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Primers for specific detection and identification of *Pseudomonas syringae* pv. *maculicola* and *P. cannabina* pv. *alisalensis*

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Abstract

Bacterial leaf spot and bacterial leaf blight are global threats to the cultivation of cruciferous vegetables, and it is necessary to develop methods to easily detect, identify, and distinguish the causative pathogens *Pseudomonas syringae* pv. *maculicola* (*Psm*) and *P. cannabina* pv. *alisalensis* (*Pca*). Here, we used the sequence specificity of the exchangeable effector loci flanking the *hrp* gene cluster to design primers that can help detect and discriminate between *Psm* and *Pca*. Primers common to both bacteria (hrpK_fw1 and hrpK_fw2) were designed within *hrpK* at the end of the *hrp* gene cluster. *Psm*-specific primers (MAC_rv1 and MAC_rv2) were designed in *hopPtoB1* and *Pca*-specific primers (ALS_rv1 and ALS_rv2) were designed in *hopX1* adjacent to *hrpK*. PCR using hrpK_fw1 and MAC_rv1 or hrpK_fw2 and MAC_rv2 amplified DNA fragments of only *Psm*, *P. syringae* pv. *tomato* (causal agent of tomato bacterial speck), and *P. syringae* pv. *spinaciae* (causal agent of spinach bacterial leaf spot), among 76 strains of phytopathogenic bacteria. PCR using hrpK_fw1 and ALS_rv1 or hrpK_2 and ALS_rv2 amplified DNA fragments of only *Pca*. Multiplex PCR with these primers could easily distinguish *Psm* and *Pca* from bacterial colonies isolated on growth media and detect the pathogen in symptomatic leaves. Multiplex nested PCR with the primers detected contamination in one *Psm*- and/or one *Pca*-infected seeds in 1000 seeds. These results suggest that these PCR primers could help detect and discriminate *Psm* and *Pca*.

Key points

- *We investigated Pseudomonas syringae pv. maculicola and P. cannabina pv. alisalensis.*
- *Novel primers common to both bacteria were designed following genome comparison.*
- *Multiplex PCR with new primers could discriminate Psm and Pca.*

Keywords Bacterial leaf spot and bacterial leaf blight · Exchangeable effector locus · Multiplex nested PCR · Specific detection from seed

Introduction

Bacterial leaf spot, an important disease in cruciferous plants (*Brassicaceae*), is caused by *Pseudomonas syringae* pv. *maculicola* (*Psm*), and it globally occurs in plants such as cabbage, cauliflower, broccoli, Chinese cabbage, turnip, radish, and Japanese radish (Peters et al. 2004; Takikawa and Takahashi 2014; Zhao et al. 2000). In 1911, McCulloch described the occurrence of a spot disease on cauliflower grown in Virginia, USA, and this was the first report of the disease (McCulloch 1911; Takikawa and Takahashi 2014). The pathogenic bacteria invades the stomata and wounds, creates small chlorotic and necrotic spots with water-soaked regions on the leaves, and rarely causes severe blight symptoms (Peters et al. 2004; Takikawa and Takahashi 2014; Zhao et al. 2000). The pathogenic bacteria are classified into three groups according to bacteriological characteristics and the host that they are isolated from (Takikawa and Takahashi 2014; Zhao et al. 2000). In addition, *Psm* is considered a heterogeneous pathovar (Peters et al. 2004; Wiebe and Campbell 1993), closely resembling pathovar *tomato* (*Psto*) in bacteriological

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characteristics and genetic similarity. As both pathogens can infect hosts of each other when artificially inoculated, it has been argued that they should be separated (Hendson et al. 1992; Takikawa et al. 1994; Wiebe and Campbell 1993). Gironde and Manceau (2012) showed that there are at least eight genetic lineages among *Psm* and *Psto* isolates and considered that there is a relationship between the genetic lineages and isolation sources.

In 2002, bacterial leaf blight was newly reported in broccoli, and *P. syringae* pv. *alisalensis* was proposed as the causative bacterium. The species was then changed to *P. cannabina* (Bull et al. 2010; Cintas et al. 2002). *P. cannabina* pv. *alisalensis* (*Pca*) has been isolated from cabbage, radish, and arugula globally (Bull et al. 2004; Ishiyama et al. 2013; Mauzey et al. 2011; Rubio et al. 2012; Sarris et al. 2010; Takahashi et al. 2013; Wechter et al. 2010). Bacterial leaf blight of cabbage, Chinese cabbage, and Japanese radish has also been reported in Japan (Ishiyama et al. 2013; Takahashi et al. 2013), and some strains isolated and once identified as *Psm* were reclassified as *Pca* (Takahashi et al. 2013; Takikawa and Takahashi 2014). Similar to *Psm*, the pathogen invades stomata and wounds, creates chlorotic and necrotic spots with water-soaked areas in the leaves, but it tends to cause large coalescing blight lesions extending up to several centimeters in length and sometimes causes severe blight symptoms (Takikawa and Takahashi 2014). *Pca* is classified into two types based on the differences in host virulence and genetic diversity (Sarris et al. 2013; Takikawa and Takahashi 2014). Furthermore, the pathogen has been reported to naturally infect bristle oat cultivated as a green manure crop, which is a problem in the rotation of cruciferous crops and bristle oat (Ishiyama et al. 2013).

In Japan, the occurrence of black-brown discoloration in the core of the stem root and the occurrence of bacterial spots on the surface of the stem root of Japanese radish caused by *Psm* and *Pca* is a problem (Horinouchi et al. 2009; Otani 2016; Takeuchi et al. 1989). The discoloration in the core of the stem root is difficult to distinguish externally, but it has been discovered in agricultural products, leading to consumer complaints. An outbreak of black-brown discoloration in the stem root is mainly caused by infection from the petiole base, and the leaf spot symptom does not always lead to black-brown discoloration in the stem root (Otani 2016). Thus, it is difficult to diagnose the occurrence of such symptoms in the field, and farmers are wary of the occurrence of bacterial leaf spots and bacterial leaf bright. Similar to other *P. syringae* group bacteria, both *Pca* and *Psm* are considered seed borne (Schofield et al. 2012; Takimoto 1931), and seed companies encounter difficulty in responding to this disease because seed transmission of the disease is suspected.

Causing disease in host plants and eliciting a hypersensitive response in non-host plants are fundamental features controlled by the *hrp* genes in *P. syringae* (Bonas 1996; He et al. 1993; Hueck 1998; Preston et al. 1995). The *hrp*/*hrc*-encoded proteins are involved in a type-III secretion pathway (Jin and He 2001; Li et al. 2002). Inoue and Takikawa (2006) reported that *P. syringae* bacterial group could be classified into five groups based on differences in gene homology within the *hrpZ* operon. According to the present study, *Psm* and *Pca* belong to different groups, and it is possible to distinguish them using designed primers (Takahashi et al. 2013). However, many pathovars are in the same group, and cannot be readily distinguished, and nonspecific amplification may occur when this primer is used for the direct detection of bacterial cells (Yoshioka et al. 2020).

Zaccardelli et al. (2005) reported a primer designed for *hrpZ* that can be used to detect *Psto*, but the specificity among pathogenic types of *P. syringae* has not been sufficiently compared. In *P. syringae*, the *hrp*/*hrc* gene cluster is flanked by a unique exchangeable effector locus (EEL) and a conserved effector locus and comprises a pathogenicity island (Alfano et al. 2000). In this region, there are differences in DNA homology among *P. syringae* pathovars (Inoue and Takikawa 1999a, 2000, 2003). There are type III effector genes in this region, and the EEL, sandwiched between *hrpK* and $tRNA^{Leu}$, has different genetic repertoires depending on the strain (Charity et al. 2003; Deng et al. 2003). Our previous studies also confirmed that the EEL region has large differences in gene sequences among strains and low homology among *P. syringae* group bacteria (Inoue and Takikawa 1999a, b, 2003).

In the present study, for the purpose of developing a gene marker that distinguishes *Psm* and *Pca*, DNA sequences of EEL of *Psm* and *Pca* were newly determined, and the DNA sequences, including published information, were compared. As a result, we designed primers that distinguished *Psm* and *Pca* and confirmed their usefulness.

Materials and methods

Bacterial strains and culture conditions

Seventy-six strains of plant pathogenic bacteria, including eight strains of *Pca*, 13 strains of *Psm*, and 30 other *P. syringae* group strains, were used (Table 1). *Xanthomonas* strains were cultured on potato semisynthetic medium (PSA) (Wakimoto 1960) at 27 °C, and other plant pathogenic bacteria were cultured on PPGA medium (Nishiyama 1978) at 25 °C or 27 °C. These cultures were suspended in sterile distilled water at an optical density of 0.3 at 600 nm (~10^8 cfu (colony-forming units)/mL in each case) and appropriately diluted for the subsequent examinations. Rifampicin-resistant strains (KMrR-R03 derived from MAFF [Ministry of Agriculture, Forestry and Fisheries, Japan] 106179 and NMH-R1 derived from MAFF 106156) were obtained by modifying the method of Glandorf et al. (1992). The medium and culture temperature were changed to YPA containing 20 mg/L rifampicin and 25 °C.

Table 1 Bacterial strains

Species and pathovar		Strain name	Souce	Host or origin	Location of isolation	Year	Lane no. of Fig. 2
Pseudomonas							
cannabina	*alisalensis*	106156[a]	MAFF	Chinese cabbage	Nagano	2010	1
		212064	MAFF	Bristle oat	Nagano	2011	2
		301419	MAFF	Japanese radish	Mie	1979	3
		730087	MAFF	Japanese radish	Aomori	N.D	4
		T-1 (#1445[b])	SUPP	Japanese radish	Shizuoka	1983	5
		NMC10311 (#2771)	SUPP	Cabbage	Nagano	2010	6
		NMR10511 (#2773)	SUPP	Japanese radish	Nagano	2010	7
		R-1 (#2924)	SUPP	Japanese radish	Aichi	1984	8
syringae	*maculicola*	106179	MAFF	Japanese radish	Kanagawa	2017	9
		301174	MAFF	Chinese cabbage	Kanagawa	1973	10
		301175	MAFF	Chinese cabbage	Hyogo	1976	11
		302539	MAFF	Chinese cabbage	Yamaguchi	1985	12
		302723	MAFF	Japanese radish	Chiba	1982	13
		302743	MAFF	Japanese radish	Chiba	1982	14
		302783	MAFF	TURNIP	Ibaraki	1983	15
		302786	MAFF	TURNIP	Ibaraki	1983	16
		730088	MAFF	Japanese radish	Yamagata	N.D	17
		90S-4 (#1331)	SUPP	CAULIFLOWER	Chiba	1990	18
		PMC8301 (#2206)	SUPP	TURNIP	Chiba	1983	19
		R101 (#2925)	SUPP	Japanese radish	Aichi	1984	20
		TLS-1 (#1447)	SUPP	Turnip	Shizuoka	1984	21
	aceris	kaede1-1 (#440)	SUPP	*Acer buergerianum*	Shizuoka	1983	22
	actinidiae	302091	MAFF	Kiwi	Kanagawa	1984	23
	aptata	301012	MAFF	Sugar beet	Kagoshima	1966	24
		302830	MAFF	Rice	Fukuoka	1976	25
	atropurpurea	301017	MAFF	Itarian ryegrass	Yamaguchi	1967	26
	castaneae	302088	MAFF	*Castanea crenata*	Shiga	1977	27
	coronafaciens	301314	MAFF	Oat	Ibaraki	1972	28
	dendropanacis	kakuremino-1 (#453)	SUPP	*Dendropropanax trifidus*	Okinawa	N.D	29
	eriobotryae	301062	MAFF	Loguat	Chiba	1952	30
	lachrymans	301315	MAFF	Cucumber	Gunma	1975	31
	magnoliae	PMG8101 (#183)	SUPP	Magnolia	Tokyo	1981	32
	mellea	302304	MAFF	Tobacco	Aomori	1968	33
	mori	810001	MAFF	Mulberry	Gunma	N.D	34
	morsprunorum	301436	MAFF	Japanese apricot	Shiga	1978	35
	myricae	301464	MAFF	Japanese bay berry	Okinawa	1978	36
	oryzae	301529	MAFF	Rice	Aomori	1983	37
	photiniae	photinia-1 (#733)	SUPP	*Photina glabla*	Shizuoka	N.D	38
	pisi	301208	MAFF	Pea	Shizuoka	1978	39
	sesami	311181	MAFF	Sesame	Ibaraki	1997	40
	solidagae	810053	MAFF	*Solidago canadensis*	Ibaraki	1998	41
	spinaciae	211666	MAFF	Spinach	Yamaguchi	1986	42
	striafaciens	301032	MAFF	Oat	Hiroshima	1967	43
	syringae	LOB2-1 (#458)	SUPP	Lilac	Nagano	1986	44
		301159	MAFF	Wheat	Fukuoka	1976	45
		301430	MAFF	Peach	Fukushima	1978	46
	tabaci	Pt7364 (#278)	SUPP	Tobacco	N.D	1973	47
	theae	302851	MAFF	Tea	Kagoshima	1993	48
	tomato	Pst-4 (#1776)	SUPP	Tomato	Nagano	1997	49
savastanoi	*glycinea*	301683	MAFF	Soybeen	Ibaraki	1981	50
	phaseolicola	301616	MAFF	Bean	Ibaraki	1981	51
cichorii		301367	MAFF	Lettuce	Kagawa	1976	52
ficuserectae		L-7 (#1391)	SUPP	*Ficus erecta*	Shizuoka	1977	53
fuscovaginae		106631	MAFF	Rice	Hokkaido	2001	54
viridiflava		301327	MAFF	Cucumber	Nagasaki	1975	55
Acidovorax							
avenae subsp. *avenae*		106618	MAFF	Rice	Toyama	N.D	56
Burkholderia							
andropogonis		302667	MAFF	Corn	Ibaraki	1981	57
caryophylli		301060	MAFF	Carnation	Kanagawa	1964	58

Table 1 (continued)

Species and pathovar		Strain name	Souce	Host or origin	Location of isolation	Year	Lane no. of Fig. 2
gladioli	*gladioli*	106671	MAFF	Rice	Ibaraki	2003	59
glumae		106747	MAFF	Rice	Akita	2005	60
plantarii		106721	MAFF	Rice	Ishikawa	2004	61
Dickeya							
dadantii		106634	MAFF	Sweet potato	Miyazaki	1996	62
zeae		212010	MAFF	Japanese ginger	Kochi	2010	63
Pantoea							
ananatis		301722	MAFF	Rice	Okayama	1980	64
Pectobacterium							
carotovorum subsp. *carotovorum*		301618	MAFF	Potato	Hokkaido	1972	65
		301941	MAFF	Ginger	Aichi	1974	66
Ralstonia							
pseudosolanacearum		107632	MAFF	Tomato	Nara	1981	67
Xanthomonas							
arboricola	*pruni*	211972	MAFF	Peach	Yamanashi	2010	68
axonopodis	*vitians*	301289	MAFF	Lettuce	Shizuoka	1976	69
campestris	*campestris*	301176	MAFF	Cabbage	Hyogo	1976	70
	incanae	302058	MAFF	Stock	Chiba	1982	71
	raphani	106181	MAFF	Japanese radish	Aomori	2014	72
citri	*citri*	673022	MAFF	*Citrus hassaku*	Aichi	1994	73
euvesicatoria	106190	MAFF	Green pepper	Ibaraki	2015	74	
oryzae	*oryzae*	311018	MAFF	Rice	Kyoto	1971	75
vesicatoria	301256	MAFF	Tomato	Tokushima	1976	76	

[a] Collection number in the Ministry of Agriculture,Forestry and Fisheries (MAFF) culture collection

[b] Collection number in the Shizuoka University Plant Pathology (SUPP) culture collection

Artificial inoculation of Japanese radish seeds

We used Japanese radish seeds (“Heian-hayabutori-tokinashi,” Takii, Kyoto, Japan), which have been found to have negligible contamination with *Psm* and *Pca*. Rifampicin-resistant strains KMrR-R03 and NMH-R1 were used for inoculation. Bacterial suspensions were prepared as described above. Approximately 5 mL of seeds was placed in a 100-mL beaker, 10 mL of the bacterial suspension was added, and air pressure was reduced at 0.02 MPa for 5 min. Thereafter, the liquid was removed, and the seeds were spread on paper towels and dried for 3 days in a clean room. To confirm the adhesion of the infected strain, an inoculated seed was immersed in 100 μL of sterile distilled water in a PCR tube and shaken for 2.5 h, and 10 μL of the immersion liquid was applied to yeast extract-peptone agar (YPA; 5 g yeast extract, 10 g peptone, 15 g agar, and 1000 mL of distilled water; pH 6.8) medium containing 20 ppm of rifampicin and 100 ppm of cycloheximide. The number of colonies formed was counted after 3 days at 25 °C. It was tested with 24 seeds.

Artificial inoculation of radish plants

In the inoculation test, radish (“Yukikomachi,” Sakata seed, Kanagawa, Japan), which is smaller than Japanese radish and shows similar lesions on the leaves, was used. Radish seeds were sown in a 7-cm pot and cultivated in a greenhouse for approximately 3 weeks. KMrR-R03, NMH-R1, and re-isolated strains from the inoculated seeds were used for inoculation. Six hours before inoculation, the plant was placed in a sealed container and placed in the dark at 22 °C. The plant was sprayed with 10 mL of approximately 10^7 cfu/mL bacterial suspension with 0.01% Tween20, which was placed in a sealed container again. The plant was kept in the dark at 22 °C for 24 h, and then incubated for 2 days under a 12-h light and 12-h dark photoperiod. Subsequently, it was taken out from the sealed container, and the occurrence of disease was investigated for 5–14 days after inoculation.

DNA manipulation

Genomic DNA for a PCR template was purified using NucleoSpin Tissue (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer’s instructions. The purified DNA was diluted in 10 mM Tris-HCl buffer or 1 mM HEPES buffer and used as a PCR template. Amplification of the EEL in *Psm* strains MAFF 106179, 90S-4, and R101, and *Pca* strains MAFF 106156 and R-1 was performed according to the method of Deng et al. (2003). The nucleotide sequence of the DNA fragment amplified by PCR was determined using ABI3130XL (Thermo Fisher Scientific, Waltham, MA) with specific primers according to the manufacturer’s instructions.

The EEL of *P. syringae* pv. *spinaciae* strain MAFF 211666 was not amplified using the method of Deng et al. (2003). Its DNA fragments were amplified by PCR using the specific primers (K0; 5′-GCAACCCAGGCCATTTACG-3′, Q004; 5′-TTTGGCCTGTGGTGCTTG-3′), and nucleotide sequences were analyzed as described above. DNA sequences have been registered in DNA Data Bank of Japan (Acc. No. LC593618- LC593623).

Primer design and PCR amplification

To design the primers, the DNA sequences derived from draft genome sequences of *Psm* PMC8301 and *Pca* MAFF 106179 (in the middle analysis), the DNA sequence of *Psm* PMC8301 (Acc. No. AB023074), *Psto* DC3000 (Acc. No. AB166796), and *Pca* ES4326 (Acc. No. CP047260) obtained from the database and determined above were used. Primers designed in the present study are shown in Table 2. Primers hrpK_fw1 and hrpK_fw2 were designed from the *hrpK* sequence of *Pca*. Primers MAC_rv1 and MAC_rv2 were designed for *hopPtoB1* of *Psm*, and the predicted PCR products using hrpK_fw1 and MAC_rv1, and hrpK_fw2 and MAC_rv2 were 591 and 256 bp, respectively. Primers ALS_rv1 and ALS_rv2 were designed for *hopX1* of *Pca*, and the predicted PCR products using hrpK_fw1 and ALS_rv1, and hrpK_fw2 and ALS_rv2 were 897 and 543 bp, respectively. As a PCR template, 5 μL of bacterial suspension or DNA solution was used. PCR amplification with hrpK_fw1, MAC_rv1, and ALS_rv1 (PsmPca_PS1) was performed as follows. The PCR mixture of volume 20 μL contained 0.5 U BIOTAQ DNA Polymerase (Bioline, London, UK), 1× reaction buffer, 1.5 mM MgCl2, 200 μM of each dNTP, 160 nM respective primers, and the template. The PCR was performed using the following thermocycling conditions: 95 °C for 3 min, followed by 30 cycles at 95 °C for 30 s, 58 °C for 25 s, and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. PCR amplification with hrpK_fw2, MAC_rv2, and ALS_rv2 (PsmPca_PS2) was performed as follows. The contents of the mixture were the same as above. The PCR was performed using the following thermocycling conditions: 95 °C for 3 min, followed by 30 cycles at 95 °C for 30 s, 58 °C for 10 s, and 72 °C for 20 s, and a final extension step at 72 °C for 5 min. The amplified PCR products were visualized by electrophoresis on 1.5% or 3% agarose gels and stained with Midori Green advance (Nippon Genetics, Tokyo, Japan) or SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, MA, USA). To improve detection sensitivity, nested PCR was also performed. For this, we used PsmPca_PS1 in the first PCR and PsmPca_PS2 in the second PCR. The first PCR was performed as described above. In the second PCR, 1 μL of the first PCR product was added to 20 μL of the reaction mixture as a template.

Table 2 Primers list

Primer set	Primer	Sequence (5′ to 3′)
PsmPca_PS1	hrpK_fw1	GTCTGGGCGGACAGATGAT
	MAC_rv1	CGCCTTCTGGTGTGCTTTAC
	ALS_rv1	CATGTTCGCGGCAGTTACC
PsmPca_PS2	hrpK_fw2	GCTGGATCAGTTCGGGATTG
	MAC_rv2	TTGGAGTCTCTGGCGCAAC
	ALS_rv2	CGTATGCTGCTTTCCAGGTC

Detection of *Psm* and *Pca* by PCR in seeds

Either of one *Pca*- or *Psm*-inoculated seed or two (one *Pca*- and one *Psm*-inoculated) seeds was added to healthy seeds for a total of 1000 seeds and placed in a 300-mL conical flask. Each seed set was prepared in triplicate. One set was added to 30 mL of wash buffer (0.85% NaCl, 0.02% Tween 20) and shaken for 2.5 h, and approximately 10 mL of the immersion liquid was collected in a conical tube. DNA was purified from 1 mL of the liquid and eluted with 100 μL of 10 mM Tris-HCl buffer. Multiplex PCR with PsmPca_PS1 was performed using DNA solution diluted five volumes as the template. Thereafter, nested PCR with PsmPca_PS2 was performed. The seed immersion liquid was also applied to two YPA plates containing 20 ppm of rifampicin and 100 ppm of cycloheximide in a volume of 100 μL to count the bacterial density in the liquid. The number of colonies formed was counted after 3 days at 25 °C. This test was repeated three times.

Colony PCR

For the colony PCR, 4, 4, and 8 colonies formed on YPA plates, derived respectively from the seed immersion liquid containing one *Psm*-inoculated seed, one *Pca*-inoculated seed, and one *Psm*- and one *Pca*-inoculated seed in the above test, were used. A colony was picked with the tip of a toothpick and immersed in 25 μL of sterile distilled water. Multiplex PCR with PsmPca_PS1 was performed using 5 μL of the suspension diluted to five volumes as a template.

Detection of *Psm* and *Pca* by PCR from inoculated plants

Infected or non-infected parts of an inoculated leaf were cut into approximately 1-cm^2 squares and ground in a 1.5-mL tube with 500 μL sterile distilled water. The suspension was centrifuged at 500×*g* for 1 min, and the supernatant was collected. DNA was purified from the supernatant and eluted with 100 μL of elution buffer. Multiplex PCR with PsmPca_PS1 was performed using DNA solution diluted in five volumes as a template. To confirm the presence of viable bacteria, a part of the supernatant was added to YPA medium. Two replicates were tested.

Results

Nucleotide sequence of EEL

The nucleotide sequences of EEL of *Psm* strains MAFF 106179, 90S-4, and R101, and *Pca* strains MAFF 106156 and R-1 were determined. The nucleotide sequence of *Psm* strain PMC8301 (Acc. No. AB023074) previously determined by us (Inoue and Takikawa 1999b) was also included in the analysis. The structure of EEL of *Psm* is shown in Fig. 1. In *Psm* strain 90S-4, an IS3-like sequence was inserted, and it had the same structure as *Psto* strain DC3000. The BLAST search showed that *Psto* strains B13-200 (Acc. No. CP019871) and PT23 (Acc. No. AB166796), *P. syringae* pv. *avii* strain CFBP3846 (Acc. No. LT963402), and *P. syringae* pv. *persicae* strain 5846 (Acc. No. AY147018) have a homologous region of length approximately 1 kbp adjacent to *hrpK*. In *Psto* strains B13-200 and PT23, there was an internal deletion of 315 bp in the front part of *hopPstB1*. In *Pca*, *hopX1* was adjacent to *hrpK*. The BLAST search showed there was no homologous gene structure other than *Pca* ES4326 (Acc. No. DQ196428) in the GenBank database. In MAFF106156, an IS66-like sequence was inserted at a position 400 bp away from t-RNA^{Leu}, but it was not present in ES4326 and R-1. Based on the analyzed nucleotide sequences described above, hrpK_fw1 and hrpK_fw2 were designed in *hrpK* of *Pca*. In *Psm*, there were a few mismatches in hrpK_fw1 and hrpK_fw2. *Psm*-specific primers MAC_rv1 and MAC_rv2 were designed in *hopPtoB1*, and *Pca*-specific primers ALS_rv1 and ALS_rv2 were designed in *hopX1*.

Specificity of primers

Conventional PCR with hrpK_fw1 and MAC_rv1, and hrpK_fw2 and MAC_rv2 amplified approximately 590- and 260-bp long DNA fragments from *Psm*, *Psto*, and *P. syringae* pv. *spinaciae* (Supplementary Fig. S1a, b). Therefore, we tried to amplify the EEL of *P. syringae* pv. *spinaciae* but could not amplify it. An analysis of the partial nucleotide sequence of the EEL of *P. syringae* pv. *spinaciae* revealed homology with *P. syringae* pv. *avii* from *hrpK* to the outside of *hopPtoB1* (Fig. 1). Conventional PCR with hrpK_fw1 and ALS_rv1, and hrpK_fw2 and ALS_rv2 amplified approximately 900- and 540-bp DNA fragments from *Pca* strains among the 76 strains of plant pathogenic bacteria, respectively (Supplementary Fig. S1c, d). Using multiplex PCR with PsmPca_PS1 or PsmPca_PS2, we identified *Psm* and related pathovars, *Pca*, and other plant pathogenic bacteria (Fig. 2). In the multiplex PCR using PsmPca_PS1 and PsmPca_PS2, amplification was confirmed up to a 5×10^3-fold dilution of the bacterial suspension (Supplementary Fig. S2). This corresponded to the presence of approximately 100 cfu per reaction. Using the nested PCR approach, the detection limits were limited to a few bacterial cells (Supplementary Fig. S3, Table S1).

Pathogen detection from disease lesion on Japanese radish

Purified DNA from lesions showing typical symptoms of *Psm* and *Pca* infections (Supplementary Fig. S4) showed specific amplification of *Psm* and *Pca* by multiplex PCR using PsmPca_PS1, respectively, but there was no amplification with DNA extracted from healthy leaf sections (Fig. 3). Each pathogen was also isolated on YPA medium from the solution extracted from each lesion, but not from healthy leaf sections.

Pathogen detection in seed samples contaminated with *Psm* and *Pca*

The number of colonies that grew from 10 μL of an inoculated seed immersion liquid varied from 0 to >500, and only one of the 24 seeds did not develop any colonies. The same results were obtained for seeds inoculated with either bacterium. There was no amplification in multiplex PCR with PsmPca_PS1 when purified DNA from the immersion liquid of 1000 seeds was used as the template. Multiplex nested PCR with PsmPca_PS2 was performed using 1 μL of the first PCR product as the template. The results showed that the length of amplified DNA fragments was 260 bp from *Psm*-contaminated seed samples, 540 bp from *Pca*-contaminated seed samples, and 260 and 540 bp from samples containing both types of contaminated seeds (Fig. 4). Conversely, no amplification was observed in samples with no contaminated seeds. Thus, multiplex nested PCR can detect the contamination of one seed with *Psm* and/or *Pca* out of 1000 seeds and can discriminate the two species. The number of target bacteria that could be cultivated on YPA medium contained in 100 μL of seed immersion liquid was 0–12, and the detection sensitivity by PCR when using this liquid was higher than that when using the bacterial suspension as a template. These colonies were used in subsequent tests.

Colony PCR

Following the multiplex PCR with PsmPca_PS1, DNA fragments of length 590 and 900 bp were amplified from colonies (four for each pathovar) isolated from the immersion liquids of *Psm*- and *Pca*-contaminated seeds, respectively (Fig. 5). Colonies derived from the seed immersion liquid containing a *Psm*-inoculated seed were only *Psm*, whereas those derived from the liquid containing a *Pca*-inoculated seed were only *Pca*. Of the eight colonies

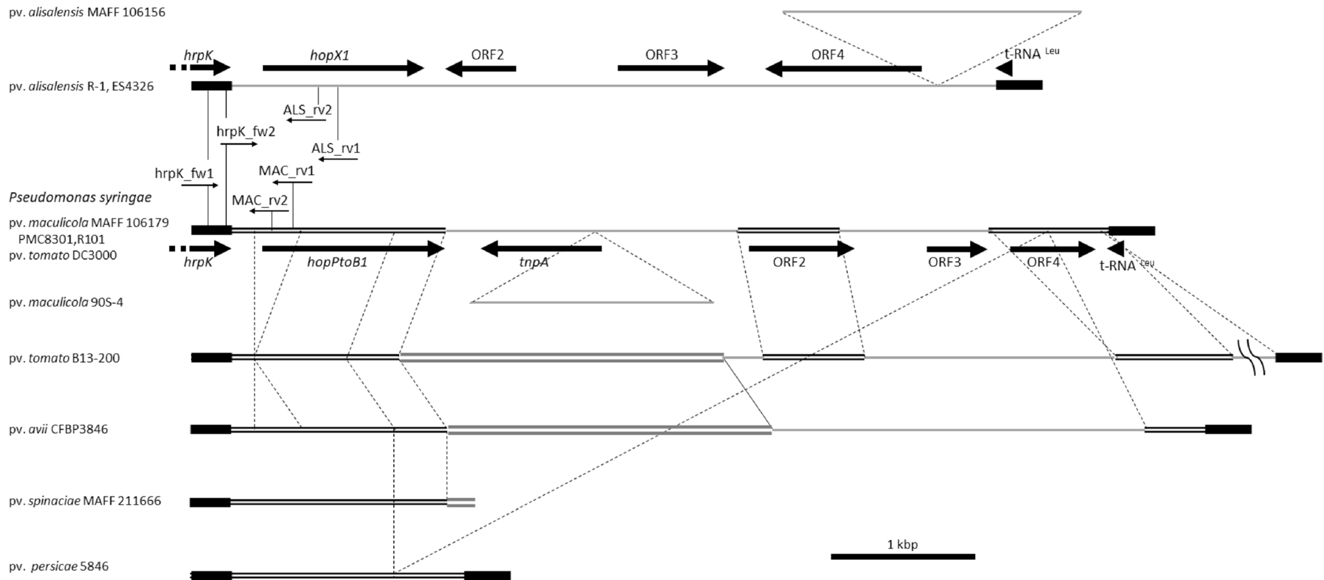

Fig. 1 Gene map of the exchangeable effector locus (EEL) of *Pseudomonas syringae* pv. *maculicola* (*Psm*)-related pathovars and *P. cannabina* pv. *alisalensis* and the location of the specific PCR primers that we designed. The horizontal lines indicate the following: thick black lines are homologous regions in all strains, black double lines are homologous regions to *Psm*, gray double lines are homologous regions other than *Psm*, and thin gray lines are not homologous. Thick arrows indicate the open reading frames and their direction and thin arrows indicate the designed primers and their direction

derived from immersion liquid containing seeds carrying both inoculants, two amplified a 590-bp DNA fragment and six amplified a 900-bp DNA fragment when subjected to multiplex PCR with PsmPca_PS1 (Fig. 5). Therefore, *Psm* and *Pca* colonies grew from the sample derived from seeds contaminated with both pathovars, and multiplex PCR with PsmPca_PS1 successfully distinguished the two. Inoculation tests using two colonies each revealed that colonies showing amplification similar to *Psm* formed typical lesions of bacterial leaf spot, and colonies showing amplification similar to *Pca* formed typical lesions of bacterial leaf bright on radish (data not shown).

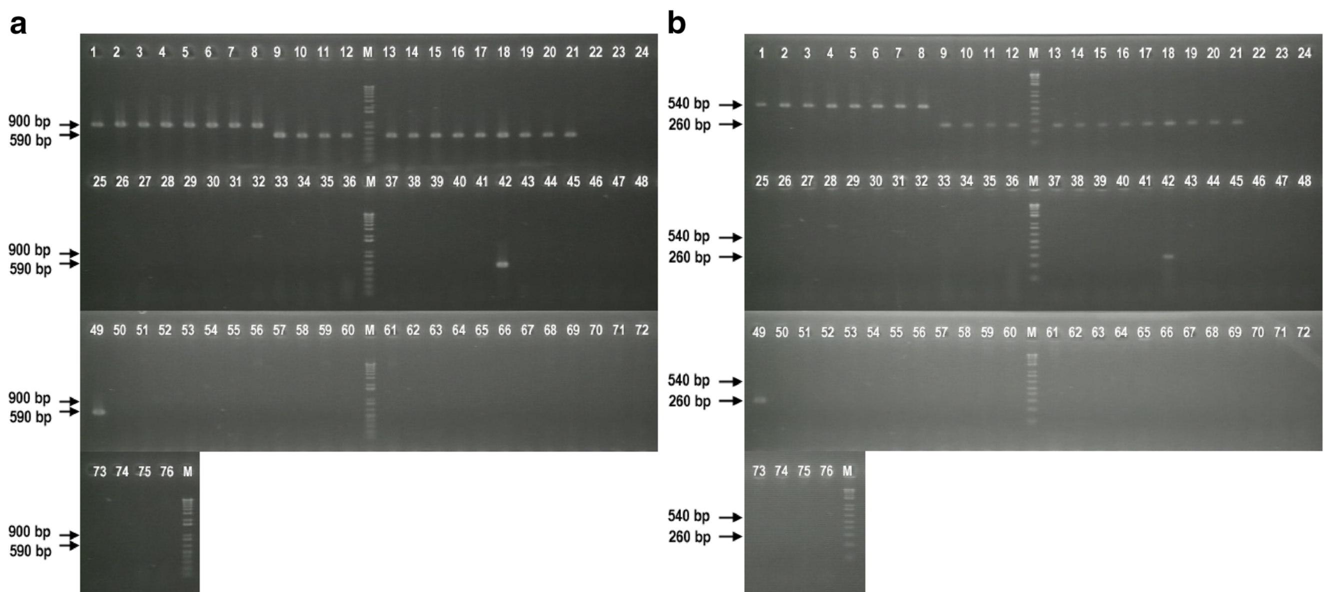

Fig. 2 Multiplex PCR amplification using the PsmPca_PS1 primer set (**a**) and PsmPca_PS2 primer set (**b**). The PCR samples were subjected to electrophoresis on 1.5% (**a**) and 3.0% (**b**) agarose gel. Lane numbers correspond to the numbers in Table 1. Lane M, DNA molecular size marker (1 Kb Plus DNA Ladder; Thermo Fisher Scientific, Waltham, MA, USA)

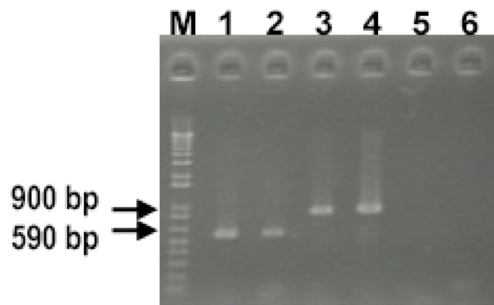

Fig. 3 Detection of *Pseudomonas syringae* pv. *maculicola* and *P. cannabina* pv. *alisalensis* from radish leaves by Multiplex PCR using the PsmPca_PS1 primer set. Lanes 1 and 2, bacterial leaf spot symptomatic leaves; Lanes 3 and 4, bacterial leaf blight symptomatic leaves; Lanes 5 and 6, symptomless leaves; Lane M, DNA molecular size marker (1 Kb Plus DNA Ladder; Thermo Fisher Scientific, Waltham, MA, USA). The PCR samples were subjected to electrophoresis on 1.5% agarose gel

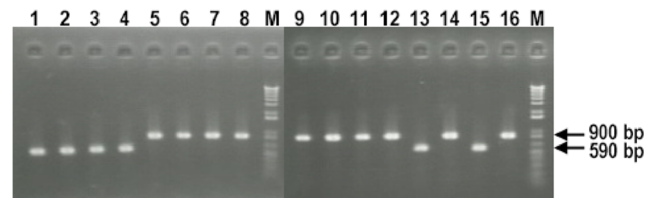

Fig. 5 Identification of *Pseudomonas syringae* pv. *maculicola* (*Psm*) and *P. cannabina* pv. *alisalensis* (*Pca*) colonies by multiplex PCR using the PsmPca_PS1 primer set. Lanes 1–4, colonies isolated from seed samples containing one *Psm*-inoculated seed; Lanes 5–8, colonies isolated from seed samples containing one *Pca*-inoculated seed; Lanes 9–16, colonies isolated from seed samples containing one *Psm*- and one *Pca*-inoculated seeds; Lane M, DNA molecular size marker (1 Kb Plus DNA Ladder; Thermo Fisher Scientific, Waltham, MA, USA). The PCR samples were subjected to electrophoresis on 1.5% agarose gel

Discussion

EEL is a genomic region where a large number of effector genes exist (Alfano et al. 2000), but the distribution of individual effector genes among pathovars and host range within a pathovar has been noted, and research on the locus has been limited in recent years. We focused on the fact that this region has different gene repertories among pathovars, considering that it is possible to develop genetic markers that distinguish the pathovars of *P. syringae* group bacteria. By designing primers in the transition site, but not in a specific effector gene, between the common and specific genes, we simultaneously detected and distinguished two pathogens with three primers. Furthermore, we improved the detection sensitivity through nested PCR by designing and using two primer sets, thus enabling simultaneously detection and identification of *Psm* and *Pca*. Although *Psm* and *Pca* were targeted here, it is possible that EEL can be used to identify other pathovars of *P. syringae* group bacteria.

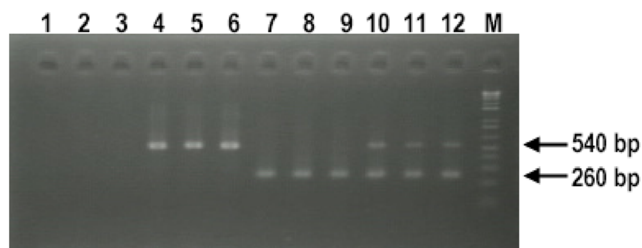

Fig. 4 Detection of *Pseudomonas syringae* pv. *maculicola* (*Psm*) and *P. cannabina* pv. *alisalensis* (*Pca*) in 1000 seeds by multiplex nested PCR. PCR using the PsmPca_PS1 primer set was performed with the DNA purified from the immersion of 1000 seeds as a template, followed by nested PCR using the PsmPca_PS2 primer set. Lanes 1–3, only healthy seed samples; Lanes 4–6, seed samples contained one *Pca*-inoculated seed; Lanes 7–9, seed samples contained one *Psm*-inoculated seed; Lanes 10–12, seed samples contained one *Psm*- and one *Pca*-inoculated seeds; Lane M, DNA molecular size marker (1 Kb Plus DNA Ladder; Thermo Fisher Scientific, Waltham, MA, USA). The PCR samples were subjected to electrophoresis on 3% agarose gel

The presence of many Type III effectors in *P. syringae* group has been reported, and their relationship with pathogenicity has been investigated (Dillon et al. 2019a; Laflamme et al. 2020; Lindeberg et al. 2012). Type III-related effector genes are mutated and distributed among various pathovars (Laflamme et al. 2020). Therefore, these distributions and the homology between genes have also been used to consider the acquisition of pathogenicity and evolution of pathogenic bacteria (Dillon et al. 2019b; Lindeberg et al. 2009). Some of the predicted genes in the EEL are of unknown function and may also be involved in plant pathogenicity.

Although *hrpK* was used as the common gene, it is possible to design a common primer using *queA*. However, in *Pca* MAFF 106156, the insertion sequence was inserted adjacent to $tRNA^{Leu}$, and therefore, the *queA* side could not be used as a gene marker in the present study. *Psm* 90S-4 also had an IS element in the EEL. EEL has many insertion sequences (Inoue and Takikawa 1999b). This indicates that this region is variable and rich in mutations. Using this region, it may be possible to clarify differences in strains within the same pathovar. In the present analysis, we confirmed that *P. syringae* pathovars *tomato*, *avii*, *persicae*, and *spinaciae* have homologous sequences with *Psm*. *Psm* has been described as a heterogeneous pathovar (Peters et al. 2004; Wiebe and Campbell 1993). *Psto* has long been discussed for differences with *Psm* (Hendson et al. 1992; Takikawa et al. 1994; Wiebe and Campbell 1993). Furthermore, pathovar *spinaciae* is the same as *Psm* (Zhao et al. 2000), but this difference must be verified in the future. The primers we designed in the present study could not distinguish *Psto* and pathovar *spinaciae* from *Psm*. Both pathovars *avii* and *persicae* have been reported as pathogens of stone fruits (Ménard et al. 2003; Young 1987), and they will also be amplified. Both pathovars were not used in the present study because there are no reports of their occurrences in Japan. *Psm* and heterogeneous pathogens can be distinguished by focusing on the specific sequence in the EEL.

Japanese radish seeds and plants were used in the present study owing to the occurrence of black-brown discoloration in

the core of the stem root in Japanese radish, a major problem in Japan (Otani 2016; Horinouchi et al. 2009; Takeuchi et al. 1989). The pathogen of this disease has been reported on *Xanthomonas campestris* pv. *raphani*, the causal agent of bacterial spot, in addition to *Psm* and *Pca* (Omi et al. 2015; Otani et al. 2014). They all cause leaf spots and are seed-borne pathogens, and a method for identifying them is required. *Psm* and *Pca* can be detected in the lesions on leaves and infected seeds using the genetic markers designed in the present study. Currently, we are developing genetic markers for the detection and discrimination of *X. campestris* pv. *raphani*.

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Author contributions YI carried out sequence alignment, designed primers, and drafted the manuscript. YT participated in the design of the study and performed a validity verification test. All authors have read and approved the final manuscript.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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