

## Specific and Sensitive Primers Developed by Comparative Genomics to Detect Bacterial Pathogens in Grains

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Accurate and rapid detection of bacterial plant pathogen is the first step toward disease management and prevention of pathogen spread. Bacterial plant pathogens *Clavibacter michiganensis* subsp. *nebraskensis* (*Cmn*), *Pantoea stewartii* subsp. *stewartii* (*Pss*), and *Rathayibacter tritici* (*Rt*) cause Goss's bacterial wilt and blight of maize, Stewart's wilt of maize and spike blight of wheat and barley, respectively. The bacterial diseases are not globally distributed and not present in Korea. This study adopted comparative genomics approach and aimed to develop specific primer pairs to detect these three bacterial pathogens. Genome comparison among target pathogens and their closely related bacterial species generated 15-20 candidate primer pairs per bacterial pathogen. The primer pairs were assessed by a conventional PCR for specificity against 33 species of *Clavibacter*, *Pantoea*, *Rathayibacter*, *Pectobacterium*, *Curtobacterium*. The investigation for specificity and sensitivity of the primer pairs allowed final selection of one or two primer pairs per bacterial pathogens. In our assay condition, a detection limit of *Pss* and *Cmn* was 2 pg/μl of genomic DNA per PCR reaction, while the

detection limit for *Rt* primers was higher. The selected primers could also detect bacterial cells up to  $8.8 \times 10^3$  cfu to  $7.84 \times 10^4$  cfu per gram of grain seeds artificially infected with corresponding bacterial pathogens. The primer pairs and PCR assay developed in this study provide an accurate and rapid detection method for three bacterial pathogens of grains, which can be used to investigate bacteria contamination in grain seeds and to ultimately prevent pathogen dissemination over countries.

**Keywords:** *Clavibacter michiganensis* subsp. *nebraskensis*, comparative genomics, *Pantoea stewartii* subsp. *stewartii*, PCR detection, *Rathayibacter tritici*

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The economic impact of plant diseases caused by bacterial pathogens escalates with global warming (Castillo and Plata, 2016) with an increase in international exchange of grains and vegetables among countries (Nutter and Madden, 2009). Comparatively, a number of bacterial pathogens prefer higher temperatures, for infection, than the fungal pathogens. The magnitude of risk for bacterial pathogens dissemination is directly proportional to the massive global exchange of the agricultural products (Pautasso et al., 2010). Therefore, precise measures for plant quarantine become important to restrict dissemination of bacterial plant pathogen globally to ensure secure agricultural production.

Bacterial pathogens, *Clavibacter michiganensis* subsp. *nebraskensis* (*Cmn*), *Pantoea stewartii* subsp. *stewartii* (*Pss*), *Rathayibacter tritici* (*Rt*, formerly *Clavibacter tritici*), are not globally distributed and are thus quarantined pathogens in many countries including Korea. *Cmn* is a

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Gram-positive bacterial pathogen causing Goss's bacterial wilt and blight of maize (*Zea mays* L.) (Vidaver and Mandel, 1974). The disease shows two different symptoms: a leaf blight and a systematic vascular wilt (Agarkova et al., 2011). Since the first report of this disease in 1969 in the USA, this disease is only limited in USA and Canada (Agarkova et al., 2011; Eggenberger et al., 2016; Vidaver and Mandel, 1974). Transmission of this disease is either through infected seeds or bacteria-infested residues. Rate of transmission via seeds is lower than bacteria-infested residues which seem to be the primary source of inoculum in disease spread (Smidt and Vidaver, 1986). Diagnosis and detection of Goss's wilt and bacterial pathogen are currently dependent on disease symptoms, immunological assays and selective medium for bacterial pathogen.

*Pss* causes Stewart's wilt of maize, mostly transmitted by the corn flea beetle (*Chaetocoenema pulicaria*) and infected seeds (Michener et al., 2002) and the disease is under plant quarantine regulations in more than 60 countries on maize seeds imports from affected areas (Pataky and Ikin, 2003). Symptoms of Stewart's wilt are somewhat similar to Goss's wilt by *Cmn* and thus a careful examination is necessary to distinguish the two diseases. To differentiate plant-associated *Pantoea* species, several methods have been developed. Immunoassay or polymerase chain reaction (PCR) primers have been described (Coplin et al., 2002; Lamka et al., 1991; Tambong et al., 2008; Wensing et al., 2010). However, those analyses cannot completely distinguish species of *Pantoea* or subspecies of *P. stewartii* (Wensing et al., 2014). Recently, Wensing et al. (2014) developed a single-nucleotide polymorphism based PCR assay to differentiate *Pss* from *P. stewartii* subsp. *indologenes*.

*Rt* is a Gram-positive coryneform bacterial pathogen causing spike blight, also called yellow ear rot or Tundu disease, in wheat and barley (Paruthi and Gupta, 1987). *Rt* infection and rapid spread is frequently associated with *Anguina tritici*, a nematode, producing seed galls (ear cockles) in certain plant varieties (Paruthi and Bhatti, 1985). Despite the wide distribution of this bacterial pathogen (Park et al., 2017), no reliable detection method has been reported so far.

DNA-based diagnostic methods such as PCR have been widely used as an effective approach to detect bacterial pathogens from diseased plants, which offer advantages over traditional antibody-based detections which are expensive and time consuming processes (Cho et al., 2011; De Boer et al., 1988; Gudmestad et al., 2009; Vincelli and Tisserat, 2008). Either the 16S rRNA gene or the spacer region between the 16S and 23S rRNA genes of bacterial

pathogen, known phylogenetic markers, have been primarily targeted for amplification by PCR (Kang et al., 2016). The possibility for use of these sequences to detect bacterial pathogens remains technically limited to sensitively distinguish bacteria at species or below species level (Baker et al., 2003; Henriques et al., 2012). Hence, there are growing efforts to search for new marker gene or sequences to be developed as detection markers for bacterial pathogens of plants with high reliability. A perpetual increase in the availability of whole genome sequences of bacterial pathogens has shifted the attention to use of comparative genome analysis, which is the most effective ways to select candidate marker gene of bacterial pathogens (Ahmed et al., 2008; Demkin et al., 2017; Papaiakevou et al., 2017; Tao et al., 2015).

Since there are no primers available to detect bacterial pathogens, *Cmn* and *Rt*, we aimed to develop specific and sensitive primers to detect bacterial pathogens in grains. In this study, by taking advantage of comparative genome analysis of bacterial pathogens, we assessed several candidate primers to detect three bacterial pathogens of grains, and selected the specific and sensitive primer pairs for these bacterial pathogens.

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## Materials and Methods

**Bacterial strains and culture condition.** Most of bacterial strains used in this study were obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB, York, UK) and the Korean Agricultural Culture Collection (KACC, Korea) (Table 1). Bacterial strains of *C. michiganensis* subsp. *capsici* (*Cmc*) (Oh et al., 2016) and *Cmm* (Yim et al., 2012) were kindly provided from Chang-Sik Oh at the Kyung Hee University, and Jae-Soon Cha at the Chungbuk National University, Korea, respectively. All of bacterial strains from NCPPB are listed as plant quarantine bacteria in Korea and many other countries, therefore, these strains were obtained under official permission from Animal and Plant Quarantine Agency, Korea for use only in a restricted area during limited time period.

Both *Clavibacter* species and *Pectobacterium* species were grown at 25°C while *Pantoea* species were grown at 30°C in nutrient broth (NB). The *Rathayibacter* species were grown in nutrient broth yeast extract broth (NBY) (Hayward, 1964) at 25°C. *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) was grown in yeast peptone glucose agar medium (Vidaver, 1967) at 30°C. Genomic DNA of bacterial strains was purified using a Wizard Genomic DNA Purification kit (Promega, Fitchburg, WI, USA) and the concentration of purified DNA was determined by us-

**Table 1.** List of bacterial strains used in this study

Strain <sup>a</sup>		Host plant	Origin <sup>b</sup>
<b>Clavibacter</b>			
<i>michiganensis</i> subsp. <i>capsici</i>	PF008 (type)	<i>Capsicum annuum</i>	Korea
<i>michiganensis</i> subsp. <i>capsici</i>	PF003	<i>Capsicum annuum</i>	Korea
<i>michiganensis</i> subsp. <i>capsici</i>	PF005	<i>Capsicum annuum</i>	Korea
<i>michiganensis</i> subsp. <i>capsici</i>	PS006	<i>Capsicum annuum</i> (seed)	China*
<i>michiganensis</i> subsp. <i>michiganensis</i>	LMG 7333 (type)	<i>Solanum lycopersicum</i>	Hungary
<i>michiganensis</i> subsp. <i>michiganensis</i>	PPP1	<i>Solanum lycopersicum</i>	Korea
<i>michiganensis</i> subsp. <i>nebraskensis</i>	NCPPB 2581 (type)	<i>Zea mays</i>	USA
<i>michiganensis</i> subsp. <i>nebraskensis</i>	NCPPB 2582	<i>Zea mays</i> (seed)	USA
<i>michiganensis</i> subsp. <i>insidiosus</i>	NCPPB 1109 (type)	<i>Medicago sativa</i>	USA
<i>michiganensis</i> subsp. <i>tesselarius</i>	NCPPB 3664 (type)	<i>Triticum aestivum</i>	USA
<b>Curtobacterium</b>			
<i>flaccumfaciens</i> pv. <i>flaccumfaciens</i>	NCPPB 1446 (type)	<i>Phaseolus vulgaris</i>	Hungry
<i>flaccumfaciens</i> pv. <i>flaccumfaciens</i>	NCPPB 2344	<i>Phaseolus vulgaris</i>	USA
<b>Pantoea</b>			
<i>agglomerans</i>	KACC 10054	<i>Wisteria floribnda</i>	Japan
<i>agglomerans</i>	KACC 15275 (type)	<i>Homo sapiens</i>	Scotland
<i>ananatis</i> pv. <i>ananatis</i>	NCPPB 3619	<i>Ananas comosus</i>	Tailand
<i>stewartii</i> subsp. <i>indologens</i>	NCPPB 2280 (type)	<i>Setaria italica</i>	India
<i>stewartii</i> subsp. <i>indologens</i>	NCPPB 2282	<i>Pennisetum glaucum</i>	India
<i>stewartii</i> subsp. <i>stewartii</i>	NCPPB 2295 (type)	<i>Zea mays</i>	USA
<i>stewartii</i> subsp. <i>stewartii</i>	NCPPB 3379	<i>Zea mays</i>	USA
<i>stewartii</i> subsp. <i>stewartii</i>	NCPPB 1553	<i>Zea mays</i>	USA
<i>stewartii</i> subsp. <i>stewartii</i>	NCPPB 3253	<i>Zea mays</i>	Italy
<b>Pectobacterium</b>			
<i>carotovorum</i> subsp. <i>carotovorum</i>	KACC 10057 (type)	<i>Solanum tuberosum</i>	Denmark
<i>carotovorum</i> subsp. <i>carotovorum</i>	KACC 10225	<i>Brassica rapa</i>	Korea
<i>carotovorum</i> subsp. <i>carotovorum</i>	KACC 10408	<i>Nicotiana tabacum</i>	Korea
<i>carotovorum</i> subsp. <i>carotovorum</i>	KACC 10421	<i>Raphanus sativus</i>	Korea
<i>carotovorum</i> subsp. <i>carotovorum</i>	KACC 10458	<i>Brassica rapa</i>	Korea
<b>Rathayibacter</b>			
<i>iranicus</i>	NCPPB 2253 (type)	<i>Triticum aestivum</i>	Iran
<i>rathayi</i>	NCPPB 2980 (type)	<i>Dactylis glomerata</i>	New Zealand
<i>tritici</i>	NCPPB 1857 (type)	<i>Triticum aestivum</i>	Egypt
<i>tritici</i>	NCPPB 1953	<i>Triticum aestivum</i>	Iran
<i>tritici</i>	NCPPB 255	<i>Triticum aestivum</i>	Egypt
<i>tritici</i>	NCPPB 471	<i>Triticum aestivum</i>	India
<i>toxicus</i>	NCPPB 3552 (type)	<i>Lolium rigidum</i>	Australia

<sup>a</sup>The (type) indicates type strain of the species or subspecies. <sup>b</sup>This strain with \* was isolated from seeds imported from China.

ing a NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

**Primer design by comparative genomic analysis.** To search for pathogen-specific genes of target bacterial pathogens, whole genome information of target bacteria and their closely related species was retrieved from National Center for Biotechnology Information (NCBI)

database (<ftp://ftp.ncbi.nlm.nih.gov/genomes/bacteria/>) at completed or scaffold level of assembly except for contig level and is listed in Table 2. We also used whole genome sequence information of *Rt* previously studied (Park et al., 2017). Based on the acquired genomic information, BLASTN searches were initially performed using 2,801 coding sequences (CDS) of *Cmn*, 4,942 CDS of *Pss* and 2,995 CDS of *Rt* as query against the constructed subject

**Table 2.** Genome sequences of bacterial strains used in this study

Strain	Accession	Size (Mb)	Assembly level	Release date	
<b>Clavibacter</b>					
<i>michiganensis</i> subsp. <i>sepedonicus</i>	ATCC33113	NC_010407.1 NC_010399.1 NC_010408.1	3.40	Completed	2008/02/12
<i>michiganensis</i> subsp. <i>michiganensis</i>	NCPPB 382	NC_009478.1-NC_009480.1	3.40	Completed	2007/05/18
<i>michiganensis</i> subsp. <i>nebraskensis</i>	NCPPB 2581	NC_020891.1	3.06	Completed	2013/04/01
<i>michiganensis</i> subsp. <i>insidiosus</i>	R1-1	NZ_CP011043.1-NZ_CP011046.1	3.41	Completed	2015/03/20
<i>michiganensis</i> subsp. <i>capsici</i>	PF008	NZ_CP011043.1-NZ_CP011046.1	3.24	Completed	2015/09/08
<b>Pantoea</b>					
<i>stewartii</i> subsp. <i>stewartii</i>	DC283	NZ_CP017581.1-NZ_CP017592.1	5.31	Completed	2017/04/10
<i>stewartii</i> subsp. <i>indologenes</i>	LMG 2632	JKO01000001-JKO01000035	4.68	Scaffolds	2014/09/23
<i>stewartii</i>	S301	NZ_LIU01000001-NZ_LIU01000027	4.49	Scaffolds	2015/10/13
<b>Rathayibacter</b>					
sp.	Leaf296	NZ_LMNR01000001-NZ_LMNR01000004	4.02	Scaffolds	2015/11/02
<i>tritici</i>	NCPPB 1953	NZ_CP015515.1	3.35	Completed	2016/05/09
<i>toxicus</i>	WAC3373	NZ_CP013292.1	2.35	Completed	2015/12/14
<i>toxicus</i>	70137	NZ_CP010848.1	2.33	Completed	2015/02/13

nucleotide sequences consisting of CDS of closely related species to the target bacterial pathogen. To eliminate genes highly matched to non-target bacteria, CDS with the lowest *e*-value < 0.1 were discarded, the filtered CDS were reran and searched against the NCBI's non-redundant (nr) DNA sequence database (Yu et al., 2010). The genes including non-matched CDS with *e*-value  $\geq$  0.05, query coverage and identity < 30% were selected as candidates of the target bacterial pathogen-specific genes.

To find a region to be amplified by PCR experiments, the specific genes more than 500 bp were compared individually to the nr database in the NCBI BLAST web site (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Subsequently, primers were designed from the unmatched region using Primer3 program (Rozen and Skaletsky, 2000). Finally, specificity of the designed candidate primer pairs were predicted *in silico* by searching against the nr sequences via Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>) of NCBI with default option.

**Polymerase chain reaction (PCR) and quantitative PCR (qPCR).** Primer pairs used for PCR and qPCR amplification reactions are listed in Supplementary Table 1. Thermal cycling for PCR was performed according to the following program: an initial denaturation for 5 min at 95°C, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at

the specified temperature (Supplementary Table 1) for 30 s, and a final extension at 72°C for 1 min. PCR reaction mixture included all required components in a 50  $\mu$ l of reaction volume with 150 ng of template genomic DNA. Negative control included PCR reaction without template DNA. When necessary for PCR sensitivity assay, template genomic DNA was serially diluted from 150 ng to 0.1 ng.

qPCR was performed with reaction mixture containing SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and various concentrations of template genomic DNA at the following program: an initial denaturation for 2 min at 95°C, followed by 39 cycles of denaturation at 95°C for 5 s, annealing at the specified temperature (Supplementary Table 1) for 20 s, and a final extension at 72°C for 30 min. The qPCR data were displayed using the CFX Manager Software ver. 3.1. Each reaction was performed in triplicate. qPCR result of individual gene was evaluated using the iCycler iQ Real-Time PCR Detection System. The  $C_t$  values of qPCR products of each gene were used to generate a standard curve against the number of gene copy (equivalent to the number of bacteria) used for qPCR (Supplementary Fig. 1-3). The number of gene (or genome) copy was obtained by dividing the amount of used DNA for qPCR (weight) with a molar weight of genomic DNA (genome size (bp)  $\times$  660 g/bp), and multiplying by  $6.02 \times 10^{23}$  molecules.

**PCR detection of bacteria on seeds.** To detect bacterial pathogen from seeds, we followed a previously described method with slight modification (An et al., 2015). Clean corn seeds and dehulled wheat seeds were purchased and used to detect bacterial pathogens in the seeds by PCR. The seeds were surface sterilized by immersing in 70% ethanol for 30 min and subsequently in 1% sodium hypochlorite solution for 10 min. Finally, the sterilized seeds were extensively washed 5 times with sterile distilled water, completely dried under a clean bench, and stored at 4°C until use. To artificially inoculate bacterial pathogens *Cmn*, *Pss*, *Rt*, 20 gram of surface sterilized corn seeds and 10 gram of wheat seeds were incubated by shaking at 100 rpm in a bacterial culture suspension (3 fold volume of seed volume with  $10^8$  cfu/ml) for 1 h. The seeds were harvested by removing the bacterial suspension and dried under clean bench prior to storage at 4°C. The surface sterilized seeds were used as negative controls.

To detect bacterial pathogens from the seeds, 10 gram of seeds was shaken at 180 rpm for 5 min in sterile distilled water containing a drop of Tween 20. The suspension was collected to determine the number of inoculated bacteria per seeds. Finally, the suspension was boiled at 95°C for 10 min, prior to PCR amplification to detect the presence of bacterial pathogen in the seeds.

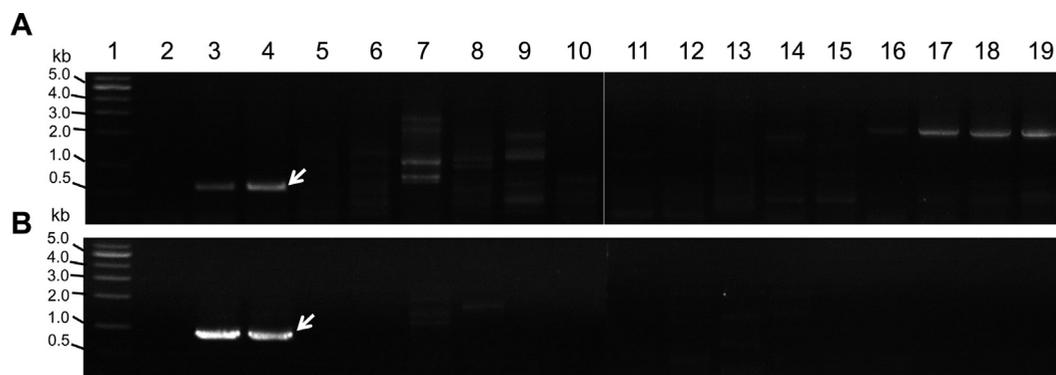
## Results and Discussion

**Candidate primers generated by comparative genomics and specific detection of bacterial pathogens.** Using comparative genomics among phylogenetically related

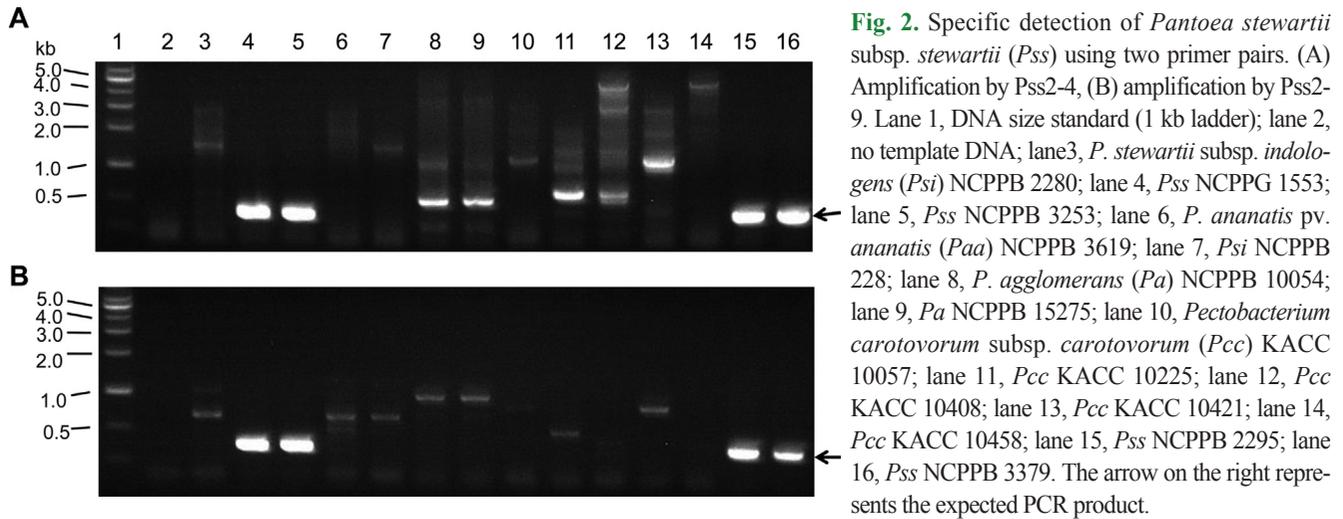
bacterial pathogens, *Cmn*, *Pss* and *Rt*, we generated 15-20 candidate primer pairs per bacterial pathogen to detect the corresponding bacterial pathogens (data not shown). Our first round of screening yielded the specific amplification of expected PCR products from the target bacterial pathogens (data not shown). All of the primer pairs which showed specific detection among the tested primers are listed in Table S1. Based on specificity and sensitivity, we further selected 2 primer pairs for each bacterial pathogen, namely: Cmn-3 (Fig. 1A) and Cmn-4 (Fig. 1B) for *Cmn*; Pss2-4 (Fig. 2A) and Pss2-9 (Fig. 2B) for *Pss*; Rt-4 (Fig. 3A) and Rt-5 (Fig. 3B) for *Rt*.

Since *Rt* was previously classified as a species of *Clavibacter* and separated from *Clavibacter* (Zgurskaya et al., 1993), we tested the primer pairs with *Clavibacter* species. Most of primer pairs designed by *Rt* genome information (Park et al., 2017) seem specific and neither detect the *Clavibacter* species nor the closely related *Rathayibacter* species such as *R. toxicus*, *R. iranicus* and *R. rathayi* (Fig. 3). *Pss* and many *Pantoea* species previously belonged to *Erwinia* species and were closely related to *Erwinia* species (Mergaert et al., 1993), which include *Pectobacterium carotovorum* (previously *E. carotovora*). Therefore, we tested *Pss* primer pairs with *Pantoea* species and *P. carotovorum* strains. PCR with Pss2-4 and Pss2-9 primer pairs resulted in the specific amplification from *Pss* strains which differed from other *Pantoea* species and *P. carotovorum* strains (Fig. 2).

Most of the primer pairs targeted amplification of genes encoding hypothetical proteins except Pss2-4, which amplified the gene encoding antiterminator Q of prophage of



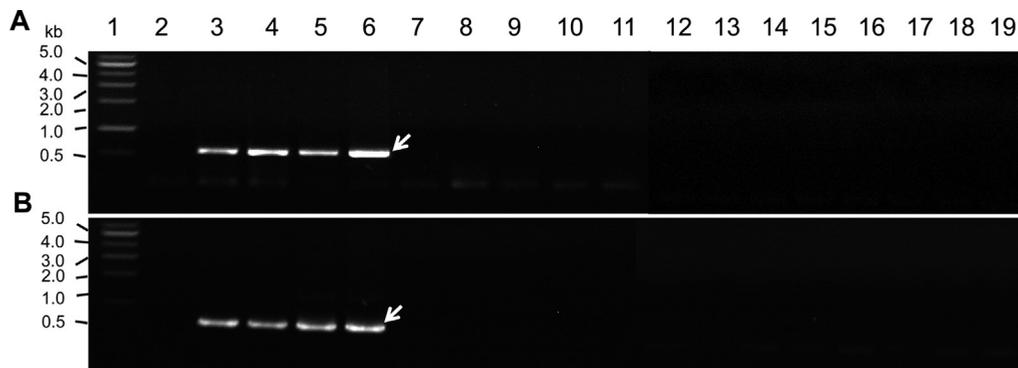
**Fig. 1.** Amplification of expected PCR product from *Clavibacter michiganensis* subsp. *nebraskensis* (*Cmn*) using two primer pairs. (A) Amplification by Cmn-3, (B) amplification by Cmn-4. Lane 1, DNA size standard (1 kb ladder); lane 2, no template DNA; lane 3, *Cmn* NCPPB 2581; lane 4, *Cmn* NCPPB 2582; lane 5, *Rathayibacter tritici* (*Rt*) NCPPB 255; lane 6, *Rt* NCPPB 471; lane 7, *R. toxicus* NCPPB 3552; lane 8, *R. rathayi* NCPPB 2980; lane 9, *R. iranicus* NCPPB 2253; lane 10, *C. michiganensis* subsp. *insidiosus* NCPPB 1109; lane 11, *C. michiganensis* subsp. *tesselarius* NCPPB 3664; lane 12, *Rt* NCPPB 1857; lane 13, *Rt* NCPPB 1953; lane 14, *C. michiganensis* subsp. *michiganensis* (*Cmm*) NCPPB LMG7333; lane 15, *Cmm* PPP1; lane 16, *C. michiganensis* subsp. *capsici* (*Cmc*) PF003; lane 17, *Cmc* PF005; lane 18, *Cmc* PS006; lane 19, *Cmc* PF008. The white arrow represents the expected PCR product.



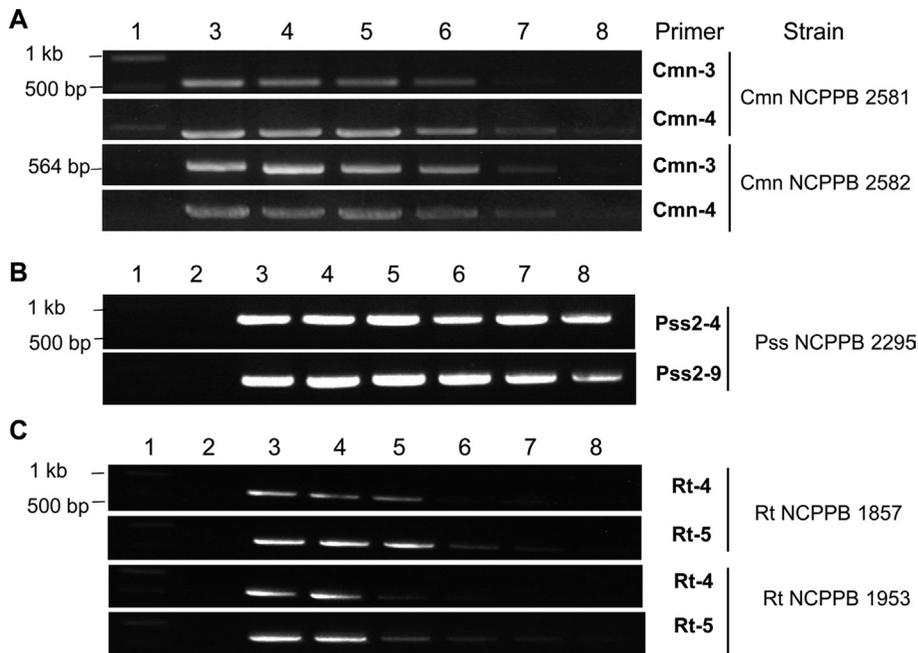
*Pss*. In case of the gene for antiterminator Q, it could be strain-specific and may not be considered suitable for specific detection. However, all of the 4 strains tested for *Pss* exhibited the specific amplification of the gene (Fig. 2A), suggesting that the gene encoding antiterminator Q would be a good marker for *Pss* detection. Other primer pairs which targeted the gene encoding hypothetical proteins should be carefully evaluated with more target bacterial strains. Genes encoding bacterial virulence determinants, such as toxins and plant cell wall degrading enzymes (CWDE), could serve as bacteria specific targets for detection. However, our preliminary comparative genome analysis indicated that genes for the virulence factors are not bacterial species or subspecies-specific, which can mislead for detection of bacterial pathogens from grains. In

fact, it was previously reported that several bacterial genes encoding toxins or CWDE are conserved among different bacterial species (Lee et al., 2016; Murray et al., 2017).

**Sensitivity of the selected primers.** While most of candidate primer pairs amplified the expected PCR products from target pathogens but not from other related bacterial strains, the sensitivity seemed to be variable from each primer pair against the target pathogens. Therefore, we tested sensitivity of the primer pairs using serially diluted templated DNA from the type strain of three bacterial pathogens *Cmn*, *Pss*, *Rt*, and an additional strain in the same species for *Cmn* and *Rt*. At the indicated PCR condition, primers *Cmn*-3 and *Cmn*-4 to detect *Cmn* (Fig. 4A), *Pss*2-4 and *Pss*2-9 to detect *Pss* (Fig. 4B), were highly sensitive to



**Fig. 3.** Specific amplification of PCR product from *Rathayibacter tritici* (*Rt*) using selected two primer pairs. (A) Amplification by *Rt*-4, (B) amplification by *Rt*-5. Lane 1, DNA size standard (1 kb ladder); lane 2, no template DNA; lane 3, *Rt* NCPPB 1857; lane 4, *Rt* NCPPB 1953; lane 5, *Rt* NCPPB 255; lane 6, *Rt* NCPPB 471; lane 7, *C. michiganensis* subsp. *nebraskensis* (*Cmn*) NCPPB 2581; lane 8, *Cmn* NCPPB 2582; lane 9, *R. toxicus* NCPPB 3552; lane 10, *R. rathayi* NCPPB 2980; lane 11, *R. iranicus* NCPPB 2253; lane 12, *C. michiganensis* subsp. *insidiosus* NCPPB 1109; lane 13, *C. michiganensis* subsp. *tesselarius* NCPPB 3664; lane 14, *C. michiganensis* subsp. *michiganensis* (*Cmm*) LMG 7333; lane 15, *Cmm* PPP1; lane 16, *C. michiganensis* subsp. *capsici* (*Cmc*) PF003; lane 17, *Cmc* PF005; lane 18, *Cmc* PS006; lane 19, *Cmc* PF008. The white arrow represents the expected PCR product.



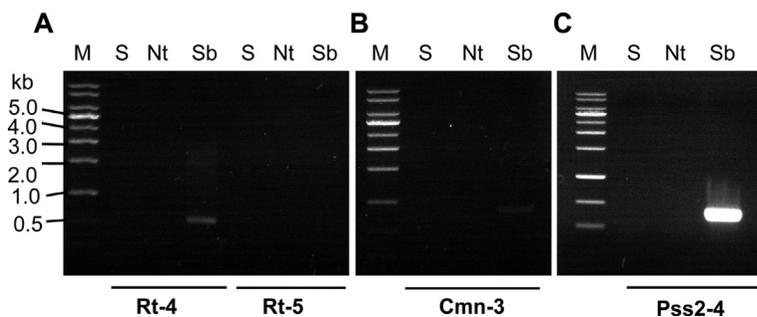
**Fig. 4.** Primer sensitivity assay using serially diluted genomic DNA of plant pathogenic bacteria. (A) Sensitive detection of *Clavibacter michiganensis* subsp. *nebraskensis* (*Cmn*), (B) sensitive detection of *Pantoea stewartii* subsp. *stewartii* (*Pss*), (C) sensitive detection of *Rathayibacter tritici* (*Rt*). Primer pairs and used bacterial strains of were indicated. Lane 1: DNA size standard; lane 2, no genomic DNA; lane 3, genomic DNA 150 ng; lane 4, genomic DNA 100 ng; lane 5, genomic DNA 50 ng; lane 6, genomic DNA 10 ng; lane 7, genomic DNA 1 ng; lane 8, genomic DNA 0.1 ng.

show apparent amplification at 0.1 ng of templated DNA ( $2 \times 10^{-3}$  ng/ $\mu$ l). However, primer pairs Rt-4 and Rt-5 showed less sensitivity to detect *Rt* at the equivalent amount of template DNA (Fig. 4C). Sensitivity of the primer pairs was further investigated by qPCR with ten-fold diluted DNA of target bacteria. The tested two primer pairs for each bacterium showed similar sensitivity against target bacteria (data not shown).

Overall, our specificity and sensitivity assay with candidate primer pairs generated from comparative genome analysis revealed that most of primers were specific to amplify the target gene from the corresponding pathogenic bacteria, suggesting the gene probing from genome-based analysis is a promising tool to provide pathogen specific markers. However, the sensitivity of primers to detect target pathogen varied, which will need additional work to select best primer pairs. It will be more complicated if we consider plant component and plant genome to detect bacterial pathogen resided inside of plant seeds.

#### Detection of bacterial pathogens in artificially inoculated seeds.

To test if the primer pairs developed in this study could be used to detect the target pathogen from seeds of corn and wheat effectively, we used seeds artificially inoculated with target bacteria. This is because all the three pathogens have not been reported in Korea, and seeds infected with *Cmn*, *Pss*, and *Rt* were not available. Among the primer pairs tested, three primer pairs, Rt-4, Cmn-3 and Pss2-4, exhibited the specific detection of bacteria from the artificially inoculated seeds (Fig. 5). The number of target bacteria on the corn seeds was enumerated for *Cmn* as  $8.8 \times 10^3$  cfu/g seed, for *Pss* as  $7.7 \times 10^4$  cfu/g seed, and for *Rt* on wheat seeds as  $7.84 \times 10^4$  cfu/g seed. These results indicated that the selected primer pairs can detect target bacterial pathogens that are inhabited on the surface of seeds at the level of  $10^3$  to  $10^4$  cells per gram of seeds. No amplification of specific bacteria was detected from the sterilized seeds without bacterial inoculation or seeds not surface sterilized (Fig. 5). The seeds were randomly pur-



**Fig. 5.** Specific detection of bacteria from the artificially inoculated seeds. (A) Detection of *Rathayibacter tritici* by Rt-4 on wheat seeds, (B) detection of *Clavibacter michiganensis* subsp. *nebraskensis* by Cmn-3 on corn seeds, (C) detection of *Pantoea stewartii* subsp. *stewartii* by Pss2-3 on corn seeds. Lane M, DNA size standard (1 kb ladder); S, seeds original; Nt, surface sterilized seeds; Sb, seeds infected with bacteria.

chased from market and it turned out they might be target pathogen free.

Although we inoculated seeds of corn or wheat with either *Cmn*, *Pss*, or *Rt*, this is not natural assay because when bacteria infest the seeds in natural disease occurrence, the seeds may be both internally and externally contaminated (Rane and Latin 1992; Tancos et al., 2013). This assay was primarily performed using bacterial cells harvested from the surface but not from the total DNA isolated from grinded seeds. If infested seeds harbour pathogenic bacteria mostly inside the seeds, we require total DNA from the grinded seeds as a template, which will interfere with the specificity and sensitivity of primers. This should further be extensively tested with the naturally infected seeds, which was not possible to do in this study because of strict regulations to handle target bacterial pathogen in Korea.

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