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Development of PCR and TaqMan PCR Assays to Detect *Pseudomonas coronafaciens*, a Causal Agent of Halo Blight of Oats

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Pseudomonas coronafaciens **causes halo blight on oats and is a plant quarantine bacterium in many countries, including the Republic of Korea. Using of the certificated seed is important for control of the disease. Since effective detection method of *P. coronafaciens* is not available yet, PCR and TaqMan PCR assays for specific detection of *P. coronafaciens* were developed in this study. PCR primers were designed from the draft genome sequence of *P. coronafaciens* LMG 5060 which was obtained by the next-generation sequencing in this study. The PCR primer set Pc-12-F/Pc-12-R specifically amplified 498 bp from the 13 strains of *P. coronafaciens* isolated in the seven different countries (Canada, Japan, United Kingdom, Zimbabwe, Kenya, Germany, and New Zealand) and the nested primer set Pc-12-ne-F/Pc-12-ne-R specifically amplified 298 bp from those strains. The target-size PCR product was not amplified from the non-target bacteria with the PCR and nested primer sets. TaqMan PCR with Pc-12-ne-F/Pc-12-ne-R and a TaqMan probe, Pc-taqman, which were designed inside of the nested PCR amplicon, generated Ct values which in a dose-dependent manner to the amount of the target DNA and the Ct values of all the *P. coronafaciens* strains were above the threshold Ct value for positive detection. The TaqMan PCR generated positive Ct values from the seed extracts of the artificially inoculated oat seeds above 10 cfu/ml inoculation level. PCR and TaqMan PCR assays developed in this study will be useful tools to detect and identify the plant quarantine pathogen, *P. coronafaciens*.**

Keywords **:** detection, halo blight, oat, PCR, TaqMan PCR

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Halo blight, an important disease of the oats is caused by *Pseudomonas coronafaciens* (Elliott, 1920; Young et al., 1978) and occurs in relatively cool and moist climates leading to substantial economic losses (Marten et al., 1984). It produces light green, oval spots on the leaves of the plant with dark water-soaked centers (Marten et al., 1984; Harder and Haber, 1992; Wallwork, 1992). *P. coronafaciens* survives on plant debris, soil and seeds (Martens et al., 1984). One of the effective control measures of the disease is prevention of pathogen transfer to the other plants using of the certificated seed (Collins, 2010). Also, *P. coronafaciens* is a plant quarantine bacterium in many countries, including the republic of Korea. For the certificated seed program and plant quarantine, the specific and effective detection method must be available.

Various tests can be used to detect the seed-borne pathogens, such as plating on selective media, ELISA, seedling grow-out tests, PCR, real-time PCR and DNA microarrays (Walcott, 2003). Among these techniques, plating on selective media and seedling grow-out tests are laborious and time-consuming and the PCR assay is more sensitive and time-saving than ELISA (Cho et al., 2010). In particular, the TaqMan PCR assay, which detects microorganisms quantitatively with a TaqMan probe, has been shown to be successful in detection and identification of seed-borne pathogens (Bella et al., 2008; Schena et al., 2004; Finetti-Sialer and Ciancio, 2005). For *P. coronafaciens,* however, effective detection method from oat seeds is not available. Any PCR assay for the specific detection of *P. coronafaciens* has not been published yet.

In this study highly specific PCR and TaqMan PCR assays for detection of *P. coronafaciens* have been developed. For development of the *P. coronafaciens* - specific PCR assays, information for the unique nucleotide sequence of *P. coronafaciens* must be available. Since there are not many gene sequences of *P. coronafaciens* available in the GenBank, in the present study, next-generation sequencing analysis was used to obtain a whole draft genome

sequence of *P. coronafaciens*. From the genome sequence, PCR primers were designed for the PCR and TaqMan PCR assays which were specific for the *P. coronafaciens*.

Materials and Methods

Bacterial strains and cultures. Strains of *P. coronafaciens* isolated in Canada, Japan, United Kingdom, Zimbabwe, Kenya, Germany and New Zealand were obtained from LMG (Belgian Coordinated Collection of Microorganisms, Laboratory of Microbiology, University of Gent, Belgium) and from KACC (Korean Agricultural Culture Collection, Rural Develpoment Administration, the Republic of Korea). For the non-target bacteria, strains related to *P. coronafaciens* in genus *Pseudomonas* or the representative strains of common plant pathogenic bacteria in genus *Acidovorax*, *Pectobacterium*, *Ralstonia*, *Rhodococcus*, *Xanthomonas*, and *Clavibacter* were collected from the various culture collections (Table 1). The bacteria were routinely grown in nutrient agar media containing 8 g of

Table 1. List of *Pseudomonas coronafaciens* strains, the non-target bacterial strains of *Pseudomonas* spp. and other plant-pathogenic bacterial strains used in this study

Strain[ab]		Host[c]	Origin[c]	Year[c]
Acidovorax				
avenae subsp. *avenae*	NCPPB 1011	*Zea mays*	USA	1958
Clavibacter				
michiganensis subsp. *incidiosus*	NCPPB 1020	*Medicago sativa*	Canada	nk
michiganensis subsp. *michiganensis*	NCPPB 1064	*Lycopersicon esculentum*	Italy	1961
michiganensis subsp. *sepedonicus*	NCPPB 2137	*Solanum tuberosum*	Canada	nk
Pectobacterium				
carotovorum subsp. *carotovorum*	NCPPB 312	*Solanum tuberosum*	Denmark	nk
Pseudomonas				
coronafacines	KACC 12133	nk	nk	nk
	KACC 13262	nk	nk	nk
	LMG 2170	*Bromus* sp.	Canada	1962
	LMG 2330	nk	nk	1966
	LMG 5030	*Lolium multiflorum*	Japan	1967
	LMG 5060[b]	*Avena sativa*	United Kingdom	1958
	LMG 5061	*Secale cereale*	Canada	1962
	LMG 5081	*Avena sativa*	Zimbabwe	1971
	LMG 5380	*Avena sativa*	Kenya	1970
	LMG 5449	*Avena sativa*	Germany	1959
	LMG 5452	*Avena sativa*	New Zealand	1969
	LMG 5536	*Avena sativa*	United Kingdom	1965
	LMG 13190	*Avena sativa*	nk	nk
savastanoi pv. *glycinea*	NCPPB 1134	*Glycine javanica*	Zimbabwe	1961
savastanoi pv. *phaseolicola*	KACC 10575	*Phaseolus vulgaris*	Poland	nk
savastanoi pv. *savastanoi*	NCPPB 639	*Olea europaea*	Yugoslavia	nk
syringae pv. *actinidiae*	KACC 10582	*Actinidia chinensis*	Rep. of Korea	1999
syringae pv. *antirrhini*	ICMP 4303	*Antirrhinum majus*	United Kingdom	1965
syringae pv. *aptata*	DSM 50252	*Beta vulgaris*	nk	nk
syringae pv. *atrofaciens*	LMG 5095	*Triticum aestivum*	New Zealand	1968
syringae pv. *berberidis*	NCPPB 2724	*Berberis* sp.	New Zealand	1972
syringae pv. *ciccaronei*	NCPPB 2355	*Ceratonia siliqua*	nk	1969
syringae pv. *delphinii*	ICMP 529	*Delphinium* sp.	New Zealand	1957
syringae pv. *dysoxyli*	ICMP 545	*Dysoxylum spectabile*	New Zealand	1949
syringae pv. *eriobotryae*	NCPPB 2331	*Eriobotrya japonica*	United Kingdom	1970
syringae pv. *helianthi*	NCPPB 1229	*Helianthus annuus*	Zambia	1962

Table 1. Continued

Strain[ab]		Host[c]	Origin[c]	Year[c]
syringae pv. *japonica*	ICMP 6305	*Hordeum vulgare*	Japan	1951
syringae pv. *lachrymans*	ATCC 11965	*Cucumis sativus*	nk	nk
syringae pv. *lapsa*	ATCC 10859	*Triticum aestivum*	nk	1978
syringae pv. *maculicola*	ICMP 3935	*Brassica oleracea*	New Zealand	1965
syringae pv. *mellea*	ICMP 5711	*Nicotiana tabacum*	Japan	1968
syringae pv. *mori*	ICMP 4331	*Morus alba*	Hungary	1958
syringae pv. *morsprunorum*	ICMP 5795	*Prunus domestica*	nk	nk
syringae pv. *myricae*	ICMP 7118	*Myrica rubra*	Japan	1978
syringae pv. *panici*	NCPPB 1498	*Panicum* sp.	nk	nk
syringae pv. *papulans*	ICMP 4040	*Malus domestica*	USA	nk
syringae pv. *passiflorae*	NCPPB 1386	*Passiflora edulis*	New Zealand	1962
syringae pv. *persicae*	NCPPB 2761	*Prunus persica*	France	1974
syringae pv. *pisi*	ICMP 4433	*Pisum sativum*	Canada	1946
syringae pv. *ribicola*	NCPPB 963	*Ribes aureum*	nk	nk
syringae pv. *sesame*	NCPPB 1016	*Sesamum indicum*	Yugoslavia	nk
syringae pv. *syringae*	NCPPB 388	*Oryza sativa*	Hungary	nk
syringae pv. *tabaci*	ICMP 2835	*Nicotiana tabacum*	Hungary	1959
syringae pv. *tagetis*	ICMP 4091	*Tagetes erecta*	Zimbabwe	1972
syringae pv. *tomato*	NCPPB 2683	*Lycopersicon esculentum*	New Zealand	1972
syringae pv. *ulmi*	NCPPB 632	*Ulmus* sp.	Yugoslavia	1958
Ralstonia				
Solanacearum	NCPPB 339	*Solanum tuberosum*	Israel	nk
Rhizobium				
radiobacter	DSM 30205	*Malus* sp.	nk	1972
rhizogenes	ATCC 11325	*Malus domestica*	nk	nk
rubi	NCPPB 1854	*Rubus ursinus* var. *loganobaccus*	USA	1942
vitis	NCPPB 3554	*Vitis vinifera*	Australia	1977
Rhodococcus				
fascians	LMG 3601	*Lillium speciosum* cv. Rubrum	Belgium	nk
Xanthomonas				
campestris pv. *campestris*	KACC 10377	*Brassica oleracea* var. *capitata*	Rep. of Korea	nk
campestris pv. *vesicatoria*	KACC 11157	*Capsicum annuum*	Rep. of Korea	1999
oryzae pv. *oryzae*	KACC 10331	*Oryza sativa*	Rep. of Korea	nk

[a]ATCC, American Type Culture Collection, USA; DSM, DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany; ICMP, International Collecti on of Micro-organisms from Plants, Landcare Research, New Zealand; KACC, Korean Agricultural Culture Collection, Rural Development Administration, Rep. of Korea; LMG, Collection of the Laboratorium voor Microbiologie en Microbiele Genetica, Ghent University, Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria, United Kingdom.
[b]This strains was used for genome sequencing by NGS.
[c]nk, not known.

nutrient broth and 15 g of agar per liter.

Genome sequencing and PCR primer design. Whole genome shotgun sequencing of *P. coronafaciens* LMG 5060 was performed using the Roche/454 pyrosequencing method on a genome sequencer with FLX titanium (Margulies et al., 2005). ORF prediction was performed with Glimmer v. 3.02 (Delcher et al., 2007), Prodigal (Hyatt et al., 2010), and GeneMark.hmm-P (Lukashin and Borodovsky, 1998). ORFs from the draft genome sequence of *P. coronafaciens* LMG 5060 with more than 500 base pairs were BLASTed in the gene bank. To design the primers, candidate ORFs were selected from the ORFs with lower than 80% nucleotide homology to any known genes. The PCR primers were designed with Primer 3 (v. 0.4.0). The specificity of the primers for *P. coronafaciens* was analyzed by PCR. Nested

primers and the nucleotide sequence for the TaqMan probe were designed from amplicon sequence of *P. coronafaciens*-specific primers. All the primers and the TaqMan probe used in the study were synthesized by Bioneer Co., Ltd (Daejeon, Korea) or NeoProbe Co., Ltd. (Daejeon, Korea). The TaqMan probe was labeled at the 5′ end with FAM and at the 3′ end with TAMRA.

PCR and TaqMan PCR assays. The genomic DNA was extracted from the bacterial cells using the GeneAll Exgene™ Cell SV kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's protocol. DNA concentrations were determined with a Qubit™ fluorometer (Invitrogen®, Carlsbad, CA, USA).

The PCR assays used 1 µl of template DNA in a 25 µl reaction mixture containing 2 mM of Tris-HCl (pH 8.0), 10 mM of KCl, 10 uM of EDTA, 100 uM of DTT, 0.05% Tween 20, 0.05% Nonidet P-40, 5% glycerol, 2.5 mM of dNTP, 5 units of Ex Taq DNA (TaKaRa Bio Inc., Shiga, Japan), and 10 pM of each primer. The reactions were performed in a T Gradient Thermal Cycler (Biometra, Göttingen, Germany) programmed for one cycle of 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, with a final extension step for 7 min at 72°C. The amplicons were analyzed by electrophoresis in a 2% agarose gel in TAE buffer.

The TaqMan PCR assay was carried out using 2 µl of DNA template, 8.5 µl of Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan), 1 µl of TaqMan probe (10 pmoles/µl), 0.5 µl (10 pmoles/µl) of each nested primer, and 12.5 µl of DEPC-DW (Bioneer, Daejeon, Korea). Real-time PCR amplifications were performed in a Smart Cycler™ System (Cepheid, Sunnyvale, CA, USA) with 40 cycles at 95°C for 30 sec and 60°C for 20 sec after initial incubation for 30 sec at 95°C.

Oat seed preparation and extraction. Prior to inoculation, the oat seeds (cultivar Samhan, provided by the National Honam Agricultural Experiment Station in Korea) were surface sterilized by soaking in 70% ethanol for 30 minute and 1.2% sodium hypochlorite solution for 1 hour. The oats seeds were washed 3 times with sterilized water and dried completely in clean bench. Twelve grams of the surface-sterilized oat seeds were artificially inoculated by shaking (120 rpm) in 45 ml of *P. coronafaciens* LMG 5060 suspension for 24 h. The seeds were dried by leaving them in a clean bench for 20 h. The bacterial pathogen was extracted from the artificially inoculated oat seeds as described previously (Maes et al., 1996). The seeds (12 g) were shaken in 12 ml of cold aqueous saline (0.85% NaCl) containing a drop of Tween 20 for 5 min on a rotator shaker (180 rpm) at room temperature. The extracts were treated at 95°C for 7 min and used in the PCR assay or stored at –20°C.

Results

Primer and PCR specificity. To obtain specific primers of the *P. coronafaciens*, the draft genome sequence of *P. coronafaciens* LMG 5060 was constructed by genome shotgun sequencing in this study. The draft genome sequence was deposited at NCBI GenBank (accession number: JSED00000000). The 36 primer sets were designed from the ORFs in total 2028 contigs which showed low homology to that of known genes in GenBank and the primer specificities for *P. coronafaciens* were checked by PCR assays with target and non-target bacterial strains. Among the primers tested, the Pc-12-F (5′-GATTGCGTCATATGCAACAT-3′) and Pc-12-R (5′-AATAGCAGATCCAGCCAAAG-3′) primer sets amplified a 498 bp in all 13 strains of *P. coronafaciens* (Fig. 1), whereas target-size DNA was not amplified in non-target bacteria, including 30 *P. syringae* pathovars, 3 *P. savastanoi* pathovars, and 16 other pathogenic bacteria (Fig. 2). The nested primers, Pc-12-ne-F (5′-AACGACGGGCTGCAGTTTAT-3′) and Pc-12-ne-R (5′-AACGTGATAGCAGCCCCACT-3′), were designed from the Pc-12-F/Pc-12-R amplicon. The PCR assay was conducted with the Pc-12-ne-F/Pc-12-ne-R nested primer set and genomic DNA amplified the 298 bp in all 13 strains of *P. coronafaciens* (supplement Fig. 1), whereas target-size DNA was not amplified in any non-target bacteria, including 30 *P. syringae* pathovars, 3 *P. savastanoi* path-

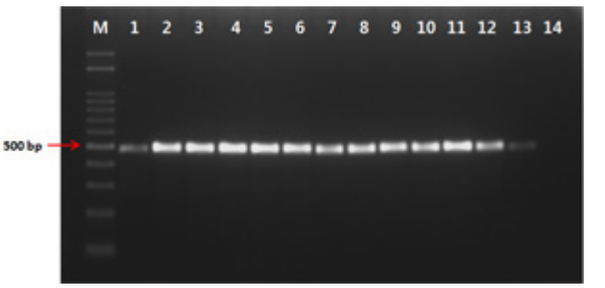

Fig. 1. Gel electrophoresis of the polymerase chain reaction products formed with primer Pc-12-F/Pc-12-R and bacterial DNA of *Pseudomonas coronafaciens* strains. Lanes 1~13, *P. coronafaciens* LMG 5060, KACC 13262, KACC 12133, LMG 2170, LMG 5030, LMG 5061, LMG 5081, LMG 5380, LMG 5449, LMG 5452, LMG 5536, LMG 13190, LMG 2330; lane 14, water as a negative control.

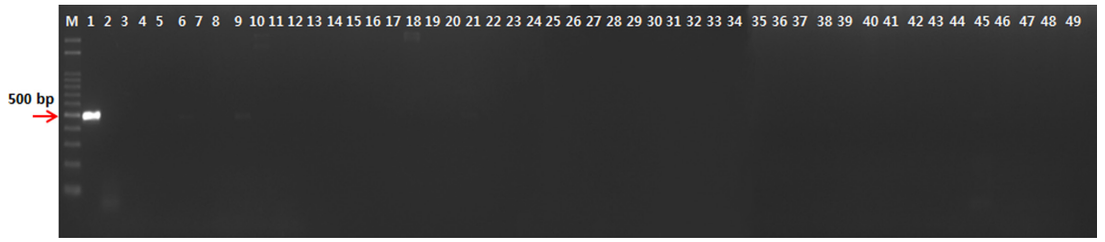

Fig. 2. Gel electrophoresis of the polymerase cain reaction products formed with primer Pc-12-F/Pc-12-R and total DNA of lane 1, *Pseudomonas coronafaciens* LMG 5060, lanes 2–31, *P. syringae* pvs, *actinidiae* KACC 10582, *antirrhini* ICMP 4303, *aptata* DSM 50252, *atrofaciens* ICMP 4394, *berberidis* NCPPB 2724, *ciccaronei* NCPPB 2355, *delphhinii* ICMP 529, *dysoxyli* ICMP 545, *eriobotyae* NCPPB 2331, *helianthi* NCPPB 1229, *japonica* ICMP 6305, *lachrymans* ATCC 11965, *lapsa* ATCC 10859, *maculicola* ICMP 3935, *mellea* ICMP 5711, *mori* ICMP 4331, *morsprunorum* ICMP 5795, *myricae* ICMP 7118, *panici* NCPPB 1498, *papulans* ICMP 4040, *passiflorae* NCPPB 1386, *persicae* NCPPB 2761, *pisi* ICMP 4433, *ribicola* NCPPB 963, *sesami* NCPPB 1016, *syringae* NCPPB 388, *tabaci* ICMP 2835, *tagetis* ICMP 4091, *tomato* NCPPB 2683, *ulmi* NCPPB 632; lanes 32~34, *P. savastanoi* pvs. *glycinea* NCPPB 1134, *pahseolicola* KACC 10575, and *savastanoi* NCPPB 639; lane 35, *Acidovorax avenae* subsp. *avenae* NCPPB 1011; lanes 36–38, *Clavibacter michiganensis* subsp. *insidiosus* NCPPB 1020, *michiganensis* NCPPB 1064, *sepedonicus* NCPPB 2137; lane 39, *Pectobacterium carotovorum* subsp. *carotovorum*, NCPPB 312; lanes 40–43, *Rhizobium radiobacter* DSM 30205, *R. rhizogenes* ATCC 11325, *R. rubi* NCPPB 1854, *R. vitis* NCPPB 3554; lane 44, *Rhodococcus fascians* LMG 3601; lane 45, *Ralstonia solanacearum* NCPPB 339; lanes 46–47, *Xanthomonas campestris* pvs. *campestris* KACC 10377, *vesicatoria* KACC 11157; lane 48, *X. oryzae* pv. *oryzae* KACC 10331; lane 1, *P. coronafaciens* LMG 5060, as a positive control; lane 49, water as a negative control.

ovars, and 16 other pathogenic bacteria (Supplement Fig. 2).

TaqMan PCR specificity. To reduce the rate of false-positives and quantitatively detect *P. coronafaciens*, a TaqMan probe, Pc-taqman (5′-TGAAACCGCCGAAACGGTCT-3′), was designed from the sequence of the Pc-12-ne-F/Pc-12-ne-R amplicon. The real-time PCR with Pc-12-ne-F/Pc-12-ne-R and Pc-taqman generated Ct values in a dose-dependent for 10 ng-10 pg of *P. coronafaciens* LMG 5060' genomic DNA. The r^2 of the linear regression was 0.994 (Fig. 3A).

The specificity of the TaqMan PCR was checked with the target and non-target bacteria. In the TaqMan PCR assays, 10-fold diluted DNAs (10 ng to 10 pg) of *P. coronafaciens* LMG 5060 were used as standard, and 10 ng of DNAs of the other bacterial strains were used. The Ct values of the 13 *P. coronafaciens* strains were 22–28 cycles (Fig. 3B and Table 2), whereas the average Ct values of the DNA of 49 non-target bacterial strains were more than 37 cycles (Table 2). The Ct values of the target and non-target bacteria were well separated into two different groups.

TaqMan PCR detection of *P. coronafaciens* from the artificially inoculated oat seeds. The TaqMan PCR assay developed in this study was applied to detect *P. coronafaciens* from the oat seeds. Since the seeds naturally infested

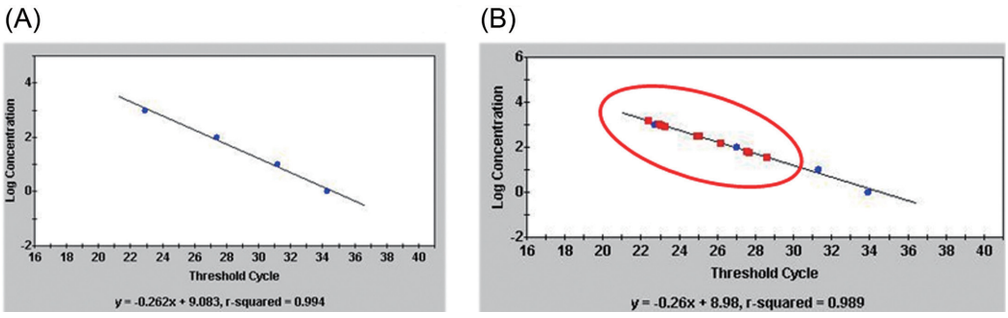

Fig. 3. Sensitivity and specificity of the TaqMan real-time PCR with primer, Pc-12-ne-F/Pc-12-ne-R and TaqMan probe, Pc-taqman. (A) The linear regression generated by ten-fold dilution of DNA of *Pseudomonas coronafaciens* LMG 5060 and (B) TaqMan PCR with DNAs (circle dot) of *P. coronafaciens* LMG 5060, and *P. coronafaciens* strains (square dot): *P. coronafaciens* KACC 13262, KACC 12133, LMG 2170, LMG 5030, LMG 5061, LMG 5081, LMG 5380, LMG 5449, LMG 5452, LMG 5536, LMG 13190, and LMG 2330.

Table 2. The Ct values of *Pseudomonas coronafaciens* strains, the non-target bacterial strains and the seed extracts from artificially inoculated oat seeds with *Pseudomonas coronafaciens* LMG 5060 which were obtained by the TaqMan PCR assays

Samples		DNA conc.	Mean Ct[a]
Pseudomonas coronafaciens	LMG 5060 (STD)	10 ng	22.8±0.1
		1 ng	28.7±2.5
		100 pg	31.3±1.5
		10 pg	36.5±3.3
	KACC 12133	10 ng	22.5±0.1
	KACC 13262	10 ng	22.7±0.2
	LMG 2170	10 ng	23.3±0.1
	LMG 2330	10 ng	27.1±0.7
	LMG 5030	10 ng	22.8±0.3
	LMG 5061	10 ng	28.0±1.0
	LMG 5081	10 ng	25.8±2.1
	LMG 5380	10 ng	24.7±0.4
	LMG 5449	10 ng	25.1±0.4
	LMG 5452	10 ng	25.5±2.0
	LMG 5536	10 ng	23.5±0.6
	LMG 13190	10 ng	27.9±1.5
Non-target bacterial strains			
Acidovorax avenae subsp. *avenae*	NCPPB 1011	10 ng	39.1±1.7
Clavibacter michiganensis subsp. *incidiosus*	NCPPB 1020	10 ng	> 40
Clavibacter michiganensis subsp. *michiganensis*	NCPPB 1064	10 ng	> 40
Clavibacter michiganensis subsp. *sepedonicus*	NCPPB 2137	10 ng	> 40
Pectobacterium carotovorum subsp. *carotovorum*	NCPPB 312	10 ng	> 40
Pseudomonas savastanoi pv. *glycinea*	NCPPB 1134	10 ng	> 40
Pseudomonas savastanoi pv. *phaseolicola*	KACC 10575	10 ng	39.5±0.9
Pseudomonas savastanoi pv. *savastanoi*	NCPPB 639	10 ng	> 40
Pseudomonas syringae pv. *actinidiae*	KACC 10582	10 ng	> 40
Pseudomonas syringae pv. *antirrhini*	ICMP 4303	10 ng	> 40
Pseudomonas syringae pv. *aptata*	DSM 50252	10 ng	> 40
Pseudomonas syringae pv. *atrofaciens*	LMG 5095	10 ng	> 40
Pseudomonas syringae pv. *berberidis*	NCPPB 2724	10 ng	37.8±1.9
Pseudomonas syringae pv. *ciccaronei*	NCPPB 2355	10 ng	39.6±0.8
Pseudomonas syringae pv. *delphinii*	ICMP 529	10 ng	39.5±0.9
Pseudomonas syringae pv. *dysoxyli*	ICMP 545	10 ng	39.3±1.3
Pseudomonas syringae pv. *eriobotryae*	NCPPB 2331	10 ng	> 40
Pseudomonas syringae pv. *helianthi*	NCPPB 1229	10 ng	39.9±0.2
Pseudomonas syringae pv. *japonica*	ICMP 6305	10 ng	> 40
Pseudomonas syringae pv. *lachrymans*	ATCC 11965	10 ng	> 40
Pseudomonas syringae pv. *lapsa*	ATCC 10859	10 ng	> 40
Pseudomonas syringae pv. *maculicola*	ICMP 3935	10 ng	> 40
Pseudomonas syringae pv. *mellea*	ICMP 5711	10 ng	38.7±1.3
Pseudomonas syringae pv. *mori*	ICMP 4331	10 ng	> 40
Pseudomonas syringae pv. *morsprunorum*	ICMP 5795	10 ng	> 40
Pseudomonas syringae pv. *myricae*	ICMP 7118	10 ng	> 40
Pseudomonas syringae pv. *panici*	NCPPB 1498	10 ng	> 40
Pseudomonas syringae pv. *papulans*	ICMP 4040	10 ng	> 40
Pseudomonas syringae pv. *passiflorae*	NCPPB 1386	10 ng	39.7±0.6
Pseudomonas syringae pv. *persicae*	NCPPB 2761	10 ng	> 40

Table 2. Continued

Samples		DNA conc.	Mean Ct[a]
Pseudomonas syringae pv. *pisi*	ICMP 4433	10 ng	> 40
Pseudomonas syringae pv. *ribicola*	NCPPB 963	10 ng	> 40
Pseudomonas syringae pv. *sesami*	NCPPB 1016	10 ng	> 40
Pseudomonas syringae pv. *syringae*	NCPPB 388	10 ng	> 40
Pseudomonas syringae pv. *tabaci*	ICMP 2835	10 ng	> 40
Pseudomonas syringae pv. *tagetis*	ICMP 4091	10 ng	> 40
Pseudomonas syringae pv. *tomato*	NCPPB 2683	10 ng	> 40
Pseudomonas syringae pv. *ulmi*	NCPPB 632	10 ng	39.8±0.3
Ralstonia solanacearum	NCPPB 339	10 ng	> 40
Rhizobium radiobacter	DSM 30205	10 ng	> 40
Rhizobium rhizogenes	ATCC 11325	10 ng	> 40
Rhizobium rubi	NCPPB 1854	10 ng	> 40
Rhizobium vitis	NCPPB 3554	10 ng	> 40
Rhodococcus fascians	LMG 3601	10 ng	> 40
Xanthomonas campestris pv. *campestris*	KACC 10377	10 ng	> 40
Xanthomonas campestris pv. *vesicatoria*	KACC 11157	10 ng	> 40
Xanthomonas oryzae pv. *oryzae*	KACC 10331	10 ng	> 40
Seed extracts from oat seeds inoculated in			
10^8 cfu/ml cell suspension		–	21.2±0.5
10^7 cfu/ml cell suspension		–	21.4±0.2
10^6 cfu/ml cell suspension		–	21.3±0.2
10^5 cfu/ml cell suspension		–	22.5±0.5
10^4 cfu/ml cell suspension		–	24.2±0.7
10^3 cfu/ml cell suspension		–	25.1±1.1
10^2 cfu/ml cell suspension		–	29.2±0.3
10 cfu/ml cell suspension		–	32.2±0.7
control (water treatment)		–	> 40

with the pathogen were not available, the artificially inoculated oat seeds were used. TaqMan PCR with the seed extracts generated a range of mean Ct values from 21.2 for 10^8 cfu/ml-inoculated seed extracts to 32.2 for 10 cfu/ml-inoculated seed extracts (Table 2). Since Ct values of all the non-target bacteria used in this study were larger than 37 cycles, the TaqMan PCR can detect *P. coronafaciens* from the seed extract of the artificially inoculated oat seeds above 10 cfu/ml inoculation level.

Estimation of the recovery number of P. coronafaciens from the artificial inoculated seed extract was tried with several different ways, but the accurate enumeration of *P. coronafaciens* from the seed extract failed because the many non-target-shape colonies were cultured on the culture plate and the selective medium for *P. coronafaciens* was not available. Surface sterilization of the oat seeds prior to artificial inoculation could not prevent growth of some non-target bacteria on the recovery culture plates.

Discussion

Highly specific PCR and TaqMan PCR assays have been developed in this study to detect *P. coronafaciens*, the halo blight pathogen of oats. Since *P. coronafaciens* is a plant quarantine bacterium in many countries and using of the certificated seed is important for the disease control, developments of these assays are significant for the management of halo blight of oats.

To obtain specific primers of the *P. coronafaciens*, a genome-wide search was conducted from the draft genome sequence of *P. coronafaciens* which was constructed by genome shotgun sequencing in this study. A specific primer, Pc-12-F/Pc-12-R, was designed from ORF 7 in contig 7 of the draft genome sequence of *P. coronafaciens* (NCBI GenBank accession number: JSED00000000). ORF 7 was 1341 nucleotides long, and no significant homologous gene was found in the NCBI GenBank database. PCRs with Pc-

12-F/Pc-12-R and Pc-12-ne-F/Pc-12-ne-R generated the target size DNA from all 13 strains of *P. coronafaciens* isolated in seven countries. The target-size DNA was not amplified in 49 strains of non-target bacteria. The Ct values of the target and non-target bacteria were well separated in the TaqMan PCR. The homology of the ORFs in which PCR was designed and the results of the PCR and TaqMan PCR indicate that the primer sequences and PCR assays developed in this study are highly specific to *P. coronafaciens*.

TaqMan PCR was applied to detect the target pathogen from artificially inoculated oat seeds. TaqMan PCR generated the *P. coronafaciens*–positive Ct values in the seed extracts obtained from oat seeds inoculated in 10 cfu/ml and above. Although detection sensitivity of this TaqMan PCR cannot compared to the previously published results because detection of *P. coronafaciens* from oat seeds has not been published, positive detection of the seed extracts obtained from oat seeds inoculated in 10 cfu/ml *P. coronafaciens* LMG 5060 suspension and above is thought to be a quite high sensitivity and can apply to seed test and plant quarantine service.

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