomato, (PTE) pathogenic on potato, tomato, and eggplant, and (PTEPe) pathogenic on all tested crops – potato, tomato, eggplant, and pepper.

2. Genome sequencing

For genomic DNA preparation, bacterial cells were grown on TZC agar medium, were cultured on LB broth (Peptone 10 g, Yeast Extract 5 g, Sodium Chloride 5 g in 1 liter distilled water) in 28℃ shaking incubator for 16 hr, and harvested by centrifugation. For Pacbio sequencing, high molecular weight genomic DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions.

The bacterial genome was sequenced using the Pacific Biosciences' Single Molecule Real Time (SMRT) Sequencing Technology using a 20-kb library, P6/C4 chemistry and one SMRT cell (www.dnalink.com, DNAlink, Korea). *de novo* assembly was conducted using the hierarchical genome assembly process (HGAP, Version 2.3) workflow (Chin et al., 2013), including consensus polishing with Quiver, and default parameters (Minimum Subread Length 500 bp, Minimum Polymerase Read Quality 0.8, and Minimum Polymerase Read Length 100 bp). After error correction based on the longest seed bases with rest shorter reads, and then assembled with error corrected reads. Gene prediction was carried out using the program Glimmer3, RNAmmer-1.2 and tRNAscan-SE and found CDS, rRNA and tRNA in the assembled genome. The annotation of each CDS was

made by homology search against Blastall2.2.26.

3. Structural genome comparisons

For comparison with reference strains, the genome sequences of GMI1000, FQY_4, CMR15, PO82, and PSI07 were obtained from NCBI database and *Ralstonia syzyggii* R24 and Blood disease bacterium (BDB) R229 were obtained from EMBL database (Table 2). To analyze the overall genome sequences similarity, Orthologous Average Nucleotide Identity (OrthoANI) tool was used (Lee et al., 2016). Pairwise whole-genome alignments were constructed and visualized using genomic similarity search tool YASS (http://bioinfo.lifl.fr/yass/yass.php) (Noe and Kucherov, 2005) with default parameters. Multiple genome alignments were performed using the Mauve software (http://darlinglab.org/mauve/mauve.html).

4. COG distribution and Pan-genome Orthologous (POG) analysis

For comparative genome analysis, pan-genome orthologous analysis was performed using the Chunlab pipeline (http://www.chunlab.com, Korea) (Chun et al., 2009). Gene prediction and annotation were analyzed using the RAST server (Aziz et al., 2008). The annotation of each coding sequence (CDS) was made by homology search

against Swiss-prot, EggNOG 4.1, SEED, KEGG databases. Clusters of Orthologous Group (COG) was analyzed using $CL_{GENOMICS}$ (http://www.chunlab.com, Korea).

5. Effectors prediction

The effectors of type III secretion systems (T3E) of Korean *Ralstonia solanacearum* strains were annotated using the RalstoT3E server (https://iant.toulouse.inra.fr/bacteria/annotation/site/prj/T3Ev2), which was the effector database of *Ralstonia solanacearum* species complex (Peeters et al., 2013).

6. Genome submission

Genome sequences of twenty-five Korean *R. solanacearum* strains were deposited in the National Center for Biotechnology Information (NCBI).

RESULTS

1. General genome features

High-quality genomes of Korean strains were constructed using Pacbio long read sequencing data. The general genomic features of *Ralstonia solanacearum* strains used in this study are summarized in Table 2. The newly sequenced twenty-five Korean *R. solanacearum* genome were completed and contained two circular contigs; one for chromosome and another for megaplasmid, except for T78 strain containing one chromosome, one megaplasmid, and one small plasmid.

The assembled genome sizes were 5.4 ~ 6.15 Mbp and GC contents were 66.4 ~ 66.9 %. The number of predicted genes were 5,071 ~ 6,811, rRNA genes were 9 or 12, and tRNA were 54 ~ 59. There were several differences in the genomes between phylotype I and phylotype IV as following: the average genome sizes of phylotype I and IV were 5.8 Mbp and 5.5 Mbp, the average GC contents were 66.91 % and 66.39 %, the average predicted CDSs were 5,551 and 5,152, the number of rRNA genes were 12 and 9, the average number of tRNA genes were 58 and 55. All genomic features (genome size and GC content, CDS, rRNA, and tRNA) of

phylotype I strains were larger than that of phylotype IV except for T42 strain, which was similar in genome size and CDS number with phylotype IV strains. However, T42 also carries 12 rRNA and 57 tRNA and had 67 % GC content, and these mean that the genome features of T42 were more close to phylotype I than phylotype IV.

Table 2. General genome features of *Ralstonia solanacearum* strains sequenced for this work.

Strain[a]	Phyl-bv[b]	Contigs	Size (bp)	GC (%)	CDSs	rRNA	tRNA	Genome Accession[c]
SL2312	IV-2	2	5,521,456	66.4	5,079	9	56	CP022796, CP022797
T12	IV-2	2	5,520,985	66.4	5,189	9	56	CP022774, CP022775
T82	IV-2	2	5,521,457	66.4	5,071	9	56	CP022763, CP022764
T101	IV-2	2	5,521,368	66.4	5,086	9	56	CP022757, CP022758
SL3022	IV-2	2	5,605,251	66.3	5,376	9	55	CP023016, CP023017
SL3175	IV-2	2	5,555,993	66.4	5,170	9	54	CP022788, CP022789
T98	IV-2	2	5,555,978	66.4	5,172	9	54	CP022759, CP022760
SL2064	IV-2	2	5,473,607	66.4	5,169	9	55	CP022798, CP022799
T11	IV-2	2	5,450,627	66.4	5,143	9	56	CP022776, CP022777
T51	IV-2	2	5,400,849	66.4	5,074	9	55	CP022770, CP022771
T95	IV-2	2	5,474,514	66.4	5,146	9	55	CP022761, CP022762
SL3103	I-4	2	5,618,133	67.0	5,257	12	58	CP022790, CP022791
SL2330	I-3	2	5,674,600	67.0	5,242	12	58	CP022794, CP022795
SL3755	I-3	2	5,792,854	66.9	5,413	12	58	CP022782, CP022783
T25	I-3	2	5,715,510	67.0	5,805	12	58	CP023014, CP023015
T110	I-3	2	5,642,243	67.1	6,811	12	57	CP023012, CP023013
SL2729	I-4	2	5,703,338	67.0	5,304	12	58	CP022792, CP022793
SL3300	I-4	2	5,903,911	66.8	5,482	12	58	CP022786, CP022787
SL3730	I-4	2	5,686,064	67.0	5,348	12	58	CP022784, CP022785
SL3822	I-4	2	5,971,831	66.8	5,558	12	59	CP022780, CP022781
SL3882	I-4	2	6,025,869	66.8	5,594	12	59	CP022778, CP022779
T42	I-4	2	5,497,698	67.0	5,133	12	57	CP022772, CP022773
T60	I-4	2	6,015,554	66.8	5,588	12	59	CP022768, CP022769
T78	I-4	3	6,147,432	66.7	5,807	12	59	CP022765, CP022766,CP022767
T117	I-4	2	5,807,463	66.9	5,378	12	59	CP022755,CP022756

Table 2. (continued- reference genome of *R. solanacearum* strains used in this work)

Strain	Phyl-bv	Contigs	Size (bp)	GC (%)	CDSs	rRNA	tRNA	Genome Accession
PSI07	IV-2	3	5,605,618	66.3	4,810	9	54	FP885906.2, FP885891.2
R. syzygii R24	IV	7	5,423,991	65.9	4,865	6	50	EMBL FR854086 - FR854092
BDB R229	IV	27	5,158,998	66.4	4,614	8	67	EMBL FR854059 - FR854085
CMR15	III	3	5,590,372	66.8	4,890	12	59	FP885895.1, FP885896.1, FP885893.1
CFBP2957	IIA-2	1	3,417,386	66.4	3,158	9	53	FP885897.1
Po82	IIB-2	2	5,430,263	66.7	4,745	9	54	CP002819.1, CP002820.1
IPO1609	IIB-2	10	5,318,522	64.9	4,659	6	31	NZ_CDGL000000000.1
GMI1000	I-3	2	5,810,922	67.0	5,055	12	57	AL646052.1, AL646053.1
FQY_4	I-4	2	5,805,250	66.8	5,068	12	51	CP004012.1, CP004013.1

[a]Strain T78 had third circular plasmid of 128,742 bp, 156 CDSs, and no rRNA and tRNA.

[b]Phylotype-biovar

[c]NCBI genome accession numbers, except *R. syzygii* R24 and Blood Disease Bacterium (BDB) R229 genome, which were EMBL genome accession numbers.

2. Structural genome comparisons: OrthoANI analysis, Pair-wise and Multiple genome alignments

To compare the overall similarity of genomes with nine reference genomes, the OrthoANI values of Korean strains had been calculated (Table 3). The results showed in the phylogenetic tree in Figure 1. Phylotype tree showed that all Korean phylotype I strains had been clustered with phylotype I reference strains, GMI1000 and FQY_4. GMI1000 had been isolated from Frensh Guyana (Salanoubat et al., 2002) and sequenced in 2002 and FQY_4 had been isolated from China and sequenced in 2013 (Cao et al., 2013). Four Korean isolates, SL2330, SL3755, T25, and T110 belonging to the biovar 3, had been clustered more closely with GMI1000 that is a biovar 3 and other ten Korean isolates in phylotype I were clustered more closed with FQY_4 that is biovar 4. In Chapter I, four Korean isolate had been determined as biovar 3 and ten isolates had been determined as biovar 4. Therefore genetic analysis showed same results with biotic analysis. Korean phylotype IV strains were clustered with phylotype IV strains of PSI07, BDB R229, and *R. syzygii* R24, which were originated from Indonesian region. Cluster for phylotype I was more compact than the cluster for phylotype IV.

To investigate the structural differences of the genome of Korean *R. solanacearum* with reference strains, pair-wise genome alignment using

YASS and multiple genome alignment using Mauve software had been performed. In pair-wise genome alignment, Korean phylotype I strains were compared with GMI1000, and the phylotype IV strains were compared with PSI07 strain (Figure 2). For phylotype IV, all Korean strains were usually co-linear with PSI07 in the chromosome and in the megaplasmid. Comparing the phylotype I strains to GMI1000, most strains had an inversion in the middle of the chromosome, except SL3300, SL3822, T25, and T117. SL3300 and T117 had double inversions in the chromosome, and SL3822 and T25 had an inversion in the megaplasmid. Comparing between GMI1000 and FQY_4, there was an inversion in the chromosome region like in the Korean strains, and two inversions in the megaplasmid. With these results, the genome of Korean phylotype I strains were co-linear with FQY_4 in chromosome, and with GMI1000 in megaplasmid.

To display the consistency among the genome of Korean *R. solanacearum* strains, multiple genome alignment was performed using Mauve tool (Figure 3). This alignment was revealed that eleven phylotype IV-biovar 2 strains (hereafter IV-2) were co-linear along the chromosome and the megaplasmid. For phylotype I strains, the phylotype I-biovar 3 (I-3) and phylotype I-biovar 4 (I-4) strains were generally consistent each other, except for SL3300 and T117, which had an inversion in the middle of the chromosome, and T25 and SL3822, which had a large inversion in the

megaplasmid, like as the results of the pair-wise alignment. Between the phylotype I and IV strains, there were many gene rearrangements in genome organization, particularly in the megaplasmid.

Table 3. Pairwise OrthoANI values among the twenty-five Korean and reference strains.

Phylotype	Strain	SL2312	T12	T82	T101	SL3022	SL3175	T98	SL2064	T51	T11	T95	R229	PSI07	R24	CMR15	CFBP2957	IPO1609	Po82	SL3103	SL2330	SL3755	T25	T110	GM1000	SL2729	SL3300	SL3730	SL3822	SL3882	T42	T60	T78	T117	FQY_4
IV	SL2312	100.0	99.9	100.0	100.0	99.3	98.5	98.5	99.3	99.3	99.3	99.3	98.5	98.5	97.9	92.2	92.5	92.1	91.9	92.6	92.6	92.5	92.5	92.5	92.5	92.5	92.5	92.5	92.5	92.5	92.4	92.5	92.4	92.6
	T12	99.9	100.0	99.9	99.9	99.3	98.5	98.5	99.2	99.3	99.2	99.2	98.5	98.5	97.9	92.3	92.5	92.0	91.9	92.6	92.5	92.4	92.5	92.5	92.4	92.5	92.5	92.3	92.6	92.5	92.4	92.6	92.4	92.5	92.5
	T82	100.0	99.9	100.0	100.0	99.3	98.5	98.5	99.3	99.3	99.3	99.3	98.5	98.5	97.9	92.2	92.5	92.0	91.9	92.7	92.6	92.5	92.5	92.5	92.6	92.5	92.6	92.5	92.6	92.5	92.5	92.4	92.5	92.4	92.6
	T101	100.0	99.9	100.0	100.0	99.3	98.5	98.5	99.3	99.3	99.3	99.3	98.5	98.5	97.9	92.2	92.5	91.9	91.8	92.6	92.6	92.5	92.5	92.5	92.6	92.4	92.6	92.4	92.5	92.5	92.4	92.5	92.4	92.5	92.6
	SL3022	99.3	99.3	99.3	99.3	100.0	98.5	98.5	99.3	99.3	99.3	99.3	98.5	98.5	97.9	92.1	92.6	91.9	91.8	92.7	92.5	92.4	92.4	92.6	92.6	92.5	92.5	92.5	92.7	92.5	92.5	92.5	92.5	92.6	92.6
	SL3175	98.5	98.5	98.5	98.5	98.5	100.0	100.0	98.5	98.5	98.5	98.5	99.3	99.4	98.0	92.0	92.2	91.9	91.7	92.4	92.3	92.3	92.3	92.3	92.2	92.3	92.3	92.3	92.3	92.2	92.3	92.3	92.4	92.3	92.2
	T98	98.5	98.5	98.5	98.5	98.5	100.0	100.0	98.4	98.5	98.5	98.4	99.3	99.5	98.0	92.0	92.2	91.9	91.8	92.4	92.3	92.3	92.3	92.3	92.3	92.3	92.2	92.3	92.3	92.2	92.3	92.3	92.4	92.4	92.3
	SL2064	99.3	99.2	99.3	99.3	99.3	98.5	98.4	100.0	99.9	100.0	100.0	98.5	98.4	97.9	92.2	92.4	91.8	91.8	92.6	92.6	92.5	92.5	92.6	92.5	92.5	92.6	92.5	92.6	92.6	92.5	92.6	92.4	92.5	92.5
	T51	99.3	99.3	99.3	99.3	99.3	98.5	98.5	99.9	100.0	99.9	99.9	98.5	98.4	97.9	92.1	92.3	91.9	91.9	92.5	92.5	92.4	92.3	92.5	92.4	92.4	92.5	92.5	92.5	92.4	92.5	92.4	92.4	92.4	92.5
	T11	99.3	99.2	99.3	99.3	99.3	98.5	98.5	100.0	99.9	100.0	100.0	98.5	98.5	97.9	92.2	92.4	91.9	91.8	92.7	92.5	92.5	92.5	92.5	92.5	92.6	92.4	92.6	92.4	92.4	92.5	92.5	92.4	92.4	92.4
	T95	99.3	99.2	99.3	99.3	99.3	98.5	98.4	100.0	99.9	100.0	100.0	98.5	98.4	97.9	92.1	92.4	91.9	91.9	92.6	92.6	92.6	92.5	92.6	92.5	92.5	92.6	92.5	92.5	92.5	92.5	92.7	92.5	92.5	92.5
	R229	98.5	98.5	98.5	98.5	98.5	99.3	99.3	98.5	98.5	98.5	98.5	100.0	99.3	97.9	92.2	92.3	91.8	91.9	92.5	92.6	92.4	92.4	92.6	92.6	92.5	92.5	92.6	92.5	92.5	92.5	92.6	92.5	92.6	92.6
	PSI07	98.5	98.5	98.5	98.5	98.5	99.4	99.5	98.4	98.4	98.5	98.4	99.3	100.0	97.9	92.0	92.3	91.8	91.7	92.4	92.4	92.4	92.3	92.5	92.3	92.4	92.4	92.4	92.4	92.4	92.4	92.4	92.4	92.3	92.3
	R24	97.9	97.9	97.9	97.9	97.9	98.0	98.0	97.9	97.9	97.9	97.9	97.9	97.9	100.0	92.1	92.4	91.9	91.6	92.5	92.4	92.4	92.3	92.4	92.4	92.3	92.5	92.4	92.4	92.3	92.4	92.3	92.4	92.2	92.4
III	CMR15	92.2	92.3	92.2	92.2	92.1	92.0	92.0	92.2	92.1	92.2	92.1	92.2	92.0	92.1	100.0	91.6	90.9	90.9	96.2	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.1	96.0	96.1	96.1	96.0	96.0	96.1	96.2
IIA	CFBP2957	92.5	92.5	92.5	92.5	92.6	92.2	92.2	92.4	92.3	92.4	92.4	92.3	92.3	92.4	91.6	100.0	96.5	96.4	91.7	91.8	91.7	91.5	91.8	91.5	91.7	91.7	91.7	91.8	91.6	91.6	91.7	91.7	91.6	91.7
IIB	IPO1609	92.1	92.0	92.0	91.9	91.9	91.9	91.9	91.8	91.9	91.9	91.9	91.8	91.8	91.9	90.9	96.5	100.0	97.4	91.1	91.0	91.1	91.0	91.1	91.0	91.0	91.1	91.0	91.1	91.1	91.1	91.1	91.0	91.0	91.0
	Po82	91.9	91.9	91.9	91.8	91.8	91.7	91.8	91.8	91.9	91.8	91.9	91.9	91.7	91.6	90.9	96.4	97.4	100.0	91.1	90.9	90.9	90.9	90.9	91.0	90.9	90.9	90.9	90.9	90.9	91.0	90.9	90.9	90.9	90.8
I	SL3103	92.6	92.6	92.7	92.6	92.7	92.4	92.4	92.6	92.5	92.7	92.6	92.5	92.4	92.5	96.2	91.7	91.1	91.1	100.0	99.1	99.2	99.2	99.1	99.1	99.7	99.6	99.6	99.6	99.6	99.7	99.7	99.6	99.6	99.4
	SL2330	92.6	92.5	92.6	92.6	92.5	92.3	92.3	92.6	92.5	92.5	92.6	92.6	92.4	92.4	96.1	91.8	91.0	90.9	99.1	100.0	99.8	99.8	99.9	99.0	99.1	99.1	99.1	99.2	99.2	99.2	99.2	99.1	99.2	99.2
	T110	92.5	92.5	92.5	92.5	92.6	92.3	92.3	92.6	92.5	92.5	92.6	92.6	92.5	92.4	96.1	91.8	91.1	90.9	99.1	99.9	99.8	99.7	100.0	99.0	99.1	99.1	99.1	99.1	99.1	99.1	99.2	99.2	99.1	99.2
	SL3755	92.5	92.4	92.5	92.5	92.4	92.3	92.3	92.5	92.4	92.5	92.6	92.4	92.4	92.4	96.1	91.7	91.1	90.9	99.2	99.8	100.0	99.8	99.8	98.9	99.1	99.2	99.2	99.2	99.2	99.1	99.2	99.2	99.2	99.2
	T25	92.5	92.5	92.5	92.5	92.4	92.3	92.3	92.5	92.3	92.5	92.5	92.4	92.3	92.3	96.1	91.5	91.0	90.9	99.2	99.8	99.8	100.0	99.7	98.9	99.1	99.1	99.2	99.1	99.2	99.2	99.2	99.2	99.2	99.2
	GM1000	92.5	92.4	92.6	92.6	92.6	92.2	92.3	92.5	92.4	92.5	92.5	92.6	92.3	92.4	96.1	91.5	91.0	91.0	99.1	99.0	98.9	98.9	99.0	100.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.1	98.9
	SL2729	92.5	92.5	92.5	92.4	92.5	92.3	92.3	92.5	92.4	92.6	92.5	92.5	92.4	92.3	96.1	91.7	91.0	90.9	99.7	99.1	99.1	99.1	99.1	99.0	100.0	99.9	99.9	99.8	99.9	99.9	99.8	99.8	99.9	99.3
	SL3300	92.5	92.5	92.6	92.6	92.5	92.3	92.2	92.6	92.5	92.4	92.6	92.5	92.4	92.5	96.1	91.7	91.1	90.9	99.6	99.1	99.2	99.1	99.1	99.0	99.9	100.0	99.9	99.8	99.8	99.9	99.8	99.8	99.9	99.3
	SL3730	92.5	92.3	92.5	92.4	92.5	92.3	92.3	92.5	92.5	92.6	92.5	92.6	92.4	92.4	96.1	91.7	91.0	90.9	99.6	99.1	99.2	99.2	99.1	99.0	99.9	99.9	100.0	99.8	99.8	99.9	99.8	99.8	99.9	99.3
	SL3882	92.5	92.5	92.5	92.5	92.5	92.2	92.2	92.6	92.4	92.4	92.5	92.5	92.4	92.3	96.1	91.6	91.1	90.9	99.6	99.2	99.2	99.2	99.1	99.0	99.9	99.8	99.8	99.9	100.0	99.9	99.9	99.9	99.9	99.3
	SL3822	92.5	92.6	92.6	92.5	92.7	92.3	92.3	92.6	92.5	92.4	92.5	92.5	92.4	92.4	96.0	91.8	91.1	90.9	99.6	99.2	99.2	99.1	99.1	99.0	99.8	99.8	99.8	100.0	99.9	99.8	99.9	99.9	99.9	99.3
	T42	92.5	92.4	92.5	92.4	92.5	92.3	92.3	92.5	92.5	92.5	92.5	92.5	92.4	92.4	96.1	91.6	91.1	91.0	99.7	99.2	99.1	99.2	99.1	99.0	99.9	99.9	99.9	99.8	99.9	100.0	99.8	99.8	99.9	99.3
	T60	92.4	92.6	92.4	92.5	92.5	92.3	92.3	92.6	92.4	92.5	92.7	92.6	92.4	92.3	96.0	91.7	91.1	90.9	99.7	99.2	99.2	99.2	99.2	99.0	99.8	99.8	99.8	99.9	99.9	99.8	100.0	99.9	99.9	99.3
	T78	92.5	92.4	92.5	92.4	92.5	92.4	92.4	92.4	92.4	92.4	92.5	92.5	92.4	92.4	96.0	91.7	91.0	90.9	99.6	99.1	99.2	99.2	99.2	99.0	99.8	99.8	99.8	99.9	99.9	99.8	99.9	100.0	99.9	99.3
	T117	92.4	92.5	92.4	92.5	92.6	92.3	92.4	92.5	92.4	92.4	92.5	92.6	92.3	92.2	96.1	91.6	91.0	90.9	99.6	99.2	99.2	99.2	99.1	99.1	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100.0	99.4
	FQY_4	92.6	92.5	92.6	92.6	92.6	92.2	92.3	92.5	92.5	92.4	92.5	92.6	92.3	92.4	96.2	91.7	91.0	90.8	99.4	99.2	99.2	99.2	99.2	98.9	99.3	99.3	99.3	99.3	99.3	99.3	99.3	99.3	99.4	100.0

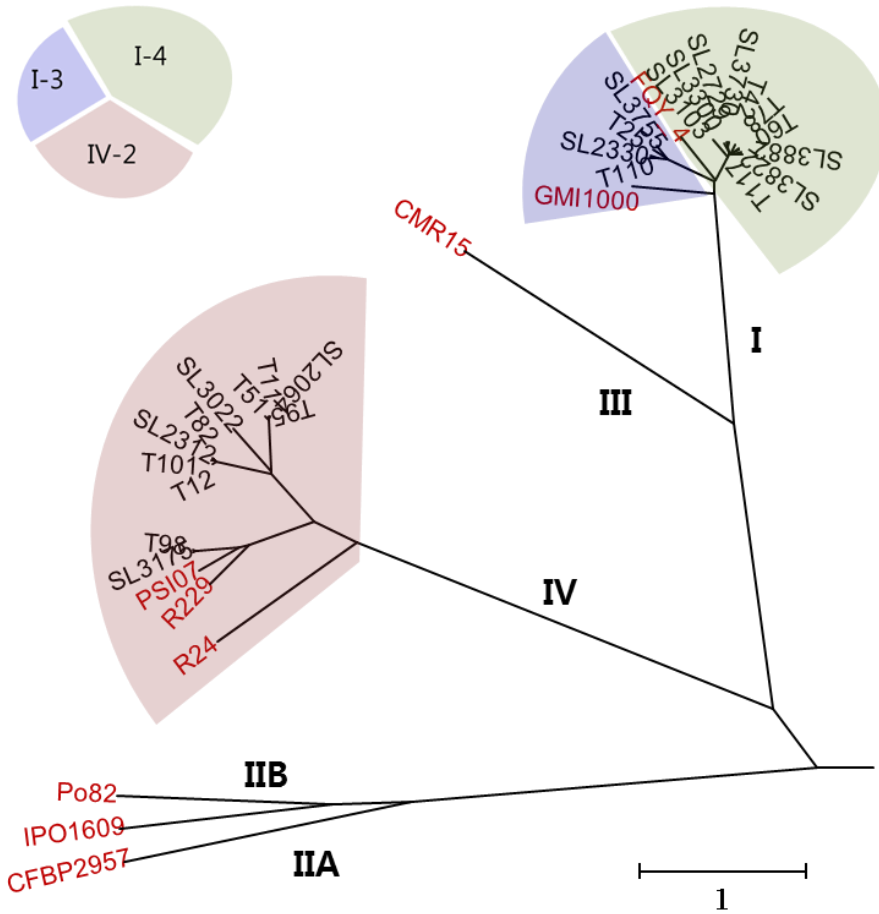


Figure 1. Phylogenetic tree using genomes of 25 Korean and 9 reference *R. solanacearum* strains based on OrthoANI value.

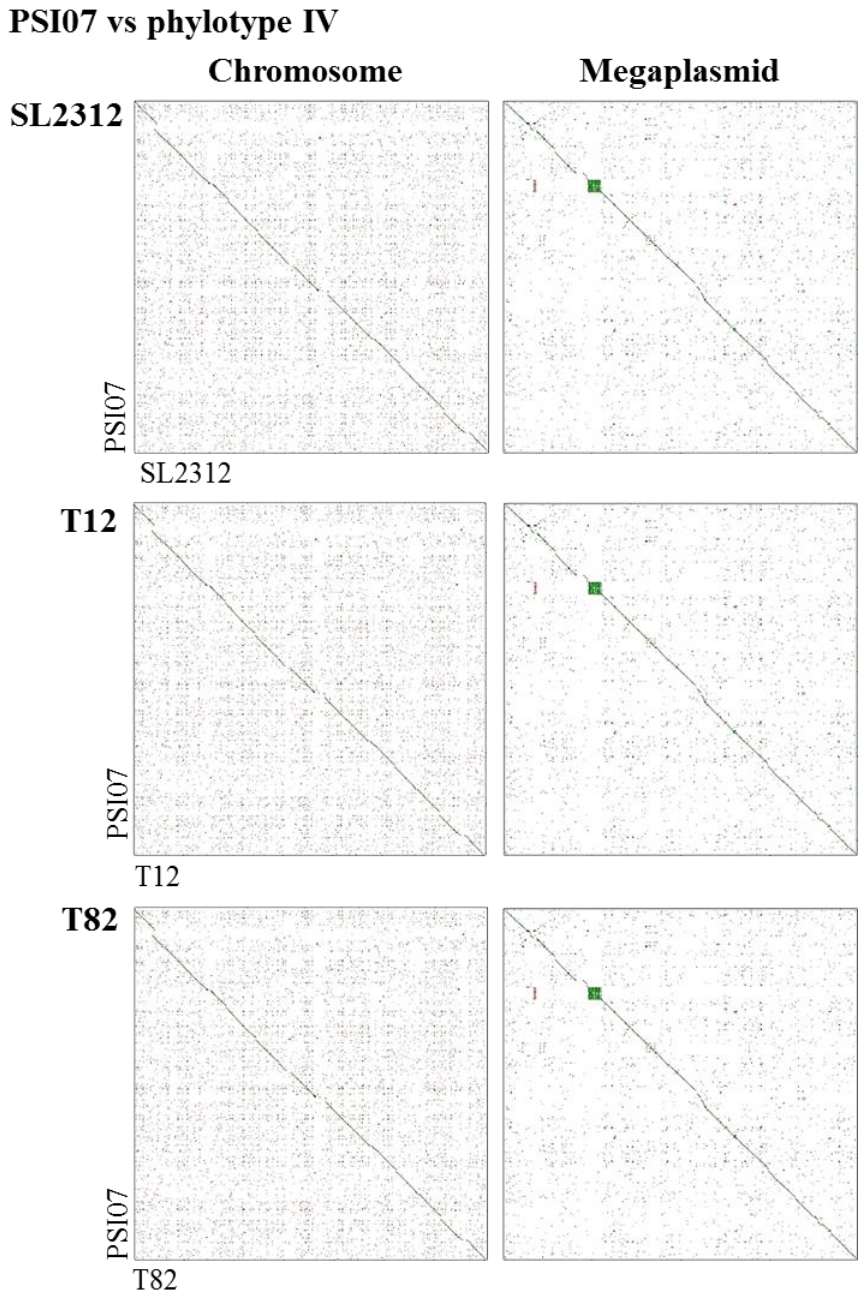


Figure 2. Pairwise genome alignments of Korean *R. solanacearum* strains to reference strain GMI1000 for phylotype I or PSI07 for phylotype IV. Descending slope indicates the positive direction of the sequences and ascending slope indicates the inversion.

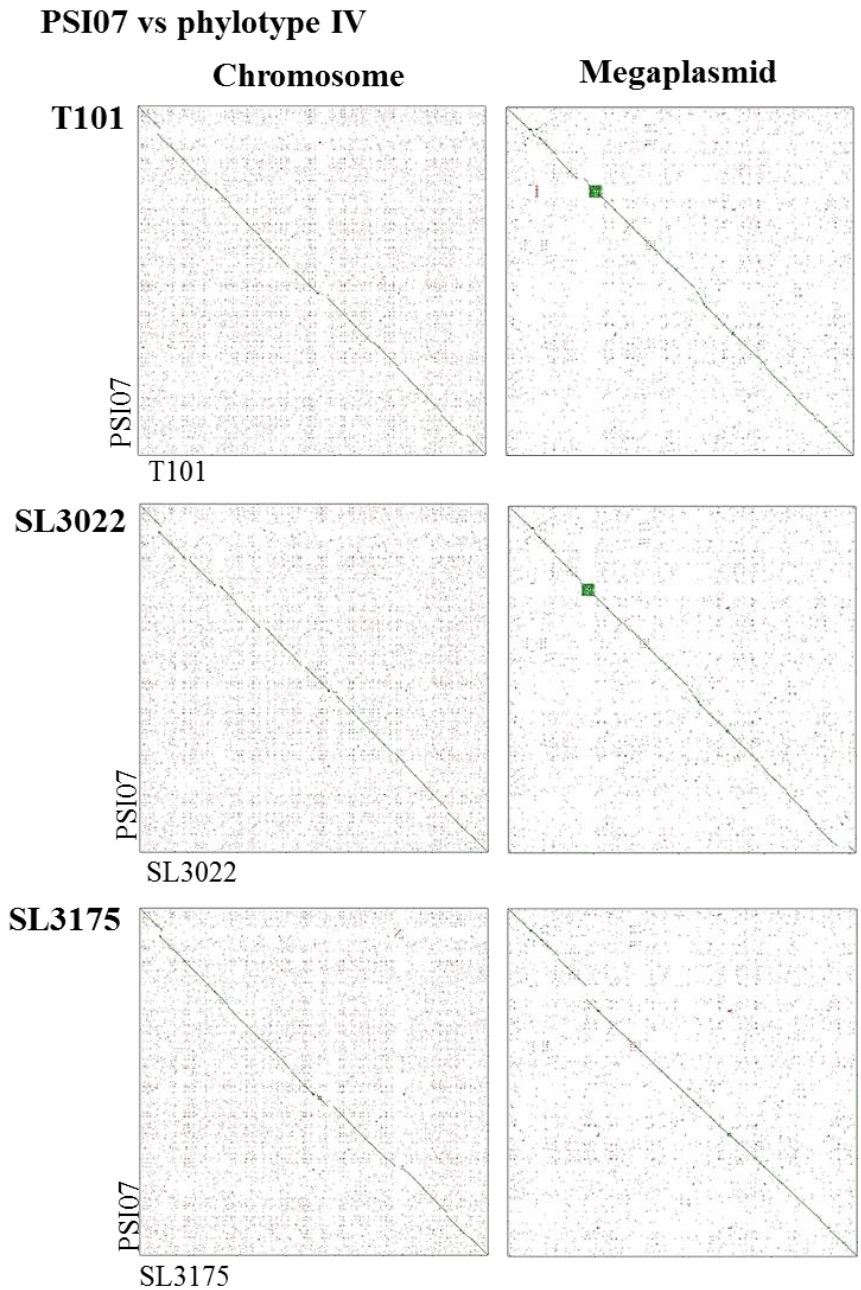


Figure 2. (continued)

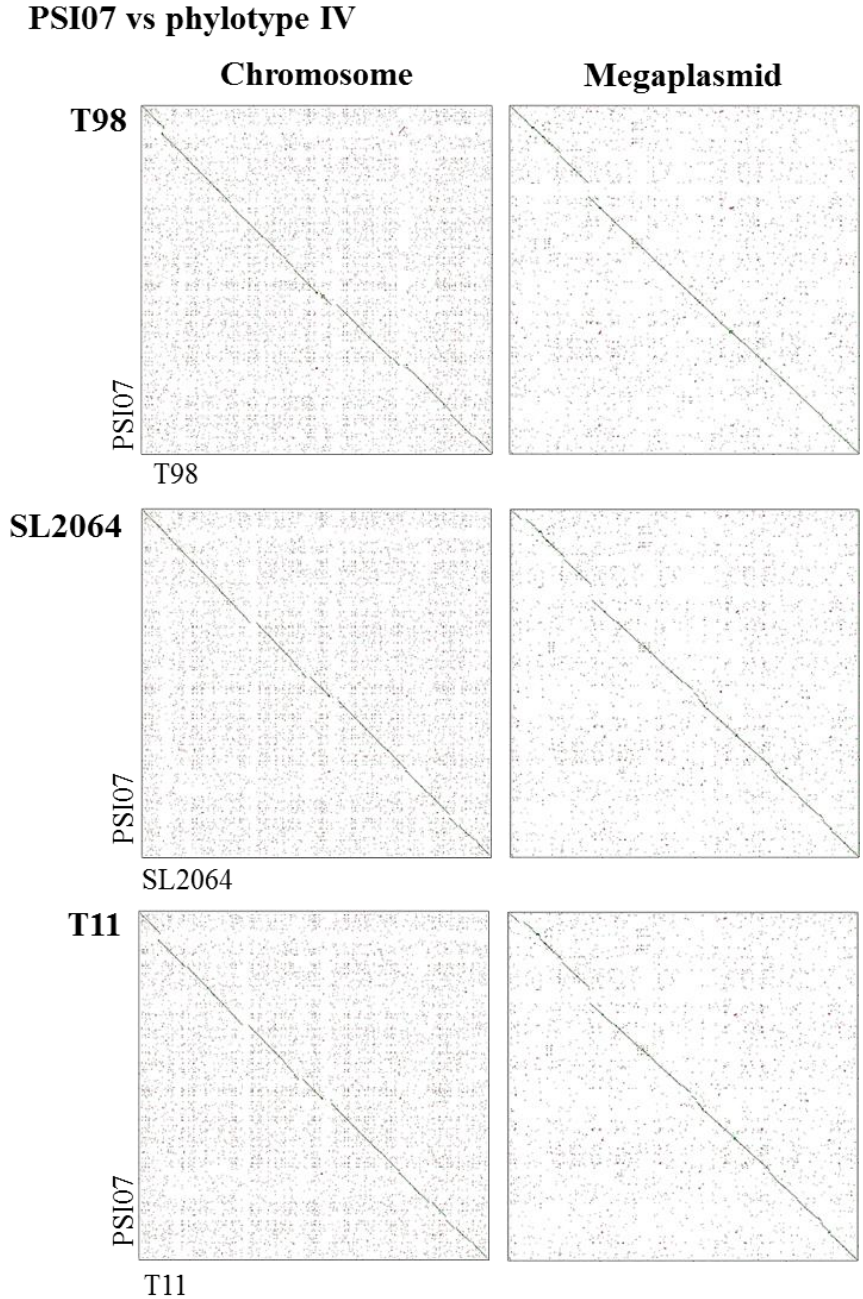


Figure 2. (continued)

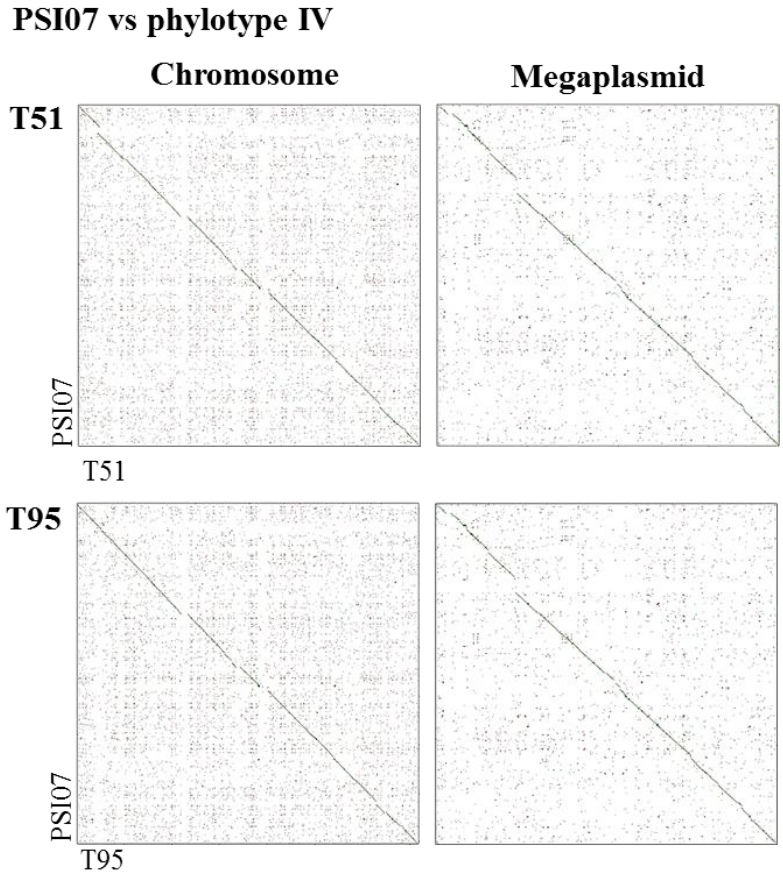


Figure 2. (continued)

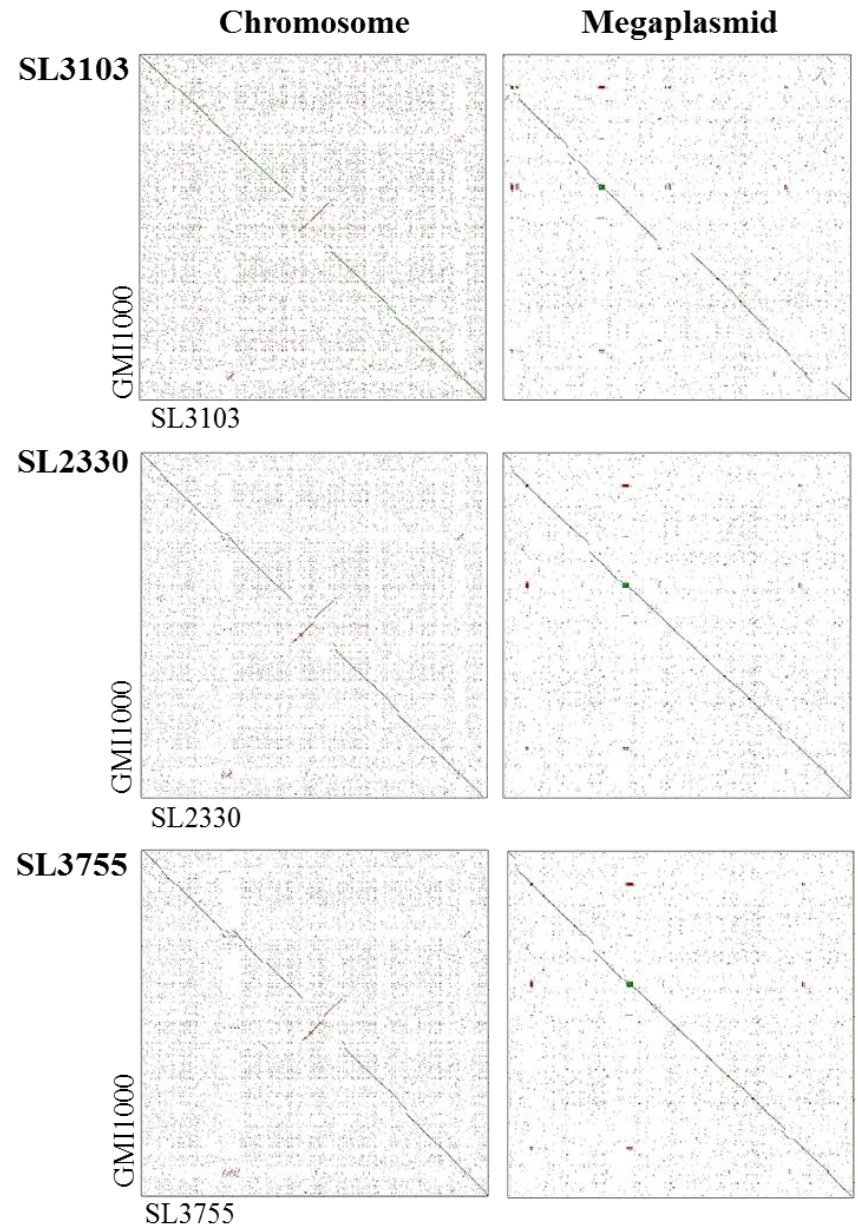


Figure 2. (continued)

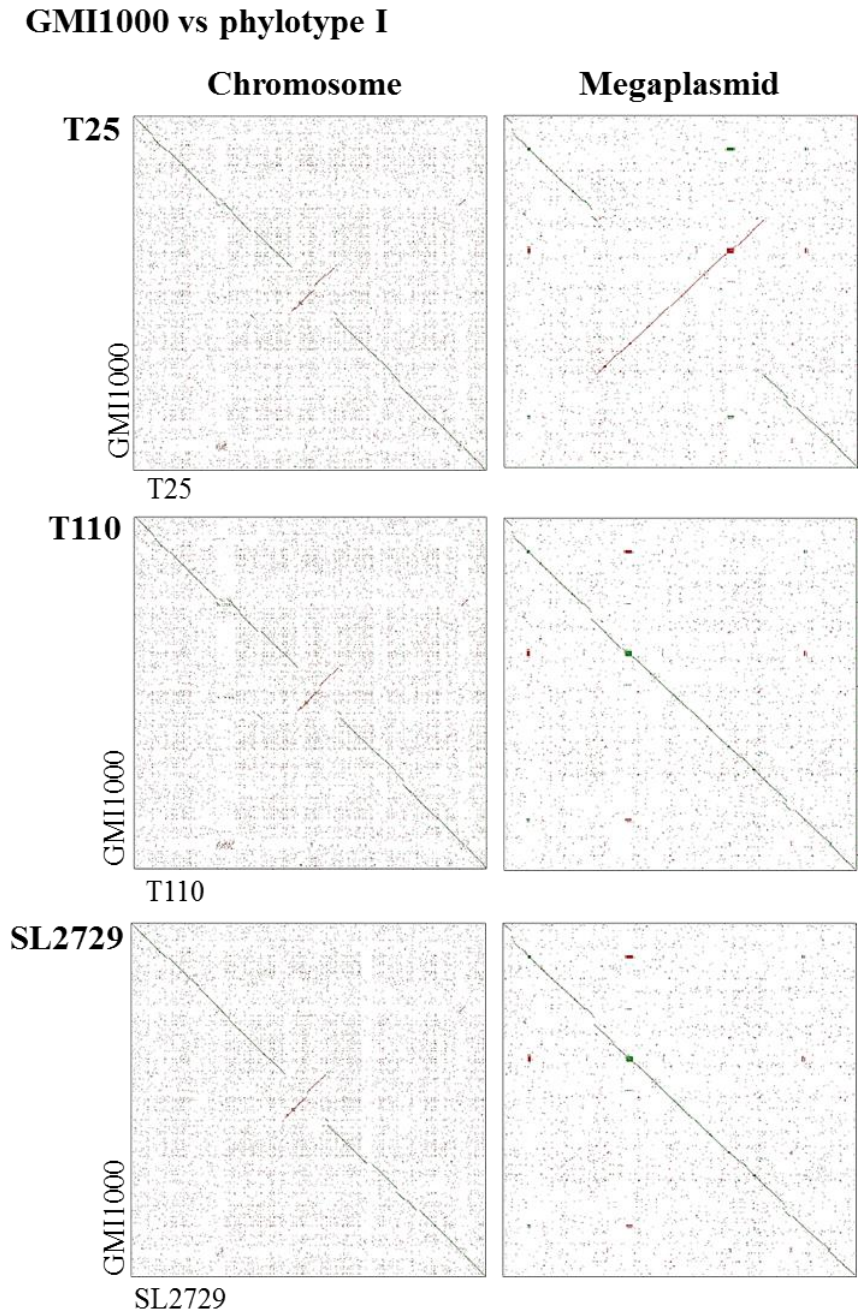


Figure 2. (continued)

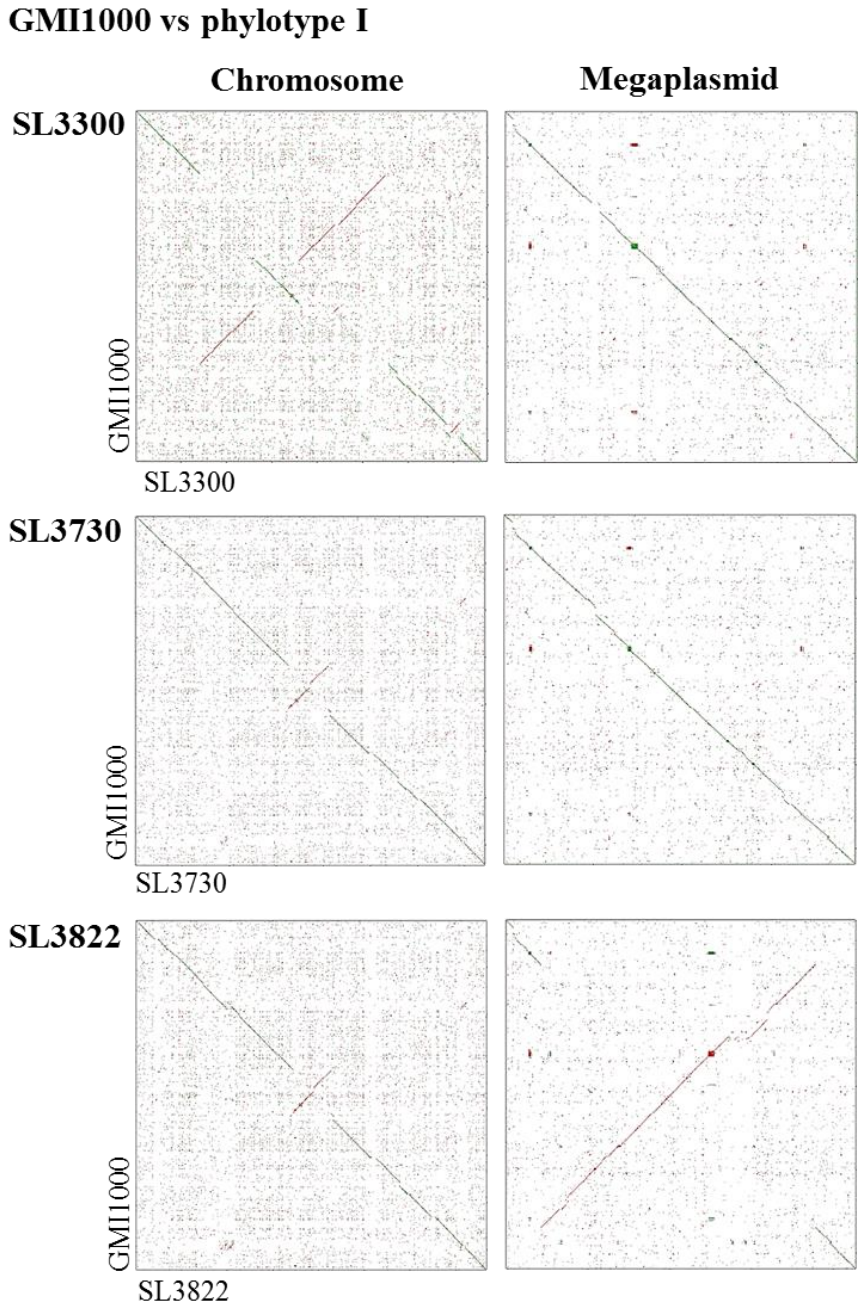


Figure 2. (continued)

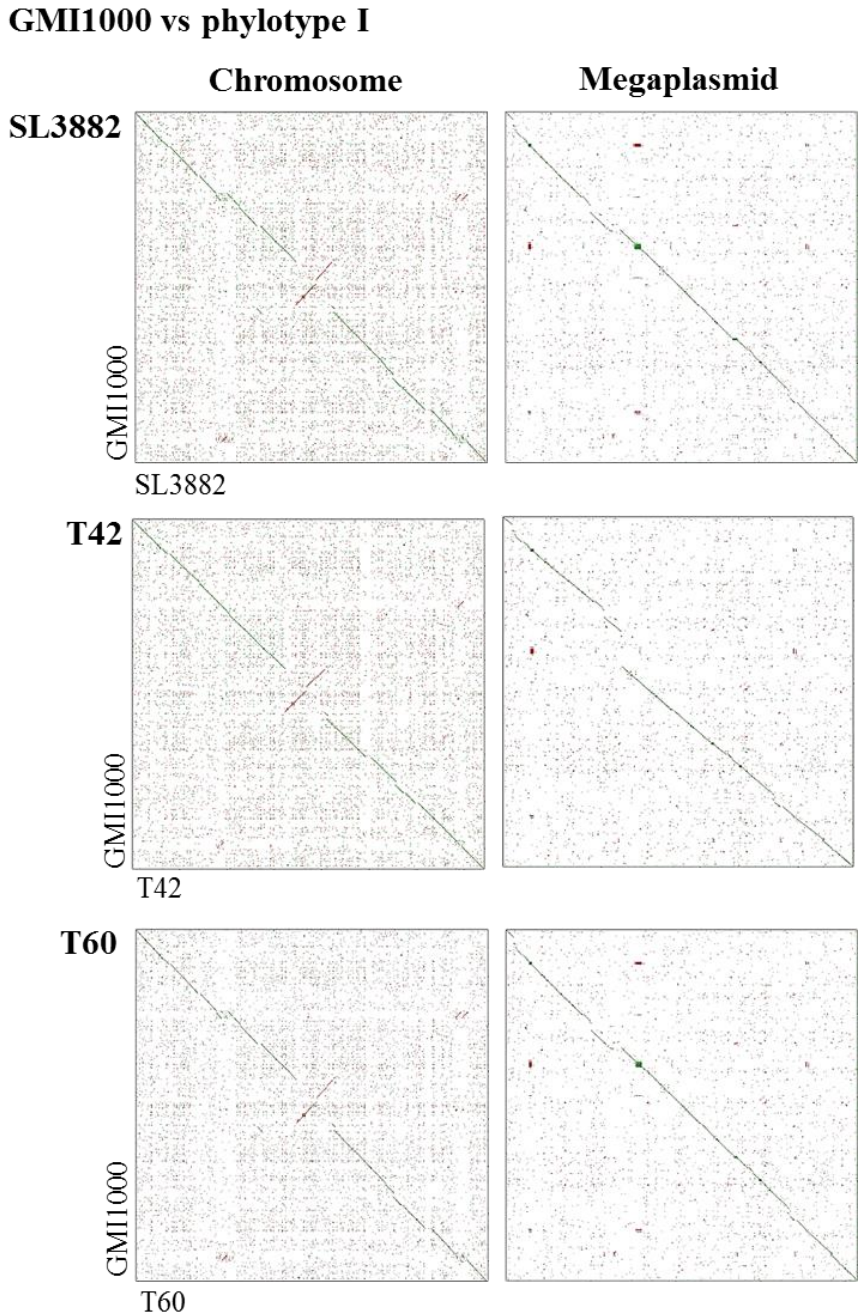


Figure 2. (continued)

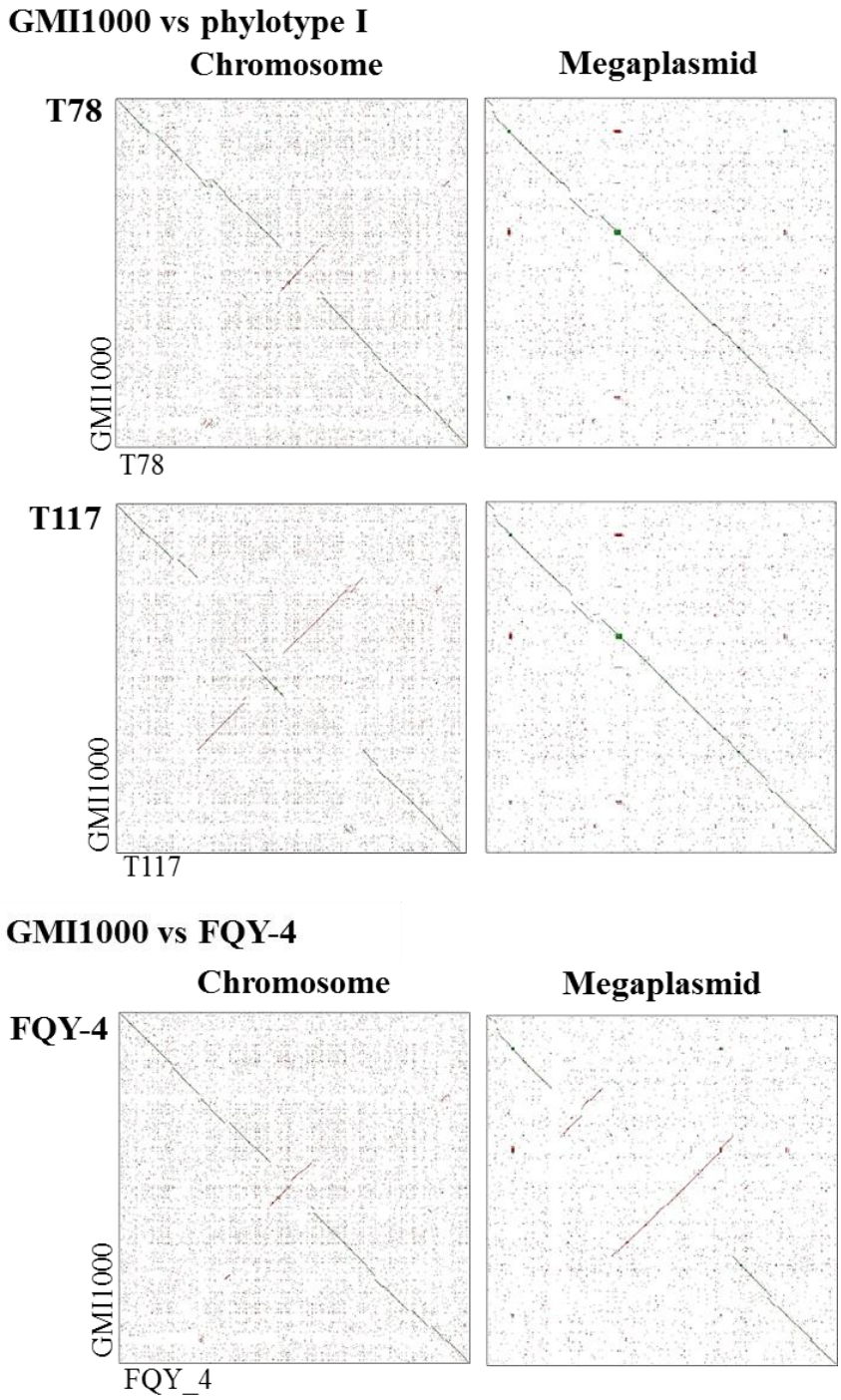


Figure 2. (continued)

A. Chromosme

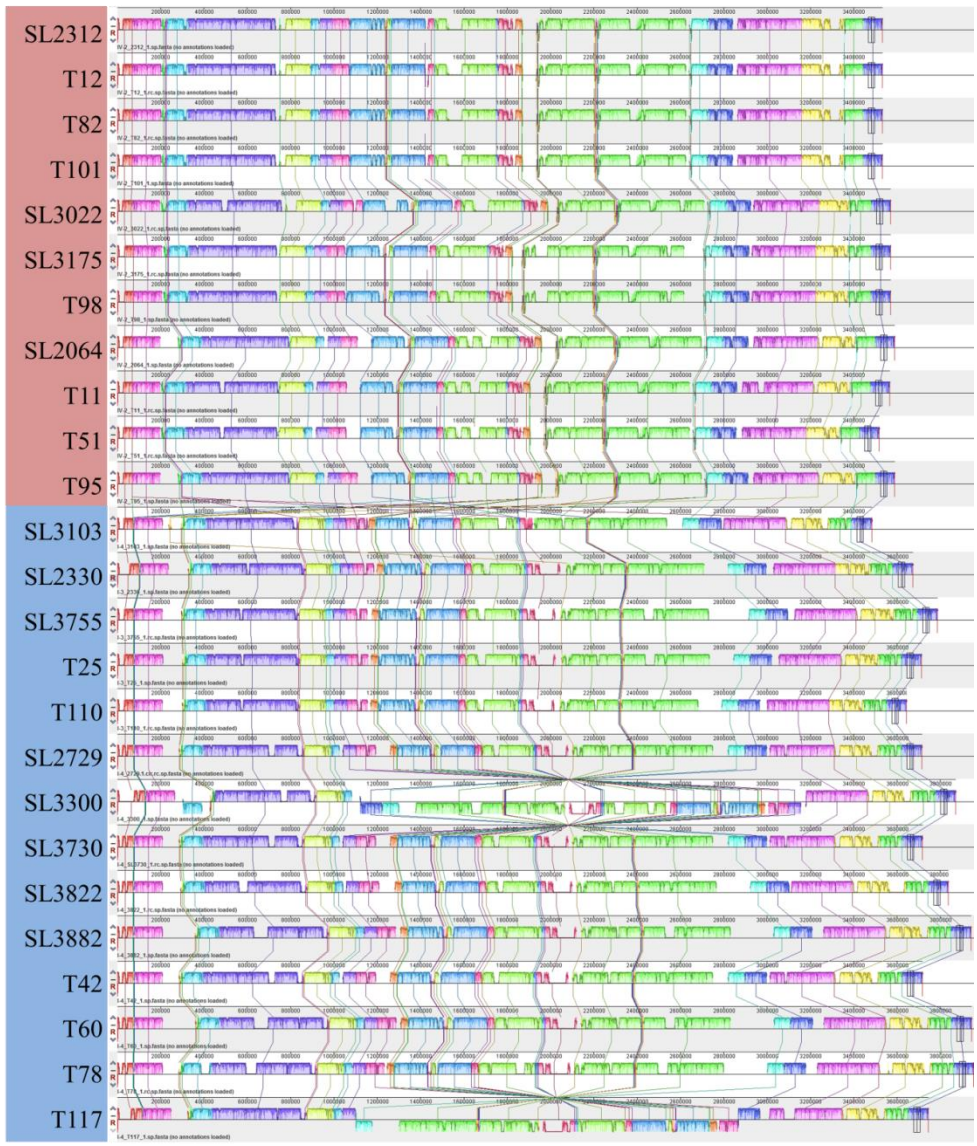


Figure 3. Multiple genome alignment for 25 Korean *R. solanacearum* strains produced using Mauve software. The sequences of chromosome (A) and megaplasmid (B) were aligned. The red box and blue box represent the phylotype IV and I, repectively. Colored lines between genomes represent rearrangements or inversions.

B. Megaplasmid

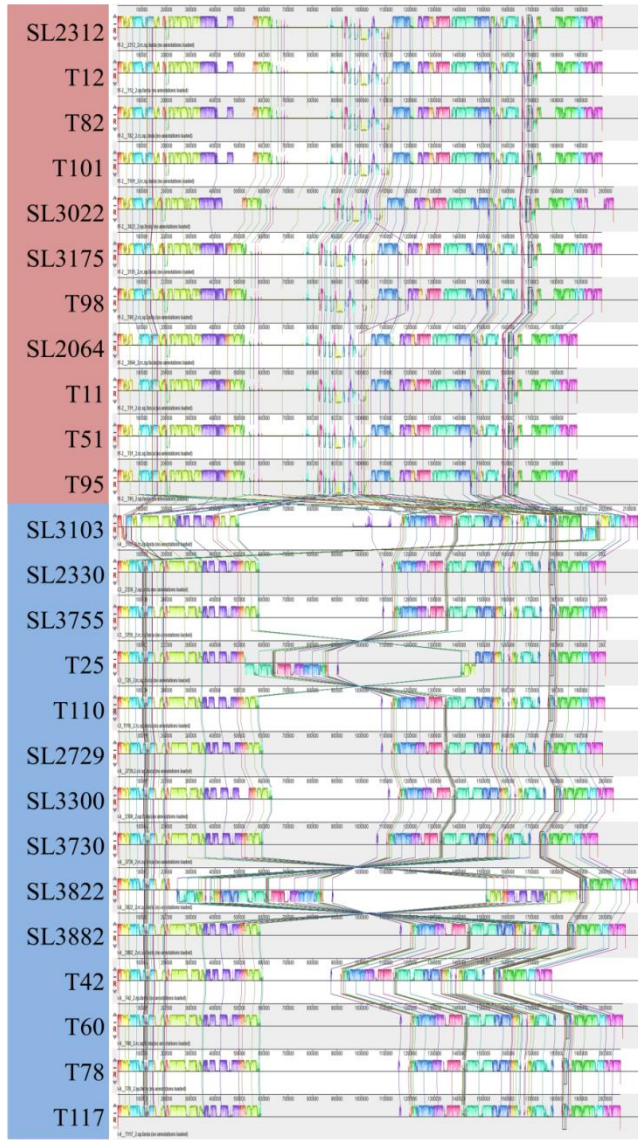


Figure 3. (continued)

3. Clusters of Orthologous Group (COG) distribution

In order to investigate the gene functions related with the host range, the functional categories of Clusters of Orthologous Group (COG) using $CL_{GENOMICS}$ (http://www.chunlab.com) had been analyzed. Among predicted CDSs, about 70 % of genes were classified into one of the 22 COG categories and about 30 % remained unknown function.

Table 4 and Figure 4 displayed the COG functional categories of the twenty-five Korean *R. solanacearum* strains. Except for function unknown genes (S), the largest functional group was the group that carried average 367 genes involved in amino acid transport and metabolism (E), followed by transcription group (K) carried average 330 genes, energy production and conversion group (C) carried average 286 genes.

There were differences of the COG distribution between phylotype I and IV strains. The bacterial strains in phylotype I had more genes than bacterial strains in phylotype IV in the category of (K) transcription, (L) replication, recombination and repair, (U) intracellular trafficking, secretion, and vesicular transport, (G) carbohydrate transport and metabolism, and (Q) secondary metabolites biosynthesis, transport and catabolism. In the categories (T) signal transduction mechanisms and (P) inorganic ion transport and metabolism, phylotype IV strains had more genes than phylotype I.

Table 4. The clusters of orthologous groups (COG) functional categories in twenty-five Korean *R. solanacearum* strains.

Isolate	J[a]	A	K	L	B	D	V	T	M	N	Z	W	U	O	C	G	E	F	H	I	P	Q	S	Total
SL2312	168	2	308	167	2	31	49	234	233	85	1	1	137	160	283	194	355	82	140	157	242	112	1,375	4,518
T12	167	2	309	171	2	31	51	239	243	88	0	1	143	163	286	205	369	88	141	162	248	124	1,405	4,638
T82	168	2	307	167	2	31	50	232	233	85	1	1	137	160	283	194	355	82	140	157	242	112	1,376	4,517
T101	168	2	306	169	2	31	50	233	235	87	1	1	139	161	285	199	359	83	141	159	244	115	1,378	4,548
SL3022	169	2	308	184	2	35	46	235	258	87	5	2	149	161	286	194	365	81	140	155	245	115	1,389	4,613
SL3175	166	2	334	177	2	33	50	228	251	70	2	1	155	161	287	197	377	82	144	153	246	111	1,438	4,667
T98	166	2	332	176	2	33	51	230	252	70	2	1	154	161	286	195	376	82	142	153	246	111	1,434	4,657
SL2064	167	2	314	163	2	32	49	232	234	66	2	0	137	166	284	194	362	81	140	152	243	99	1,443	4,564
T11	167	2	313	160	2	33	49	228	235	66	2	1	135	163	285	196	362	81	140	152	243	99	1,407	4,521
T51	167	2	312	157	2	32	49	231	235	66	2	1	135	161	283	194	361	81	140	152	242	99	1,390	4,494
T95	167	2	314	165	2	32	49	232	234	66	2	1	138	166	284	194	362	81	140	152	242	100	1,442	4,567
SL2330	166	2	343	228	2	32	55	207	242	67	1	1	154	171	288	211	370	80	143	158	232	115	1403	4,671
SL3755	166	2	346	230	2	32	57	208	244	67	1	1	164	172	288	212	373	80	143	159	231	115	1,489	4,782
T25	169	2	352	230	2	33	55	210	249	67	1	1	169	173	295	222	382	82	145	171	238	131	1,473	4,852
T110	172	2	354	244	2	32	61	222	257	77	1	1	178	185	305	230	386	88	156	172	248	166	1,466	5,005
SL3103	164	2	323	359	2	32	49	215	238	66	2	1	149	163	281	204	361	83	141	160	228	114	1,382	4,719
SL2729	165	2	338	254	2	32	51	216	239	67	1	1	152	165	288	211	366	83	141	160	226	116	1,447	4,723
SL3300	165	2	346	295	2	32	55	216	241	67	2	1	152	169	287	213	370	83	142	160	226	116	1,532	4,874
SL3730	165	2	340	259	2	32	53	219	241	67	1	1	156	167	287	214	370	83	145	158	231	115	1,467	4,775
SL3822	165	2	346	271	2	32	54	214	243	67	1	1	165	172	285	211	369	84	144	160	228	120	1,556	4,892
SL3882	166	2	349	265	2	32	56	215	243	68	1	1	165	174	288	213	369	83	142	161	229	118	1,644	4,986
T42	164	2	323	250	2	32	48	212	235	67	1	1	151	164	281	206	355	82	127	149	216	104	1,413	4,585
T60	166	2	352	268	2	32	56	215	243	68	1	1	163	174	291	213	370	83	142	161	230	117	1,638	4,988
T78	166	2	355	273	2	33	56	219	247	67	1	1	179	172	288	214	371	83	142	161	231	115	1,696	5,074
T117	165	2	338	240	2	32	53	216	244	67	1	1	155	170	287	212	370	83	141	163	229	115	1,513	4,799

[a]J; Translation, ribosomal structure and biogenesis, A; RNA processing and modification, K; Transcription, L; Replication, recombination and repair, B; Chromatin structure and dynamics, D; Cell cycle control, cell division, chromosome partitioning, V; Defense mechanisms, T; Signal transduction mechanisms, M; Cell wall/membrane/envelope biogenesis, N; Cell motility, Z; Cytoskeleton, W; Extracellular structures, U; Intracellular trafficking, secretion, and vesicular transport, O; Posttranslational modification, protein turnover, chaperones, C, Energy production and conversion, G; Carbohydrate transport and metabolism, E, Amino acid transport and metabolism, F; Nucleotide transport and metabolism, H; Coenzyme transport and metabolism, I; Lipid transport and metabolism, P; Inorganic ion transport and metabolism, Q; Secondary metabolites biosynthesis, transport and catabolism, S; Function unknown.

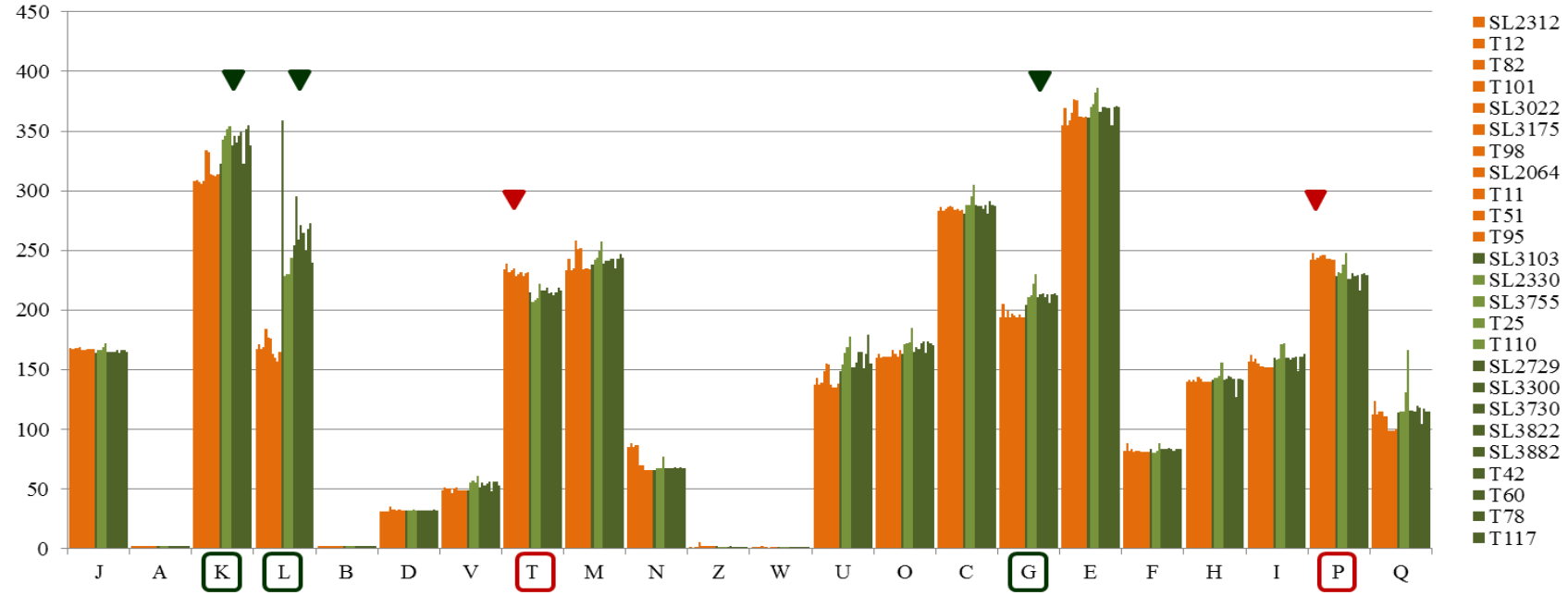


Figure 4. Graph of COG functional categories of twenty-five Korean strains. Orange color presented phylotype IV strains, and green and olive color presented the phylotype I strains. Red triangle and box indicate the category of more genes in phylotype IV than phyloytpe I, and green triangle and box indicate the category of more genes in phylotype I than phyloytpe IV. Category S of unknown function were excluded in the graph. (J; Translation, ribosomal structure and biogenesis, A; RNA processing and modification, K;

Transcription, L; Replication, recombination and repair, B; Chromatin structure and dynamics, D; Cell cycle control, cell division, chromosome partitioning, V; Defense mechanisms, T; Signal transduction mechanisms, M; Cell wall/membrane/envelope biogenesis, N; Cell motility, Z; Cytoskeleton, W; Extracellular structures, U; Intracellular trafficking, secretion, and vesicular transport, O; Posttranslational modification, protein turnover, chaperones, C, Energy production and conversion, G; Carbohydrate transport and metabolism, E, Amino acid transport and metabolism, F; Nucleotide transport and metabolism, H; Coenzyme transport and metabolism, I; Lipid transport and metabolism, P; Inorganic ion transport and metabolism, Q; Secondary metabolites biosynthesis, transport and catabolism, S; Function unknown.)

4. Functional genome comparison: phylotype-biovar

Twenty five Korean *R. solanacearum* isolates with full genome sequence were belonged to phylotype I and IV. Total 14 isolates were in phylotype I and it could be divided into biovar 3 and biovar 4 depends on the utilization of carbohydrates. All 11 phylotype IV isolates were belonged to biovar 2. Since 25 isolates could be divided into 3 different biovars, the pan- and core genomes within or between 3 different groups had been analyzed.

The pan-genome of twenty-five strains comprises 8,299 genes and core genome for essential for all *R. solanacerum* comprises 3,339 genes (Figure 5). Number of genes present only in the phylotype IV biovar 2 was 363. Five genes shared phylotype IV-biovar 2 and phylotype I-biovar 3 but none of genes shared phylotype IV-biovar 2 and phylotype I-biovar 4. Total 279 genes were common in phyotype I and 41 genes present only in biovar 3 and 33 genes present only in biovar 4.

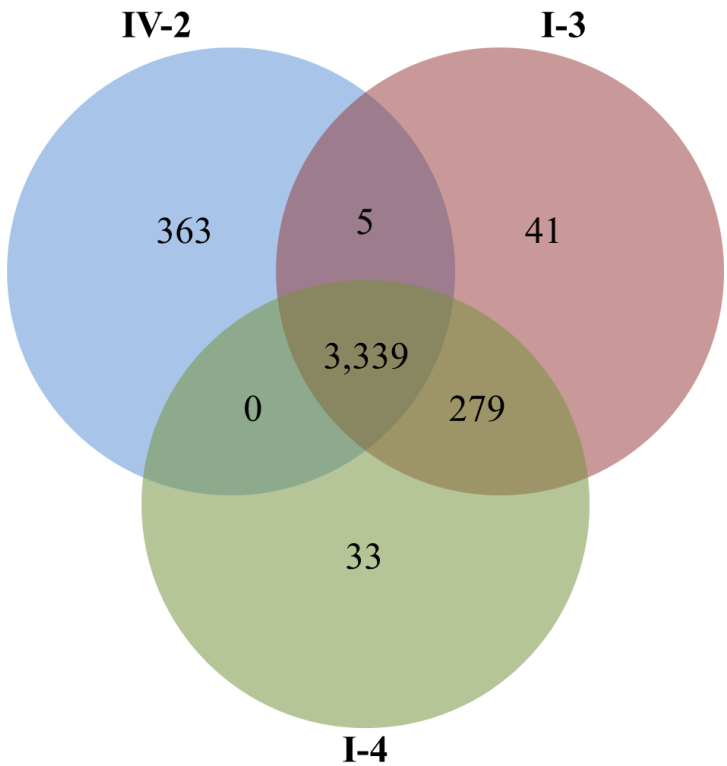


Figure 5. Venn diagrams of predicted genes for 25 Korean *R. solanacearum* according to the phylotype -biovar.

5. Functional genome comparison: host specificity

To find out responsible genes for host specificity, comparative genome analyses were performed based on the pathotypes. In the previous chapter, Korean *R. solanacearum* strains isolated from potato bacterial wilt were divided into 4 different pathotypes based on the pathogenicity on 4 different crops; potato, tomato, eggplant, and pepper. Though *R. solanacearum* isolates in phylotype I were belonged to pathotype PTEPe except isolate SL3103, *R. solanacearum* isolates in phylotype IV were divided into 3 different pathotypes; P, PT, and PTE. The number of analyzed genes for host specificity were shown in Table 5.

Table 5. Specific gene numbers of tomato, eggplant, and pepper-pathogenic and nonpathogenic *R. solanacearum* strains.

Strains	Phylotype -biovar	Original Host	Pathotype[a]	Specific genes		
				Tomato	Eggplant	Pepper
SL2312	IV-2	Potato	P	127	5	1
T12	IV-2	Potato	P			
T82	IV-2	Potato	P			
T101	IV-2	Potato	P			
SL3022	IV-2	Potato	PT	8	7	34
SL3175	IV-2	Potato	PT			
T98	IV-2	Potato	PT			
SL2064	IV-2	Potato	PTE			
T11	IV-2	Potato	PTE			
T51	IV-2	Potato	PTE			
T95	IV-2	Potato	PTE			
SL3103	I-4	Potato	PTE			
SL2330	I-3	Potato	PTEPe			
SL3755	I-3	Potato	PTEPe			
T25	I-3	Potato	PTEPe			
T110	I-3	Potato	PTEPe			
SL2729	I-4	Potato	PTEPe			
SL3300	I-4	Potato	PTEPe			
SL3730	I-4	Potato	PTEPe			
SL3822	I-4	Potato	PTEPe			
SL3882	I-4	Potato	PTEPe			
T42	I-4	Potato	PTEPe			
T60	I-4	Potato	PTEPe			
T78	I-4	Potato	PTEPe			
T117	I-4	Potato	PTEPe			

[a]Pathotype: (P) only pathogenic on potato, (PT) pathogenic on potato and tomato, (PTE) pathogenic on potato, tomato, and eggplant, and (PTEPe) pathogenic on all tested crops – potato, tomato, eggplant, and pepper.

Number of genes specific for each crop had been counted. Total 127 genes present only in four isolates that could not infect tomato (Table 6). Most of them were hypothetical and few functionally designated genes had relations with mobile elements such as bacteriophage infection or insertional elements. Three genes showed homology with CRISPR proteins; Cas9, Cas1, and Cas2 (Figure 6-A). CRISPR (clustered regularly interspaced short palindromic repeat) is an adaptive immune system that provides protection against mobile genetic elements (viruses, transposable elements, and conjugative plasmids). Four genes showed homology with Mu-like prophage proteins (Figure 6-B). Besides Mu-like prophage FluMu proteins, one capsid protein and another Mu-like virus tape measure protein had been listed in gene group specific for tomato non-pathogen. Two copies of insertional element IS476 were also shown in the list (Table 6 and Figure 6-C).

Table 6. The list of 127 genes present specifically only in 4 tomato nonpathogenic strains.

POG name	Other name	EggNog ID	Kegg ID	Product
POG_2312_00006		S:ENOG410Y9EE		hypothetical protein
POG_2312_00007				hypothetical protein
POG_2312_00008				hypothetical protein
POG_2312_00009		S:ENOG410ZA79		hypothetical protein
POG_2312_00010				hypothetical protein
POG_2312_00011		U:COG3505	K03205	hypothetical protein
POG_2312_00013	hsdS	V:COG0732	K01154	Type I site-specific deoxyribonuclease
POG_2312_00014		S:ENOG410Y2DX		Anticodon nuclease
POG_2312_00016		S:ENOG410YZZK		hypothetical protein
POG_2312_00018				hypothetical protein
POG_2312_00019		L:COG3293	K07492	hypothetical protein
POG_2312_00020		S:ENOG410YK2Z		hypothetical protein
POG_2312_00021		L:COG3293	K07492	hypothetical protein
POG_2312_00022		L:COG3293	K07492	hypothetical protein
POG_2312_00249		S:ENOG4111HFU		hypothetical protein
POG_2312_00555		S:ENOG41103VQ	K08732	Ral guanine nucleotide dissociation stimulator
POG_2312_00868		S:ENOG41128X2		hypothetical protein
POG_2312_00869		S:ENOG4112DDQ		hypothetical protein
POG_2312_00870				hypothetical protein
POG_2312_00871		L:ENOG4111N20	K04763	hypothetical protein
POG_2312_00872		S:ENOG4111G9I		hypothetical protein
POG_2312_00873		S:ENOG410Y7TT		hypothetical protein
POG_2312_00874		S:ENOG410YRYJ		hypothetical protein
POG_2312_00875				hypothetical protein
POG_2312_00879				hypothetical protein
POG_2312_00882		S:ENOG410YCI7		hypothetical protein
POG_2312_00883		O:COG2214	K05516	DnaJ like protein subfamily A member
POG_2312_00884		S:ENOG410ZTSE		hypothetical protein
POG_2312_00885		S:COG4248		uncharacterized protein
POG_2312_00886		S:ENOG4111YVJ		uncharacterized protein
POG_2312_00887		S:COG4245		uncharacterized protein
POG_2312_00888	DPO3E,dnaQ	S:ENOG4111RWV	K02342	DNA-directed DNA polymerase
POG_2312_00889				hypothetical protein
POG_2312_00894				hypothetical protein
POG_2312_00895				hypothetical protein
POG_2312_00896		K:COG1396		hypothetical protein
POG_2312_00897				hypothetical protein
POG_2312_00898	RTCB,rtcB	S:COG1690	K14415	RNA ligase (ATP)
POG_2312_01205		S:ENOG410Y502		hypothetical protein
POG_2312_01266		K:ENOG4112BE8		hypothetical protein
POG_2312_01267		S:ENOG4111UPI		hypothetical protein
POG_2312_01377				hypothetical protein
POG_2312_01378				hypothetical protein
POG_2312_01785				hypothetical protein
POG_2312_01786		S:COG2916	K03746	hypothetical protein

Table 6. (continued)

POG name	Other name	EggNog ID	Kegg ID	Product
POG_2312_01792				hypothetical protein
POG_2312_01793				hypothetical protein
POG_2312_01794				hypothetical protein
POG_2312_01797		S:ENOG410XYF1		hypothetical protein
POG_2312_01798				hypothetical protein
POG_2312_02168	csn1, cas9	L:COG3513	K09952	CRISPR-associated endonuclease Cas9
POG_2312_02169		L:COG1518	K15342	CRISPR-associated endonuclease Cas1
POG_2312_02170		L:COG3512	K09951	CRISPR-associated endoribonuclease Cas2
POG_2312_02171				hypothetical protein
POG_2312_02208				hypothetical protein
POG_2312_02209				hypothetical protein
POG_2312_02210				hypothetical protein
POG_2312_02211		S:ENOG410ZXI2		Glycine-rich cell wall structural protein
POG_2312_02416		S:ENOG4111MFE		hypothetical protein
POG_2312_02445				hypothetical protein
POG_2312_02447				hypothetical protein
POG_2312_02549		S:ENOG41121N0	K15125	Epstein-Barr nuclear antigen
POG_2312_02550				hypothetical protein
POG_2312_02754		S:ENOG410ZQPZ		hypothetical protein
POG_2312_02755		S:ENOG410XXXN		hypothetical protein
POG_2312_02758				hypothetical protein
POG_2312_03252				hypothetical protein
POG_2312_03449				hypothetical protein
POG_2312_03489		S:COG3501	K11904	hypothetical protein
POG_2312_03573				hypothetical protein
POG_2312_03649				hypothetical protein
POG_2312_03650				hypothetical protein
POG_2312_03732		K:COG1396		hypothetical protein
POG_2312_03733		K:COG2932		hypothetical protein
POG_2312_03734				hypothetical protein
POG_2312_03735		L:COG2801	K07497	hypothetical protein
POG_2312_03736		L:ENOG4111GUQ		hypothetical protein
POG_2312_03737				hypothetical protein
POG_2312_03738		S:ENOG41120IM		hypothetical protein
POG_2312_03739		S:ENOG410XUH0		hypothetical protein
POG_2312_03740				hypothetical protein
POG_2312_03741				hypothetical protein
POG_2312_03742				hypothetical protein
POG_2312_03743		S:ENOG4111UM7		hypothetical protein
POG_2312_03744				hypothetical protein
POG_2312_03745		S:ENOG4111NEI		hypothetical protein
POG_2312_03746				hypothetical protein
POG_2312_03747				hypothetical protein
POG_2312_03748		S:ENOG4111YCS		hypothetical protein
POG_2312_03750		S:ENOG410XWSJ	K07132	hypothetical protein

Table 6. (continued)

POG name	Other name	EggNog ID	Kegg ID	Product
POG_2312_03751		S:ENOG4111U4Z		hypothetical protein
POG_2312_03752		S:ENOG410Z6ZM		hypothetical protein
POG_2312_03753		S:ENOG410ZUH5		hypothetical protein
POG_2312_03754		T:COG1734		hypothetical protein
POG_2312_03755				hypothetical protein
POG_2312_03756		S:ENOG4111RKF		hypothetical protein
POG_2312_03757		S:ENOG410XNW6		hypothetical protein
POG_2312_03758		S:COG4383		Portal protein
POG_2312_03759		S:COG2369		Putative capsid assembly protein
POG_2312_03760		S:COG5005		hypothetical protein
POG_2312_03762		S:ENOG41126PV		hypothetical protein
POG_2312_03763		S:COG4397		Mu-like prophage FluMu major head subunit
POG_2312_03764				hypothetical protein
POG_2312_03765		S:COG4387		Mu-like prophage FluMu protein gp36
POG_2312_03766				hypothetical protein
POG_2312_03767				hypothetical protein
POG_2312_03769		S:ENOG410ZDIZ		hypothetical protein
POG_2312_03770		S:ENOG410XYVK	K03646	Probable tape measure protein
POG_2312_03775		S:ENOG410XXU2		hypothetical protein
POG_2312_03877				hypothetical protein
POG_2312_04004		T:COG0639	K01090	Protein-serine/threonine phosphatase
POG_2312_04005				hypothetical protein
POG_2312_04006				hypothetical protein
POG_2312_04092				hypothetical protein
POG_2312_04133				hypothetical protein
POG_2312_04220		U:COG3210	K15125	hypothetical protein
POG_2312_04221		U:COG3210	K15125	hypothetical protein
POG_2312_04247		L:COG2801	K07497	hypothetical protein
POG_2312_04248		L:COG2801	K07497	Insertion element IS476
POG_2312_04249		L:COG2801	K07497	Insertion element IS476
POG_2312_04250		L:COG2801	K07497	hypothetical protein
POG_2312_04251		T:COG2204	K10126	Alginate biosynthesis transcriptional regulatory protein AlgB
POG_2312_04252		S:ENOG410XPGJ		hypothetical protein
POG_2312_04252				hypothetical protein
POG_2312_04261		S:ENOG411292U		hypothetical protein
POG_2312_04263				hypothetical protein
POG_2312_04467		L:COG3293	K07492	hypothetical protein
POG_2312_04613				hypothetical protein

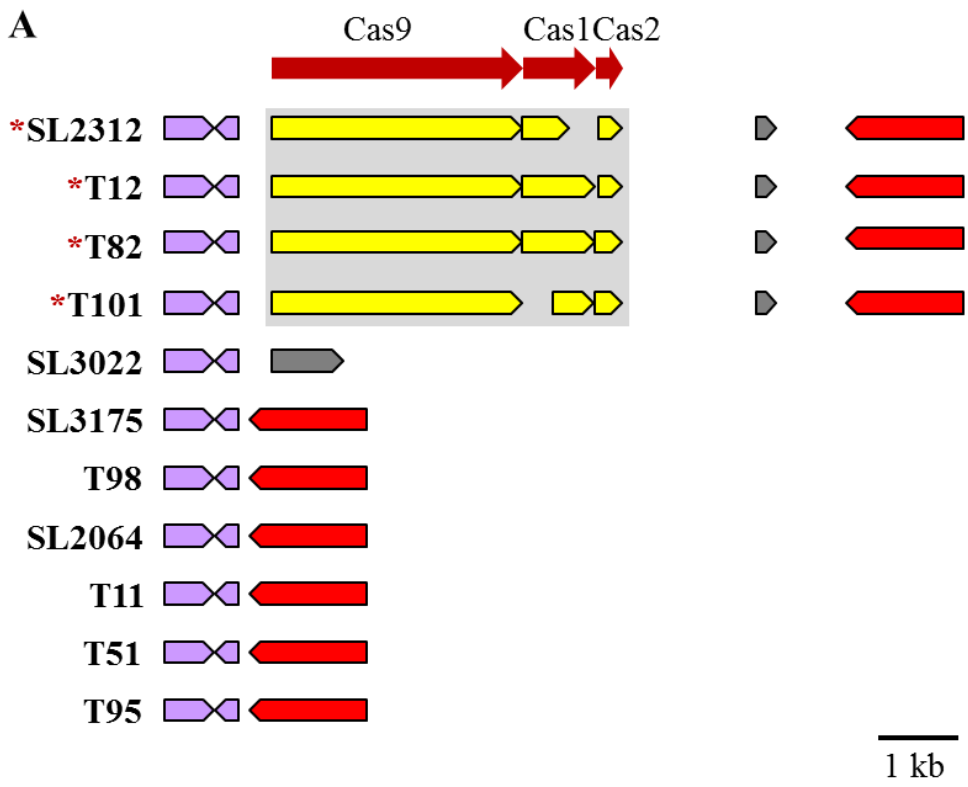


Figure 6. The unique genetic region for tomato nonpathogenic phylotype IV strains. (A) Cluster region of POG_SL2312_02168~02170 containing CRISPR-cas, (B) cluster region of POG_2312_03732~03775 containing virus particles, and (C) cluster region of POG_2312_04247~04252 containing two insertional sequence 476 (IS476). * indicates tomato nonpathogenic strains.

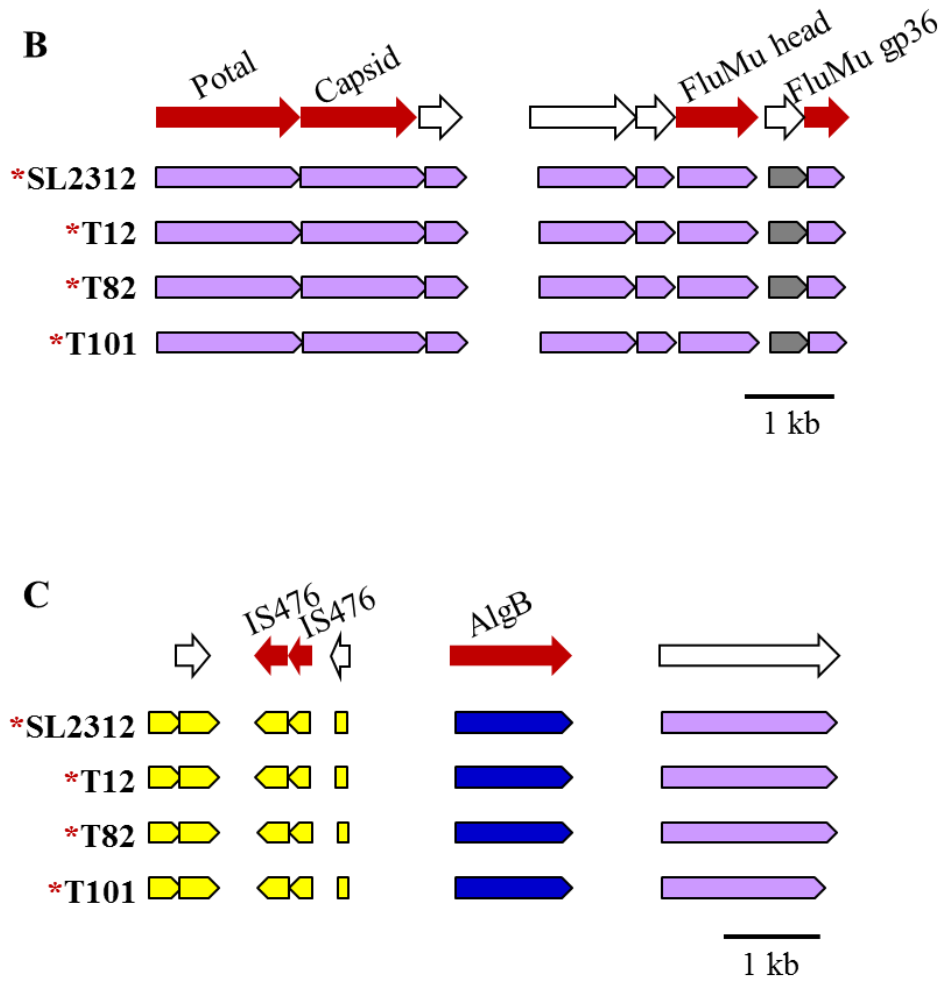


Figure 6. (continued)

Twenty-one tomato-pathogenic strains shared 8 genes (Table 7 and Figure 7). Most of genes with putative functions were related with protein secretion or cell attachment. Four isolates in phylotype IV that could not infect tomato did not carry these 8 genes.

In the case of eggplant, 7 *R. solanacearum* isolates that could not cause disease on eggplant shared 5 genes. Four of them were hypothetical but one is a putative RipA that was known as a transcriptional regulator for type III secretion with helix-turn-helix DNA binding motif (Table 8 and Figure 8-A, B). Seven genes were required for eggplant pathogenicity. None of them showed similarity with genes with known function (Table 9). For pepper plant, thirteen pathogenic strains shared 34 genes and twelve nonpathogenic strains shared 1 gene (Table 10 and Table 11). Total 11 *R. solanacerum* isolates that could not cause disease on pepper shared only one gene but the putative function of that gene is not known. Total 34 genes were required for the infection on pepper. Most of genes with known functions were related with metabolism. The most interesting feature is that isolate SL3103 which is in phylotype I-biovar 4 but could not infect pepper. All Korean *R. solanacearum* isolates in pylotype I could infect pepper except SL3103. Though SL3103 was in phylotype I, it could not infect pepper and only SL3103 did not carry those 34 genes among Korean phylotype I isolates (Figure 9).

Table 7. The list of 8 genes present specifically in 21 tomato pathogenic strains.

POG name	Other name	EggNog ID	Kegg ID	Product
POG_2064_00116		U:COG2165	K02456	Type II secretion system protein
POG_2064_00118		U:ENOG410ZZ6R	K02456	Fimbrial protein
POG_2064_00122		U:COG4796	K02453	Type IV pilus bioproteinis and competence protein PilQ
POG_2064_00123		M:COG3209		Probable deoxyribonuclease RhsB
POG_2064_02102		S:COG1376		hypothetical protein
POG_2064_04196	pcaL	S:ENOG410XRF3	K14727	3-oxoadipate enol-lactonase
POG_2064_04377		S:ENOG410YAST		hypothetical protein
POG_2064_04625		S:ENOG410ZHXP		hypothetical protein

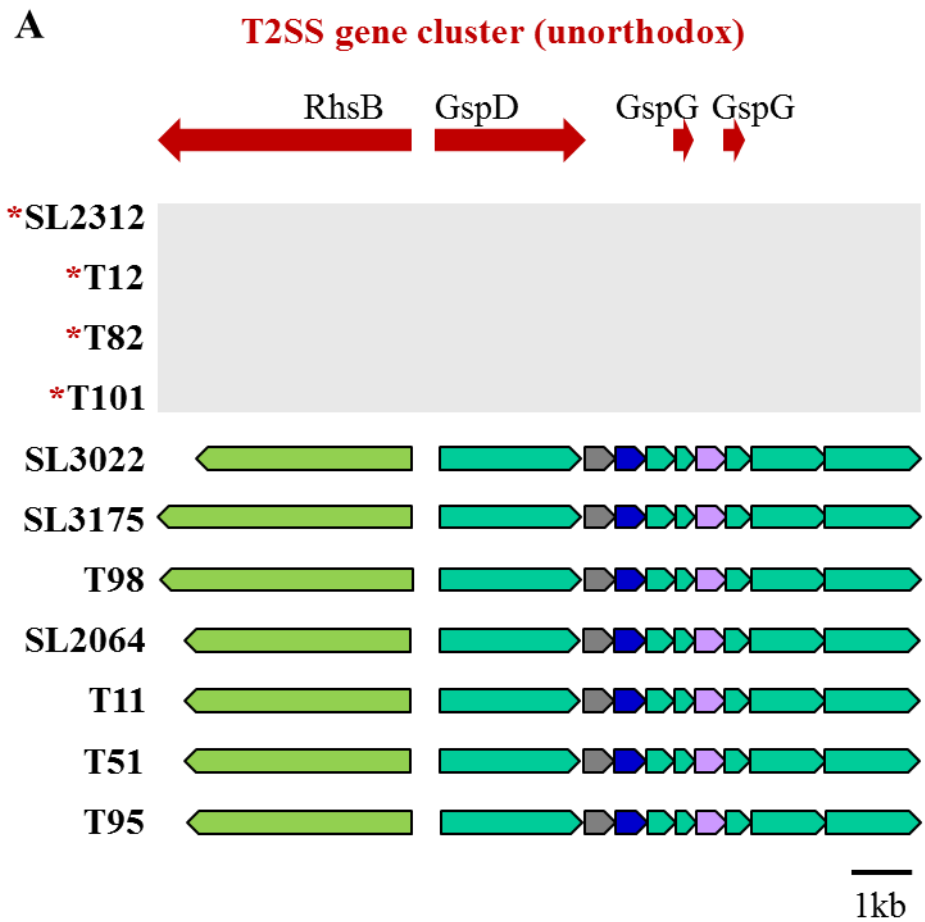


Figure 7. Genetic region of specific gene of tomato pathogenic strains. Cluster region of POG_2064_00116 ~00123 containing type II secretion system of phylotype IV (A) and I (B). * indicates tomato nonpathogenic strains.

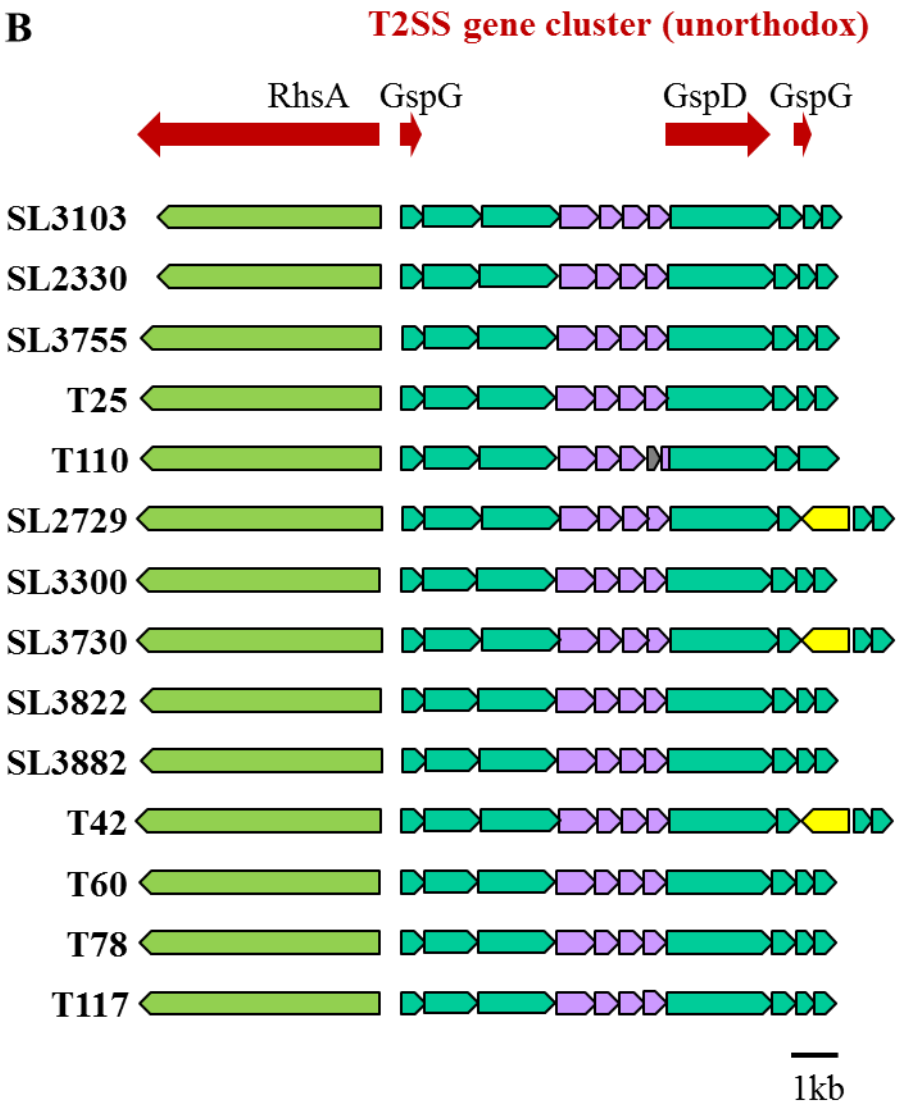


Figure 7. (continued)

Table 8. The list of 5 genes present specifically only in 7 eggplant nonpathogenic strains.

POG name	Other name	EggNog ID	Kegg ID	Product
POG_2312_00644				hypothetical protein
POG_2312_02082		S:ENOG4111UCD		hypothetical protein
POG_2312_02083		K:ENOG4111G2V	K02099	HTH-type transcriptional regulator RipA
POG_2312_03580		GM:COG0702		hypothetical protein
POG_2312_04000				hypothetical protein

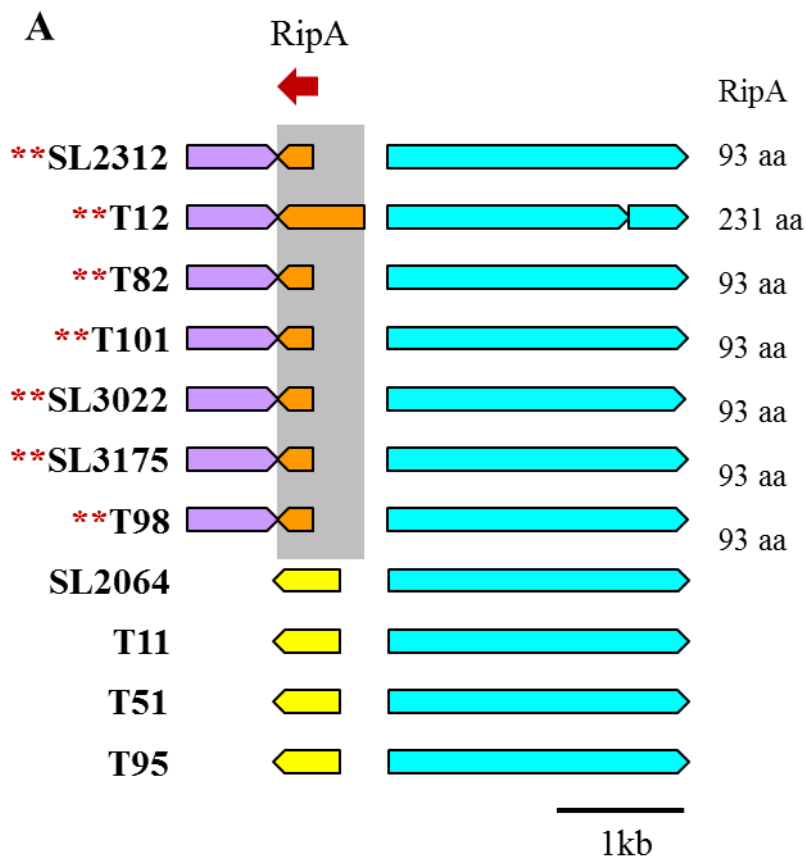
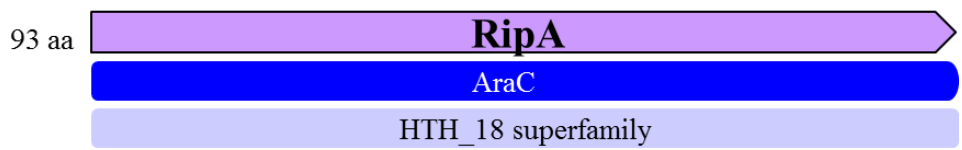


Figure 8. Genetic region of specific gene of eggplant nonpathogenic phylotype IV strains POG_2312_02083 (A) and (B) predicted functional domain. ** indicates eggplant nonpathogenic strains.

B



AraC : AraC-type DNA-binding domain

HTH_18 : Helix-turn-helix domain

Figure 8. (continued)

Table 9. The list of 7 genes present specifically in 18 eggplant pathogenic strains.

POG name	Other name	EggNog ID	Kegg ID	Product
POG_2064_00338		S:ENOG410Z5VH		hypothetical protein
POG_2064_02276		S:ENOG4112C5E	K06938	hypothetical protein
POG_2064_04357				hypothetical protein
POG_2064_04358		S:ENOG410XV72		hypothetical protein
POG_2064_04360				hypothetical protein
POG_2064_04361				hypothetical protein
POG_2064_04362		S:ENOG410Y4MZ		hypothetical protein

Table 10. The list of 34 genes present specifically in 13 pepper pathogenic strains.

POG name	Other name	EggNog ID	Kegg ID	Product
POG_2330_00090		S:COG1073	K06889	uncharacterized protein
POG_2330_00602				hypothetical protein
POG_2330_01806		I:COG2267		hypothetical protein
POG_2330_01807		K:COG1522	K03719	Bkd operon transcriptional regulator
POG_2330_01808		G:ENOG410XQE0	K07552	Uncharacterized MFS-type transporter YdeG
POG_2330_01811		L:COG2963	K07483	Transposase InsN for insertion sequence element IS911A
POG_2330_01856		S:ENOG410Z8US		hypothetical protein
POG_2330_01857		S:ENOG4111XGZ		hypothetical protein
POG_2330_03514		S:ENOG410YEIK		hypothetical protein
POG_2330_03515	iscS, NFS1	E:COG1104	K04487	Cysteine desulfurase
POG_2330_03516	cfa	Q:COG0500	K00574	Cyclopropane-fatty-acyl-phospholipid synthase
POG_2330_03526	pgl	G:COG2706	K07404	6-phosphogluconolactonase
POG_2330_03527	ACO, acnA	C:COG1048	K01681	Aconitate hydratase
POG_2330_03530		C:COG0667	K05275	Pyridoxine 4-dehydrogenase
POG_2330_03869		S:COG5525		hypothetical protein
POG_2330_04180	cmtC, dhbA	E:COG0346	K10621	hypothetical protein
POG_2330_04181	cmtD, dhbB	Q:COG0235	K10622	hypothetical protein
POG_2330_04182	dmpC, xylG	C:COG1012	K10217	Aminomuconate-semialdehyde dehydrogenase
POG_2330_04183	mhpD	Q:COG3971	K02554	2-oxopent-4-enoate hydratase
POG_2330_04184	dmpH, xylI, nahK	Q:COG3971	K01617	2-oxo-3-hexenedioate decarboxylase
POG_2330_04185	praC, xylH	S:COG1942	K01821	2-hydroxymuconate tautomerase
POG_2330_04186	mhpF	Q:COG4569	K04073	Acetaldehyde dehydrogenase (acetylating)
POG_2330_04187	mhpE	E:COG0119	K01666	4-hydroxy-2-oxovalerate aldolase
POG_2330_04189		S:ENOG410Z9MC		hypothetical protein
POG_2330_04190		O:COG0526	K03673	Thiol:disulfide interchange protein DsbA
POG_2330_04191	hpaH	Q:COG3971	K02509	hypothetical protein
POG_2330_04214		K:COG1309		hypothetical protein
POG_2330_04215		S:ENOG4111GFK		hypothetical protein
POG_2330_04702		Q:COG3319	K13611	Mycosubtilin synthase subunit
POG_2330_04732				hypothetical protein
POG_2330_04740		S:COG1574	K07047	Putative amidohydrolase YtcJ
POG_2330_04748				hypothetical protein
POG_2330_04749		C:COG4313		hypothetical protein
POG_2330_04750		S:COG3584		hypothetical protein

Table 11. A gene presents specifically only in 12 pepper nonpathogenic strains.

POG name	Other name	EggNog ID	Kegg ID	Product
POG_2064_00408		M:COG3209	K11904	hypothetical protein

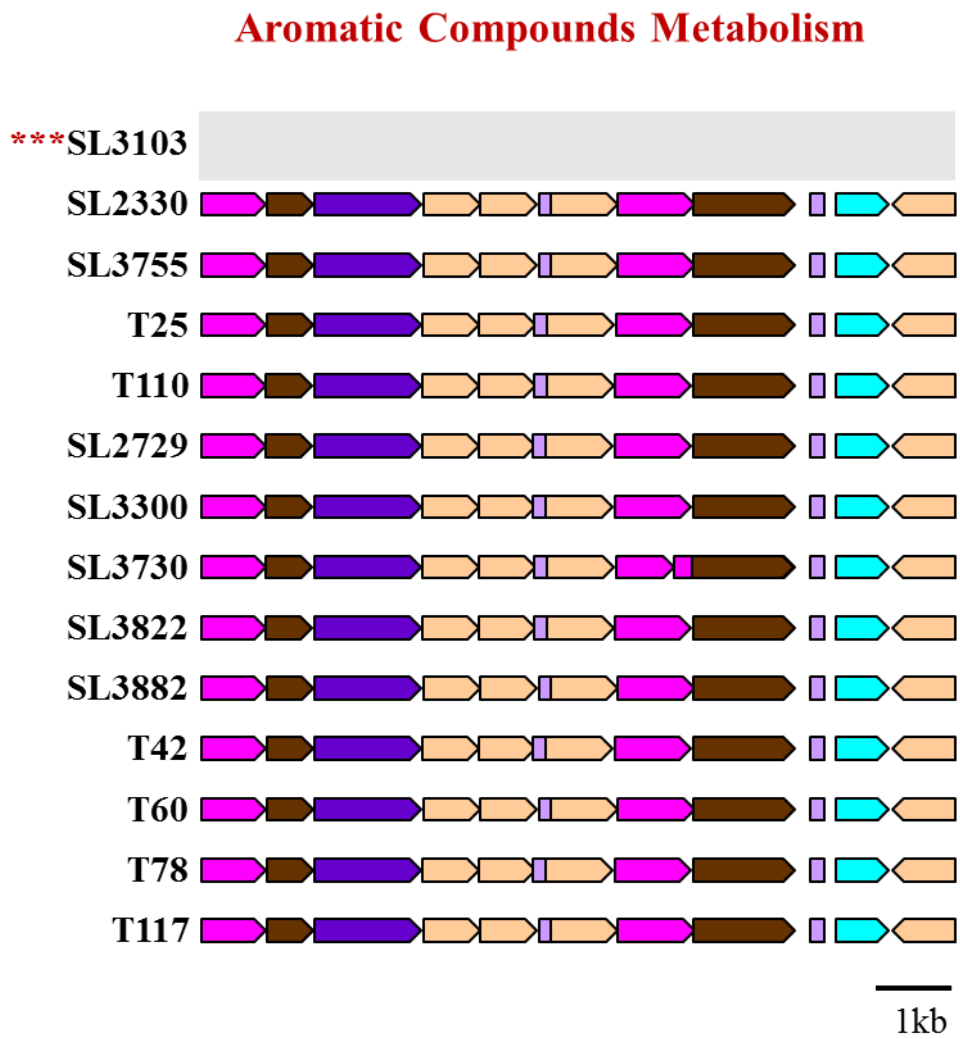


Figure 9. Genetic region of POG_2330_04180~04191 specific genes for pepper pathogenic phylotype I strains. *** indicates pepper nonpathogenic strain.

6. Functional genome comparison: T3SS effector

It is known that T3SS is deeply involved in the restriction of host range and *R. solanacearum* carries the most abundant T3SS effectors that secreted through T3SS. Total 25 Korean *R. solanacearum* strains had been divided into 4 pathotypes and 1 to 127 genes had been shown to be specific for the pathogen to respond against tomato, eggplant, and pepper but the functions of most of those genes were unknown. Therefore, I analyzed the T3SS effectors (T3Es) of Korean *R. solanacearum* using the RalstoT3E annotation server and analyzed the relations between the pathogenicity and effectors (Figure 10).

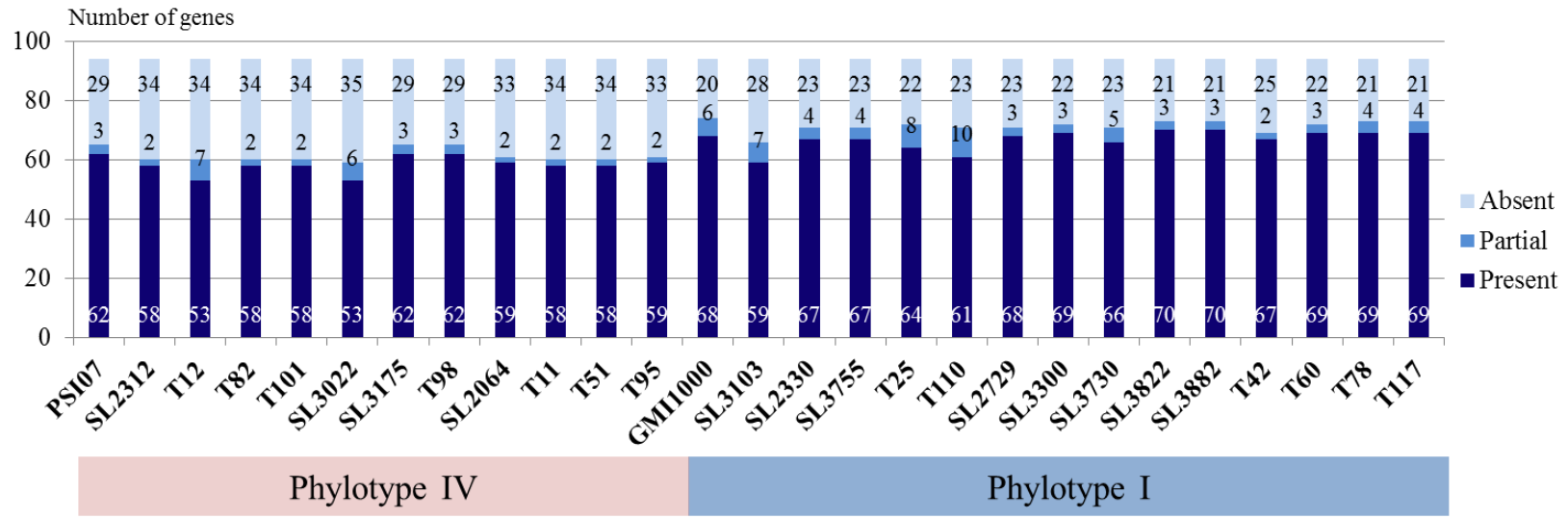


Figure 10. Graph of predicted effector gene numbers. The colors indicate as following: dark blue, presence of a gene; blue, partial gene, light blue; absent of a gene.

Among total 94 T3E repertoires of *R. solanacearum*, 82 effectors were found in the twenty-five Korean strains as a full or partial. In general, strains in phylotype I had more effetors than strains in phylotype IV. The distinct features could be found between phylotype I and IV (Figure 11). Four I-3 (phylotype I-biovar 3) strains had 68 effectors, ten I-4 (phylotype I-biovar 4) strains had 70 effectors, and eleven IV-2 (phylotype IV-biovar 2) strains had 70 effectors. For sharing of effectors among phylotype-biovar groups was as following: the group of I-3 strains presented 59 effectors; the group of I-4 strains, 57 effectors; the group of IV-2 strains, 41 effectors; the group of I-3 and I-4, 51 effectors; the group of I-4 and IV-2, 33 effectors; the group of I-3 and IV-2, 32 effectors; and the group of I-3, I-4, and IV-2, 30 effectors. Total 30 effectors were present in all sequenced *R. solanacearum* strains and 12 effectors were absent in all sequenced Korean strains (Figure 12). Eight effectors were present only in phylotype I and 6 effectors had been found only in phylotype IV. The presence of each effectors in phylotype I showed more conserved pattern than that in phylotype IV. For example the strains SL3175 and T98 in phylotype IV showed different effector pattern comparing with other strains in phylotype IV.

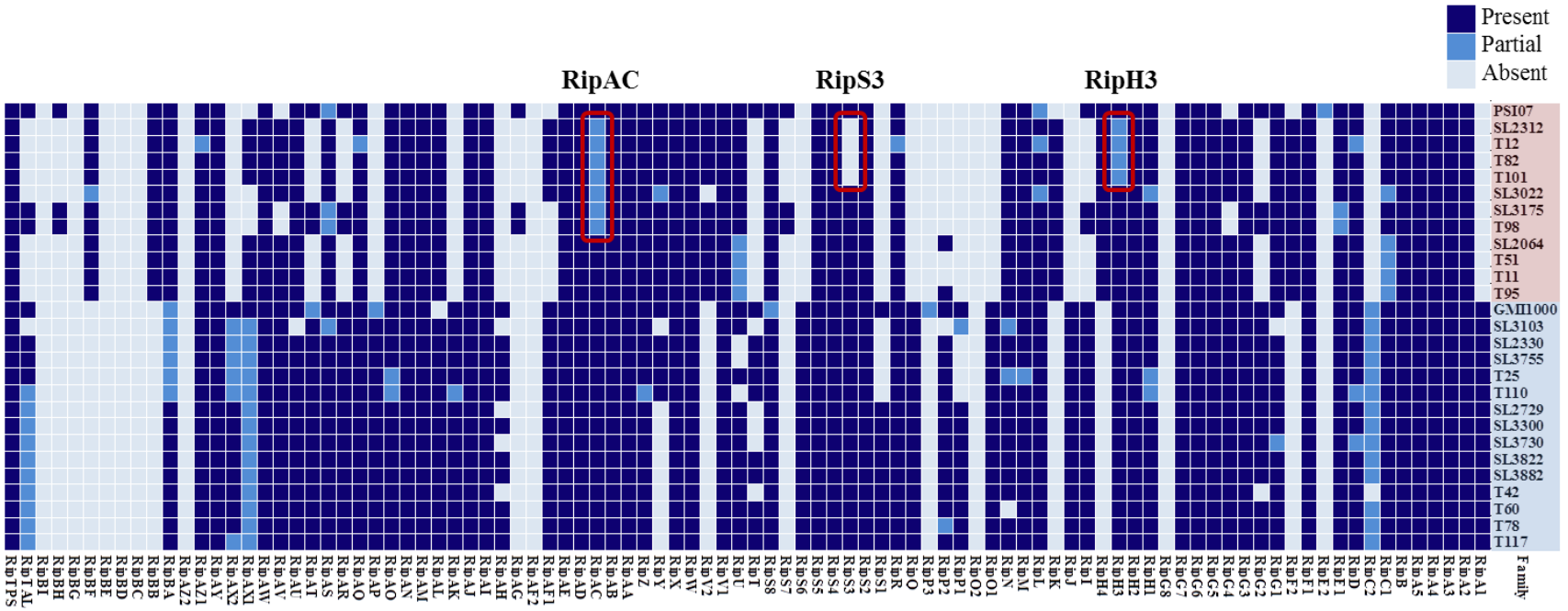


Figure 11. Distribution of the type III effectors genes of 25 Korean strains and reference GMI1000 and PSI07 strain. The colors indicate as following: dark blue, presence of a gene; blue, partial gene, light blue; absent of a gene. Pink box represent phylotype IV strains and blue box represent phylotype I strains.

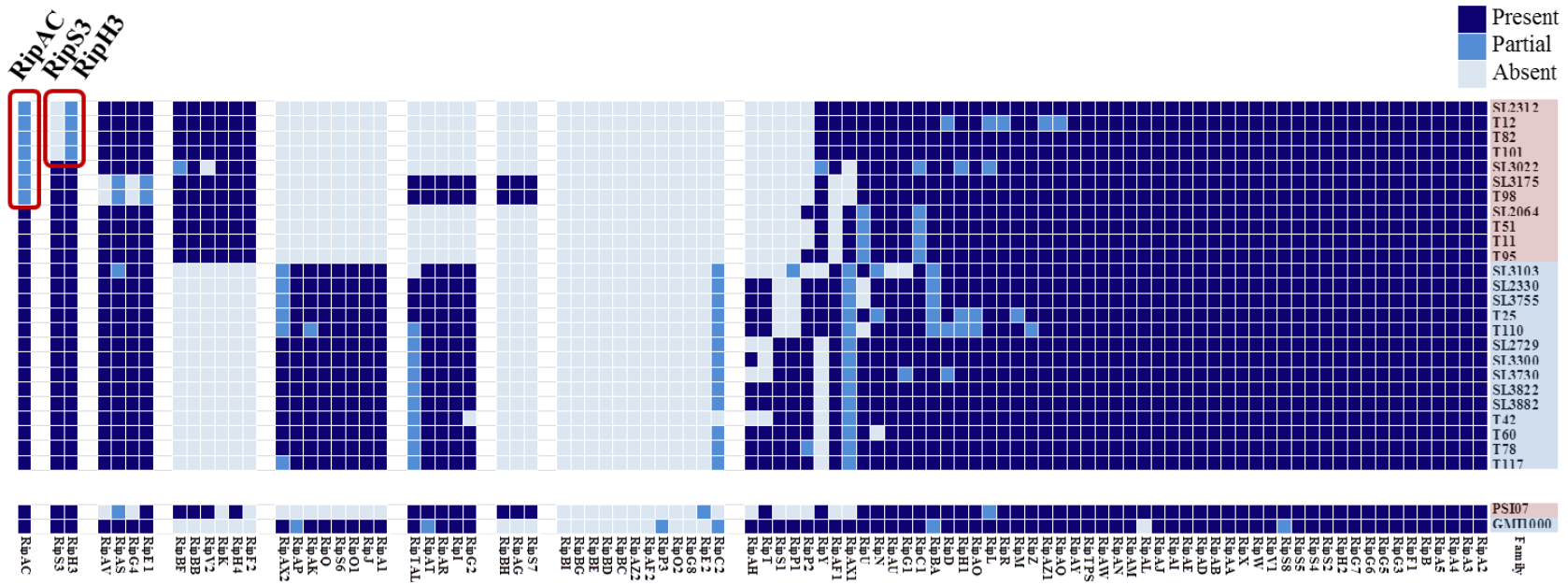


Figure 12. Data showed the rearrangement of the presence of T3SS effectors in twenty-five Korean strains and reference GMI1000 and PSI07 strains. The colors indicate as following: dark blue, presence of a gene; blue, partial gene, light blue; absent of a gene.

I checked the presence of effectors related to the host specificity – specific effectors for infection of tomato, eggplant, or pepper plant. Among the studied effectors, I found two specific effectors for tomato infection (RipH3 and RipS3) and one effector for eggplant (RipAC). Genetic regions of these proteins coding region were showed in Figure 13 and Figure 14, repectively. RipS3 (other name Skwp3) was present in all twenty-one tomato-pathogenic strains but was absent in the four tomato-nonpathogenic strains (SL2312, T12, T82, and T101). The gene function of RipS3 is unknown but it carries nucleotidyltransferase domain of RelA (Figure 13-C). RipH3 presented as the complete form in all the tomato-pathogenic strains, but presented as partial in the nonpathogenic strains. SL2312 had C-terminal truncated form and T12, T82, and T101 had N-terminal truncated form. Though promoter of the gene RipH3 showed PIP box, putative protein RipH3 did not show any known functional domain in it. RipAC was presented as the complete form in all eighteen eggplant-pathogenic strains but was presented as the C-terminal truncated partial form in the seven eggplant-nonpathogenic strains (SL2312, T12, T82, T101, SL3022, SL3175, and T98). The alternative name of effector RipAC is PopC which is known as an effector that carries 2 tandem leucine-rich repeats (LRR). The LRR domain of PopC protein formed a consensus that perfectly mached the predicted eukaryotic cytoplasmic LRR consensus (Figure 14).

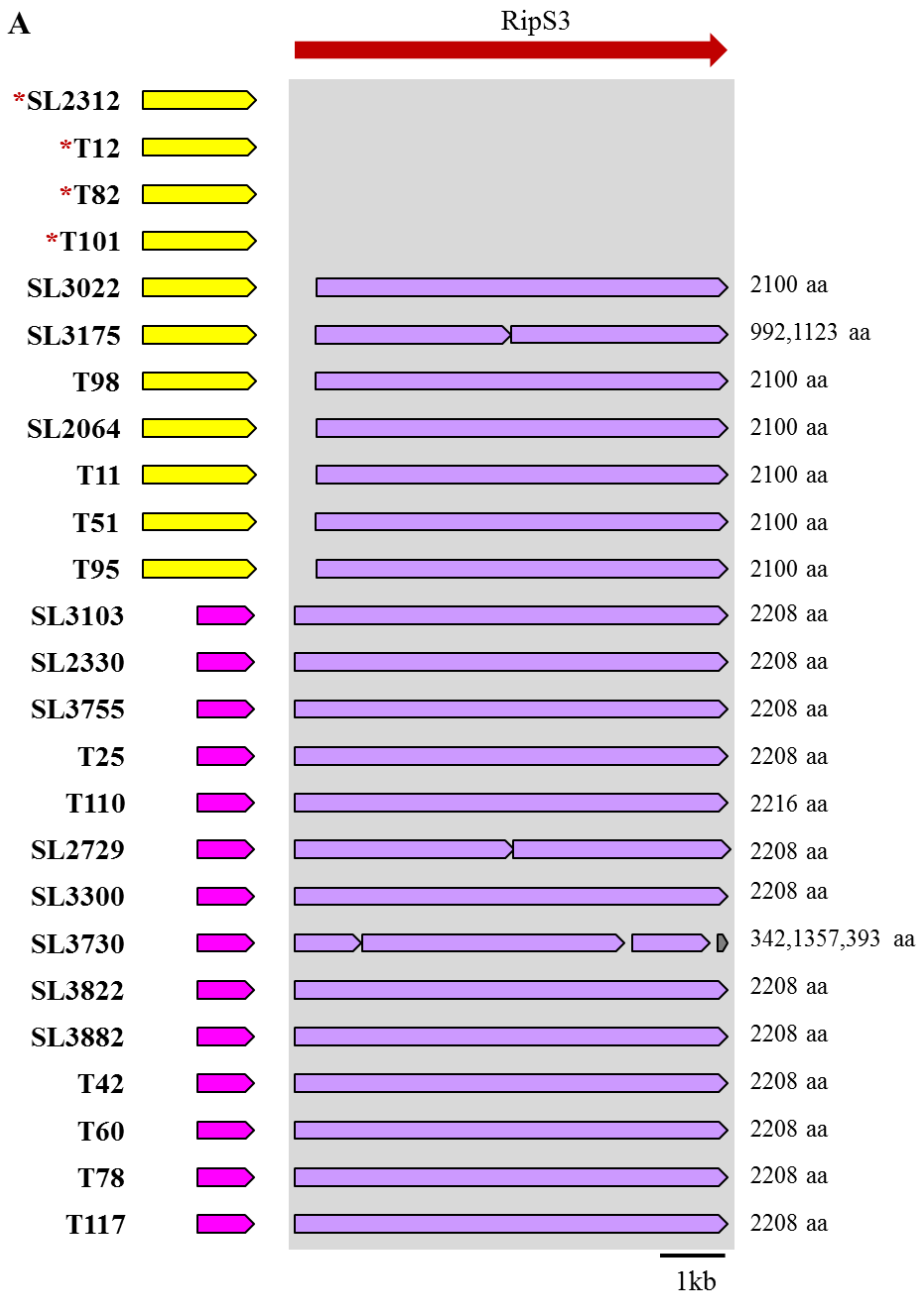


Figure 13. Genetic regions of RipS3 (A) and RipH3 (B) coding gene specific for tomato pathogenic strains, and predicted functional domain of RipS3 (C). * indicates nonpathogenic strains for tomato.

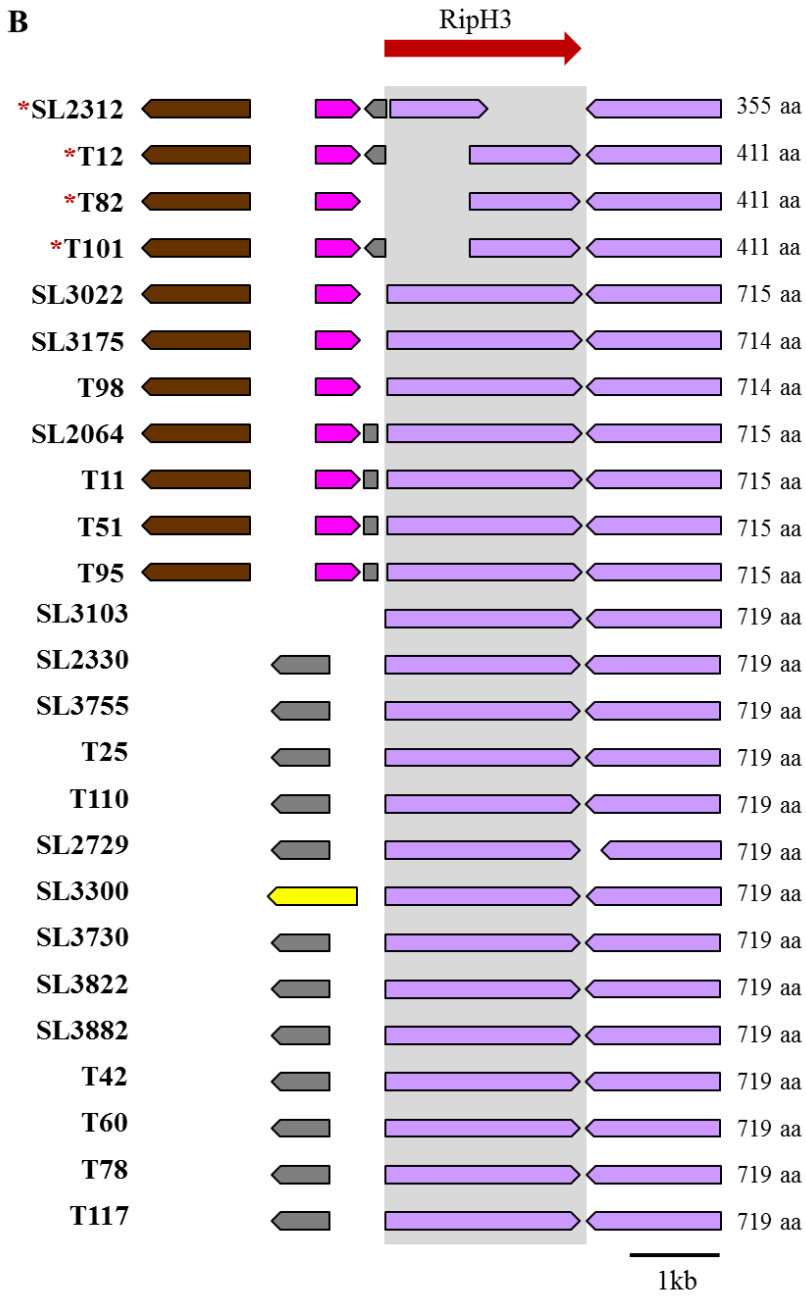
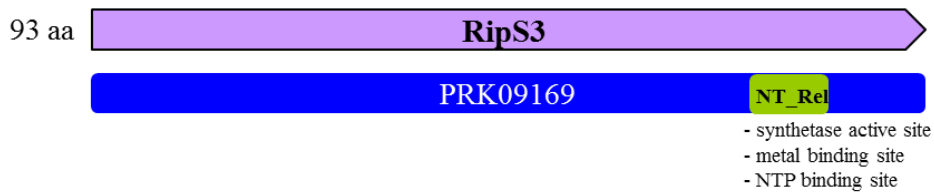


Figure 13. (continued)

C



PRK09169 : hypothetical protein

NT_Rel : Nucleotidyltransferase (NT) domain of RelA-like ppGpp synthetases and hydrolases

Figure 13. (continued)

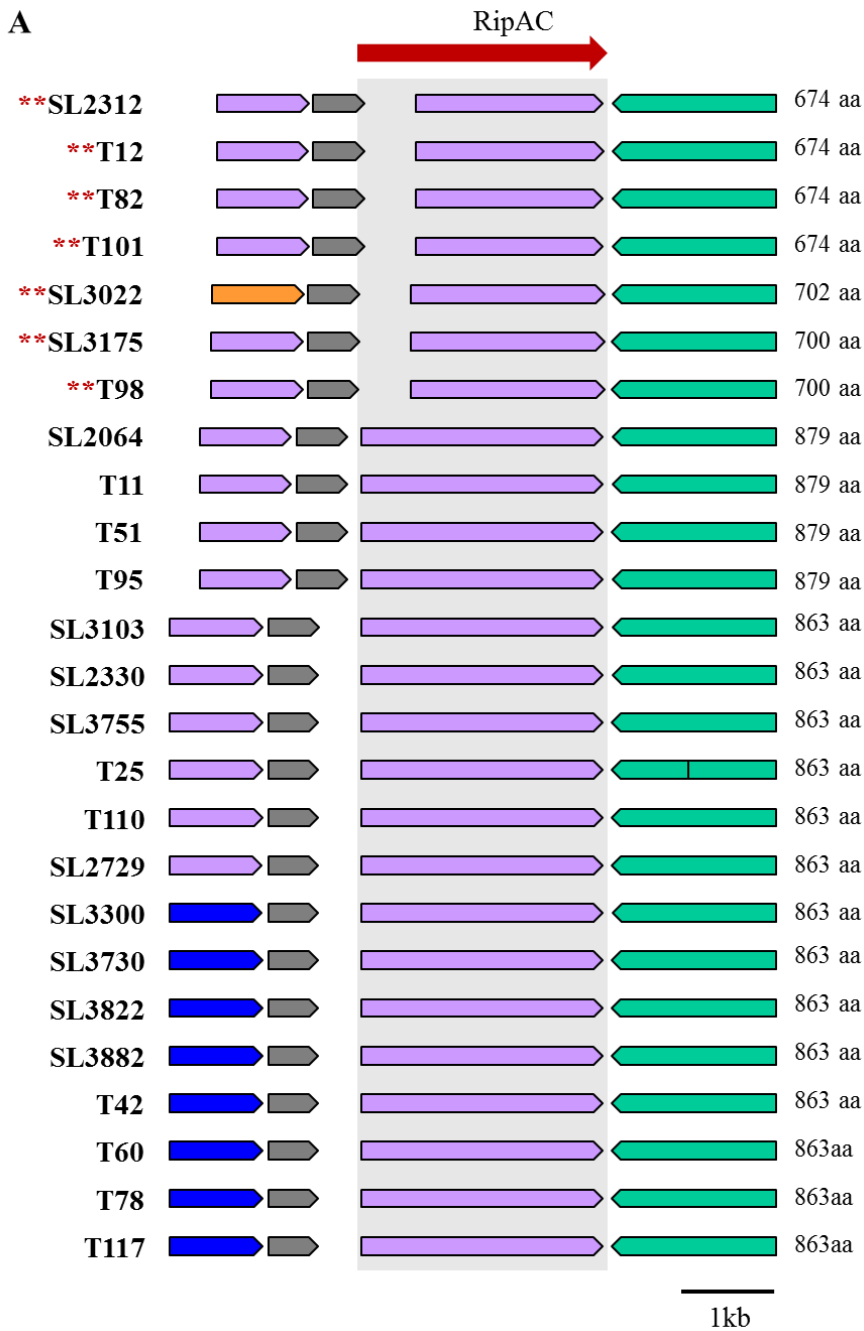


Figure 14. Genetic regions of RipAC coding gene (A) specific for eggplant pathogenic strains and predicted functional domain (B). ** indicates nonpathogenic strains for eggplant.

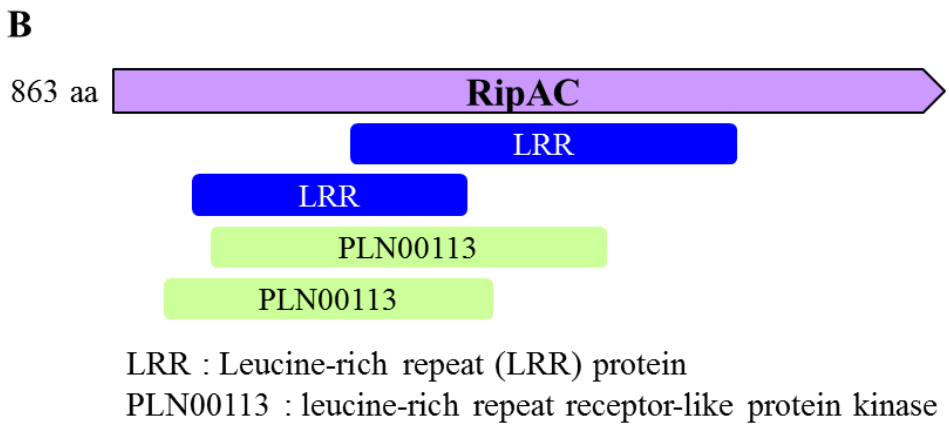


Figure 14. (continued)

DISCUSSION

Ralstonia solanacearum species is sophisticate phytopathogen with unusual broad host range and wide geographical distribution. In this study, the whole genome of twenty-five Korean *R. solanacearum* strains had been sequenced completely using Pacbio long read sequencing platform. All sequenced 25 Korean *R. solanacearum* strains had been fall into 2 phylotypes; phylotype IV and I and phylotype I showed destructive features on tomato, eggplant, and pepper. Among phylotype I strains, T42 strain had the smallest genome size. The genome size of 5.5 Mbp and 5,133 predicted genes were similar with those of phylotype IV strains, while 67 % GC content, 12 rRNA, and 57 tRNA were similar with those of other phylotype I strains. Because the T42 strain had pathogenicity on potato, tomato, and eggplant as well as pepper, this strain seemed the minimal genes to infect pepper plant.

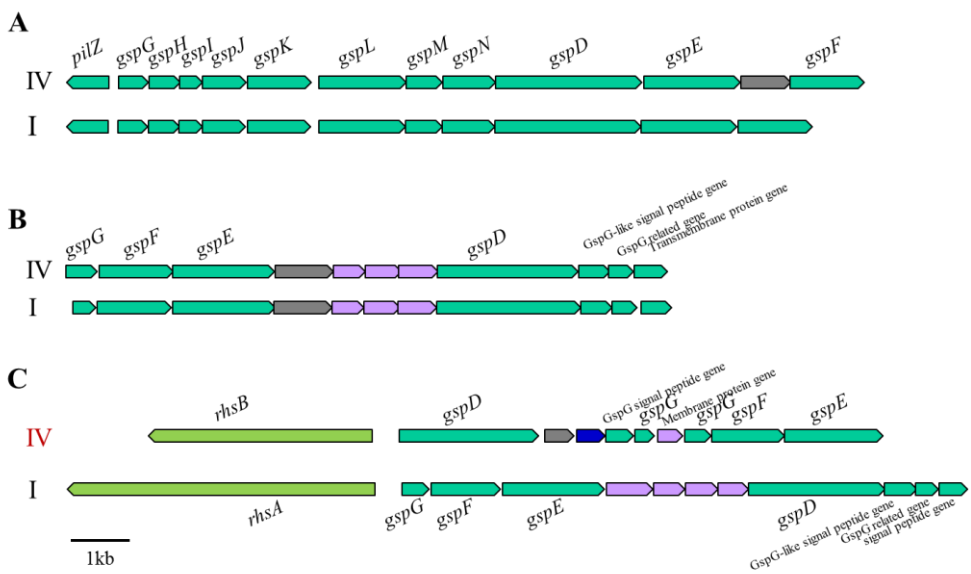
All genomic features (genome size and GC content, CDS, rRNA, and tRNA) of phylotype I strains were larger than those of phylotype IV strains. Previously Li et al. (2016) suggested that phylotype I strains might have evolved from phylotype IV strains. The genome of phylotype I strains have distinctive features compared with those of other representative

phylotype II (Po82, IPO1609), III (CMR15), and IV strains (PSI07, R24, and R229); large genome size, high GC contents, and more rRNA and tRNA genes. These genomic features tell us that phylotype I strains accepted foreign genome fragments leading to increased GC contents and genomes size, and among the accepted genome, there might be exist various virulence factors or effector proteins to infect the new host.

The results of genome comparisons using ANI, pair-wise and multiple alignment, Korean phylotype IV strains were very consistent each other compared to the phylotype I strains, which had variations of genome size, inversion, deletion, insertion, etc. With these results I could infer that the phylotype IV strains recently introduced to Korea than the phylotype I strains. Among phylotype I strains, strain SL3300 and T117 had an inversion in the chromosome, SL3822 and T25 had an inversion in the megaplasmid, T42 had deletion, and SL3103 had deletion and insertion.

To investigate responsible genes for host specificity of *Ralstonia solanacearum*, pan-genome orthologous analysis of twenty-five strains had been carried out and these genetic information had been analyzed based on host specificity. This analysis suggested that many genes were involved in the tomato infection. Total 127 genes present only in four isolates that could not infect tomato. Most of them are hypothetical and few genes functionally

designated had relations with mobile elements such as bacteriophage infection or insertional elements. It suggested that several genes disrupted by IS elements or phage infection could be required for tomato infection. This result may be consistent with the results that twenty-one tomato-pathogenic strains carry specific 8 genes including components of type II secretion system or type IV secretion system, flagella, etc. When I investigated type II secretion systems in those 4 tomato nonpathogenic strains in depth, those 4 strains did carry less type II secretion system related genes than the other 21 strains that could infect tomato (Figure 15).



* T2SS_C-IV : does not exist in the strains of tomato nonpathogen (SL2312, T12, T82, and T101)

Figure 15. Genetic organization of three T2SS gene clusters in phylotype IV and I strain of *R. solanacearum*. (A) orthodox T2SS cluster, (B) and (C) unorthodox T2SS cluster. (IV) phylotype IV, (I) phylotype I.

Also the results from T3effector analysis showed similar results that RipS3 and RipH3 were missing in those 4 tomato nonpathogenic strains. The eggplant specific infection genes were 7 genes but the functions of them were not known. However, RalstoT3E prediction system showed that RipAC (PopC) had eggplant specificity. Without functional RipAC *R. solanaceraum* could not cause disease on eggplant. RipAC had two leucine rich repeat (LRR) domains, which expected to bind to some host protein. It had been reported that there were polymorphism of the *ripAC* gene of *R. solanacearum* Moko strain and brown rot strain (Ailloud et al., 2015). With a competitive index assay using wild type and mutants, 12 effector genes were identified as contributing to bacterial fitness in eggplant leaves (Macho et al., 2010). Among those *popC* was there. All these results suggest that *popC* may be a good candidate gene to be involved in the pathogniecity of *R. solanacearum* on eggplant. For pepper-infection strains, predicted 34 specific genes having enzymatic activities in metabolism. These genes are considered as secondary factors to give virulence effect on host but not the main key factors. It has been reported that Rsa1 having aspartic protease activity involved in the pepper pathogenicity. When this *rsa1* gene was expressed from the pepper pathogenic strain, *rsa1* containing strain could not cause disease on pepper but *R. solanacearum* strains with dis-functional Rsa1 could infect pepper (Jeong et al., 2011). *rsa1* gene was present in all

sequenced phylotype IV strains but not present in phylotype I. However, strain SL3103 which showed unique feature that other phylotype I strains. Though strain SL3103 is in phylotype I, it could not infect pepper and it did not have *rsa1* gene. It suggested that Rsa1 may work as a pepper specific pathogenicity factor with unknown protein(s). This is consistent with that 34 genes were involved in the pepper pathogenicity.

This extensive comparative genomic analysis uncovered several genes associated with the pathogenicity of *R. solanacerum* on different crops. Though biological functions should be proved, these data could contribute to understand host specificity.

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solanacearum phylotype IV strains as *Ralstonia syzygii* subsp. *indonesiensis* subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. *celebesensis* subsp. nov. and *R. solanacearum* phylotype I and III strains as *Ralstonia pseudosolanacearum* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 64:3087-3103.

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한국의 세균성 풋마름병 감염 감자에서 분리한

*Ralstonia solanacearum*의 유전체 해독 및 비교분석

조희정

요약(국문초록)

*Ralstonia solanacearum*은 토마토, 감자, 고추 등의 가지과 작물을 비롯해 바나나, 뽕나무, 땅콩, 참깨, 들깨 등 단자엽·쌍자엽, 초본·목본을 가리지 않고 다양한 식물에서 풋마름병을 일으키는 병원균이다. 토양 속에서 오랜 시간 생존하다 적당한 기주를 만나면 뿌리의 상처를 통해 침입하고, 식물의 물관에서 증식하면서 물의 이동을 방해해 식물이 고사하게 된다. 이러한 세균성 풋마름병은 열대, 아열대, 온대 지역에서 만연하고, 기주 범위가 넓기 때문에 세계적으로 가장 피해를 주고 있다. 이 논문에서는 국내에서 감자풋마름병이 최초로 보고되었던 1998년부터 2003년까지 전국의 감자풋마름병 발생지역에서 수집되었던 *R. solanacearum* 93 균주를 대상으로 생화학적 특성 검정 및 유전자 염기서열 분석으로 분류학적 위치를 파악하고 다양한 가지과 작물에 대한 병원성 검정으로 각 균주의 기주 범위를 확인하였다. 그 결과, 3종의 이당

류와 3개의 육탄당 알코올 이용 여부를 통한 biovar 검정을통해, biovar 2가 22 균주, biovar 3가 8 균주, biovar 4가 63 균주로 분석되었으며, 이중 biovar 2는 phylotype IV로, biovar 3와 biovar 4 균주는 모두 phylotype I에 속하는 것을 확인하였다. 이들 균주를 이용하여 경제적으로 중요한 감자, 토마토, 가지, 고추에 병 검정을 실시한 결과, 다음과 같은 4 가지 병원형을 보였다 – 감자에만 병을 일으키는 경우 (P형, Potato), 감자와 토마토에만 병을 일으키는 경우 (PT형, Potato, Tomato), 감자, 토마토, 가지에만 병을 일으키는 경우 (PTE형, Potato, Tomato, Eggplant), 그리고 감자, 토마토, 가지, 고추 모두에 병을 일으키는 경우 (PTEPe형, Potato, Tomato, Eggplant, Pepper). P형은 phylotype IV형 11 균주, PT형은 phylotype IV형 4 균주, PTE형은 phylotype IV형 7 균주, phylotype I형 5 균주가 나왔고, 검정한 기주 식물 모두에 병을 일으켰던 PTEPe형은 phylotype I형에서만 59 균주가 나왔다. 이러한 기주 범위를 기반으로 25 균주를 선발하고 유전체를 해독하여 비교분석을 수행하였다. 새롭게 해독된 국내 균주의 유전체 정보와 이전에 발표된 국외의 9개 레퍼런스 게놈 정보와의 유전체를 비교분석한 결과 구조적으로는 국내외 phylotype I 또는 phylotype IV 균주 간에 높은 상동성을 보여주었다. *R. solanacearum*의 기주 특이성을 알아보기 위해, 토마토 감염/비감염 균주 집단, 가지 감염/비감염 균주 집단, 고추 감염/비감염 균주 집단으로 나누어 유전체 정보 비교 분석하였

다. 그 결과 토마토 감염 균주 집단에만 있는 유전자 8 개, 비감염 균주 집단에만 있는 유전자 127개, 가지 감염 균주 집단에만 있는 유전자 7 개, 비감염 균주 집단에만 있는 유전자 5 개, 고추 감염 균주 집단에만 있는 유전자 34 개, 비감염 균주에만 있는 유전자 1 개의 유전자 정보를 얻었다. 또한 기주식물로 분비되어 기주의 병 방어 시스템을 약화시키는 effector 유전자 중, 토마토에 감염하는 균주에는 RipH3와 RipS3가 있었으며, 가지에 감염하는 균주에는 RipAC가 있었다. 이 연구는 *R. solanacearum*의 기주 범위가 특정 effector 단백질 이외에도, 대사작용, 분비 시스템, 분해 효소 등 다른 병원성 인자와의 종합적인 작용이 필요함을 보여주었다.

주요어 : *Ralstonia solanacearum*, 감자풋마름병, 기주 범위, 병원형, 유전체, 유전체 비교분석

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