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Race- and Isolate-specific Molecular Marker Development through Genome-Realignment Enables Detection of Korean *Plasmodiophora brassicae* Isolates, Causal agents of Clubroot Disease

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Clubroot is one of the most economically important diseases of the *Brassicaceae* family. Clubroot disease is caused by the obligate parasite *Plasmodiophora brassicae*, which is difficult to study because it is non-culturable in the laboratory and its races are genetically variable worldwide. In Korea, there are at least five races that belongs to four pathotype groups. A recent study conducted in Korea attempted to develop molecular markers based on ribosomal DNA polymorphism to detect *P. brassicae* isolates, but none of those markers was either race-specific or pathotype-specific. Our current study aimed to develop race- and isolate-specific markers by exploiting genomic sequence variations. A total of 119 markers were developed based on unique variation exists in genomic sequences of each of the races. Only 12 markers were able to detect *P. brassicae* strains of each isolate or race. Ycheon14 markers was specific to isolates of race 2, Yeoncheon and Hoengseong. Ycheon9 and Ycheon10 markers were specific to Yeoncheon isolate (race 2, pathotype 3), ZJ1-3, ZJ1-4 and ZJ1-5 markers were specific to Haenam2 (race 4) isolate, ZJ1-35, ZJ1-40, ZJ1-41 and ZJ1-49 markers were specific to Hoengseong isolate and ZJ1-56 and ZJ1-64 markers were specific to Pyeongchang isolate (race 4, pathotype 3). The PCR-based sequence characterized amplified region (SCAR) markers developed in this study are able to detect five Korean isolates of *P. brassicae*. These markers can be utilized in identifying four Korean *P. brassicae* isolates from different regions. Additional effort is required to develop race- and isolate-specific markers for the remaining Korean isolates.

Keywords : clubroot, isolate-specific markers, *Plasmodiophora bassicae*, SCAR markers, sequence comparison

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Clubroot is a serious disease of the Brassicaceae that is increasingly problematic worldwide (Wang et al., 2008). This disease is caused by the soil-borne biotrophic cercozoan *Plasmodiophora brassicae* Woronin (Nikolaev et al., 2004). This pathogen lives in soil for up to 20 years as resting spores (Karling, 1968; Wallenhammar, 1996) that are difficult to control with anti-microbial chemicals or crop rotation (Tsushima, 2000; Voorrips, 1995). Root hairs are the primary target of this pathogen, but the infection eventually spreads to the cortical region of the roots. The infected roots swell to form of galls or clubs that severely reduce the quality and production of *Brassica* crops, such as cabbage (Dixon, 2009; Kim et al., 2009). Several factors increase the likelihood of *P. brassicae* infection, including acidification of soils, changes in climate that favor the growth of this pathogen, an increase in cultivated area devoted to *Brassica* crops, and transportation of crucifer-

ous crop products and residues (Li et al., 2013). The resting spores multiply exponentially in the newly infected soil if they parasitize a cruciferous crop.

Understanding of clubroot disease has been limited by several aspects of the pathogen's biology. *P. brassicae* is non-culturable in laboratory conditions and is difficult to clone and sequence due to its high genomic similarity with host plants (Bryngelsson et al., 1988; Laila et al., 2017; Schwelm et al., 2015). Moreover, several pathotypes (a group of organisms that have the same pathogenicity on a specified host) and races of *P. brassicae* exist worldwide (Buczacki et al., 1975; Hatakeyama et al., 2004; Kuginuki et al., 1999; Somé et al., 1996; Williams, 1966). Kim et al. (2016) re-classified Korean *P. brassicae* isolates into four pathotypes (Jo et al., 2011; Kim et al., 2016). Development of race-specific markers would enable more effective and precise detection of this pathogen. Such molecular markers have been used effectively in plant disease diagnostics for many years, including limited methods in *P. brassicae* based on PCR amplification of DNA from galls or resting spores (Faggian and Strelkov, 2009; Ito et al., 1997, 2008; Li et al., 2017; Wallenhammar and Arwidsson, 2001; Yang et al., 2002). Ito et al. (1997) cloned and sequenced 1.4 kb DNA fragment from 19 different Japanese *P. brassicae* isolates and found that the markers covering that sequence do not amplify fungi and bacterial DNA they studied. Thus, the authors suggested two markers PBTZS-3 and PBTZS-4 for detecting 19 *P. brassicae* isolates. This research group also developed a single-tube nested polymerase chain reaction method for detecting *P. brassicae* isolates from soil samples. In another study conducted in Sweden, Wallenhammar and Arwidsson (2001) found two markers of 389 bp and 507 bp were able to detect *P. brassicae* isolates from soil sample of both Sweden and UK by a nested PCR method. The authors, however, found that isolates from two different countries had variation in 18s and ITS1 (inter-transcribed spacer 1) regions indicating that those markers are unable to differentiate any isolate specific variations. In a recent study in China, Li et al. (2017) successfully detected an unknown *P. brassicae* isolate after targeting *D85819* and *Pro1* gene-specific primers. However, none of those PCR based methods could distinguish between the different *P. brassicae* isolates. *P. brassicae* isolates in Korea differ in their level of pathogenicity and virulence (Laila, 2018). It might be also possible that two or more isolates of from adjacent geographical regions might co-exist in a particular location. Furthermore, isolates from one region may translocate to another region due to transportations of green plant products. Therefore, an effective detection protocol of existing Korean isolates is important for their race-specific and isolate-specific identity. Laila et al. (2017) attempted to distinguish 11 Korean *P. brassicae* isolates by creating ribosomal DNA (rDNA) markers. This approach produced four high resolution melting-based markers targeting smaller subunit region of ribosomal DNA that can detect two groups of the 11 *P. brassicae* isolates. However, none of these markers could distinguish between the Korean isolates. In this study, we compared all available genomic sequences of *P. brassicae* to obtain isolate-specific molecular markers.

Materials and Methods

Retrieval and alignment of *P. brassicae* genomic DNA sequences. Genomic sequences of *P. brassicae* isolates e3 and ZJ-1 were retrieved from NCBI (Genbank accession numbers GCA_001049375.1 and GCA_002093825.1, respectively). The sizes of the e3 and ZJ-1 genomes were 24.0508 and 24.8311 Mb, respectively (Table 1). We recently sequenced partial genomic DNA sequences of four additional *P. brassicae* isolates (21 Mb each): Daejeon, Gangneung 1, Haenam-2, and Yeoncheon (SRA accession: SRP150814). Genomic sequences of these four isolates were compared to identify variable nucleotide positions. These six sequences were aligned using the "Align with progressiveMauve" approach in Mauve (v. 2.4.0) (Darling et al., 2010) and visualized in Geneious (v. 11.0.3, Kearse et al., 2012). This comparison identified variable regions

Table 1. *Plasmodiophora brassicae* isolates used for comparing genome sequences. Sequences of e3 and ZJ-1 isolates were compared to four Korean isolates

Isolate	Sequence	Race	Country	Genome size	Reference
e3	Genome	Race unknown	Sweden	24.0508 Mb	Schwelm et al. (2015)
ZJ-1	Genome	Race 1	China	24.8311 Mb	Bi et al. (2016)
Daejeon	Whole genome re-seq	Race 5	Korea	21 Mb	
Gangneung 1	Whole genome re-seq	Race 9	Korea	21 Mb	SRA accession: SRP150814
Haenam-2	Whole genome re-seq	Race 4	Korea	21 Mb	
Yeoncheon	Whole genome re-seq	Race 2	Korea	21 Mb	
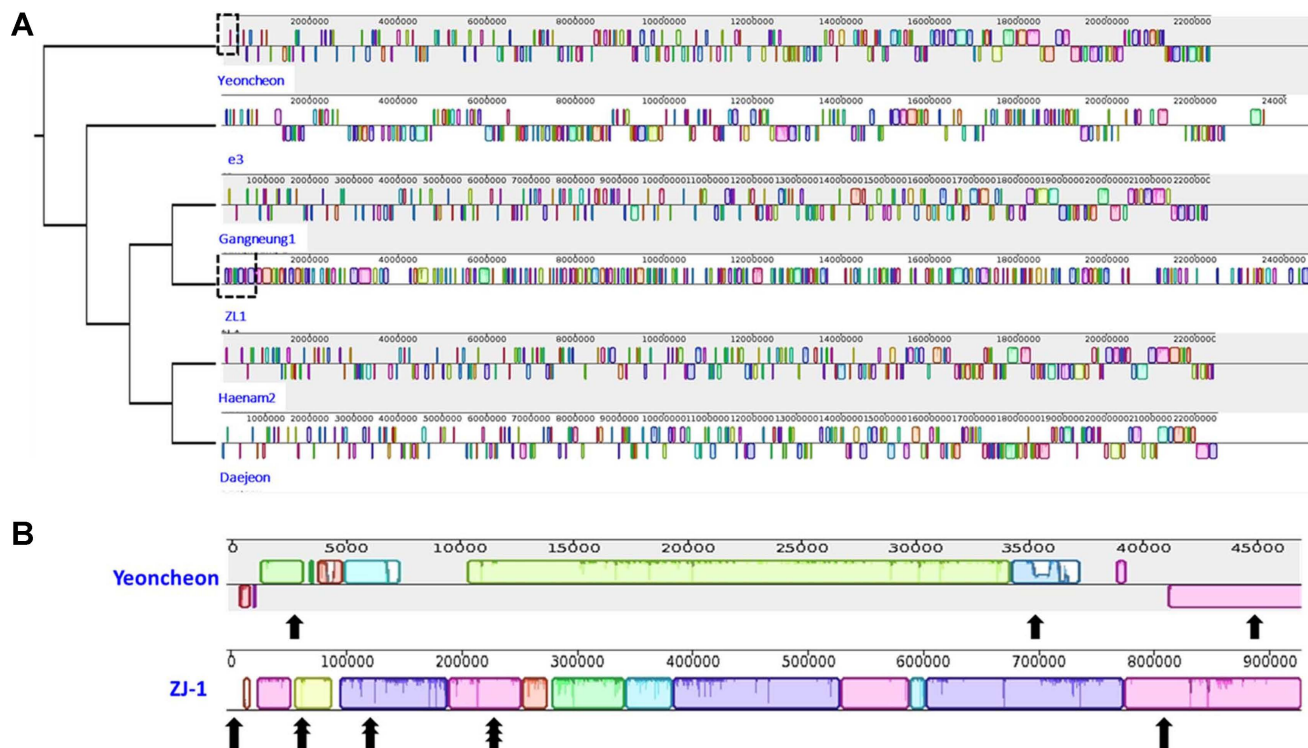
Fig. 1. Alignment of whole genome sequences of *Plasmodiophora brassicae*. (A) Alignment of five isolates: the dotted rectangles in Yeoncheon and ZL1 indicate the regions where isolate-specific markers were targeted. (B) Identified Yeoncheon- and ZJ1-specific markers regions are highlighted by black arrows.

among these isolates that were used to develop isolate-specific primers (Fig. 1).

Design of isolate-specific primers. Isolate-specific primers were designed using Primer3 software (Koressaar and Remm, 2007; Untergasser et al., 2012). Primer specificity was tested following Robin et al. (2016). A total of 115

primers were designed (Table 2, Supplementary Table 1).

Collection of *P. brassicae* infected isolates. We infected Chinese cabbage variety Bullam-3-ho with 11 previously collected *P. brassicae* isolates: Gangneung (1 and 2), Yeoncheon, Daejeon, Haenam (1 and 2), Seosan, Pyeongchang, Hoengseong, Geumsan, and Goesan (Jo et al.,

Table 2. Forward and reverse primer sequences used to identify Korean *Plasmodiophra brassicae* isolates

Sequence No.	Given primer name	Forward sequence	Reverse sequence	Product Size (bp)	Genomic positions	
					Start	End
>LCB455.5	Ycheon_9	ACAGTGCGGGTAGTCTATACG	ACAGTGCGGGTAGTCTATACG	1317	35690	34374
>LCB460.5	Ycheon_10	TCGAACCAGAAACCGTGAAC	TTCGGCACATCCCCATACAT	1589	44998	43410
>LCB975.5	Ycheon_14	GGTATGATTGTACAGGTCGCC	CACTTTCCGGCAAACTACGC	1459	3247	1789
>LCB888.2	ZJ1_3	TGGACGAGGCTGACAATCTT	CAAAAGCTCACAACGGTCGA	1073	224268	223196
>LCB1098.2	ZJ1_4	GTCGGCGATCTGGAGAACTA	CACGACGAGCTTCATTCCAG	1060	66622	65563
>LCB1098.2	ZJ1_5	CACCAGTATTTCGCGCTTCA	AGGTTTGATCCGCCTGTACA	1118	65497	64380
>LCB608.2	ZJ1_35	CTCCAACTTGAAAGGCAGAGG	GATGCAACGAACGCTTTGAC	547	761	1307
>LCB679.2.1	ZJ1_40	TGAATACGAACCGATGCTGC	CGTACACAATGGTTCGGCTG	517	224996	224480
>LCB679.2.2	ZJ1_41	CGGAGTTGAACGAGACTAGC	GCATACCTAATTGAGCGCGT	722	222677	222019
>LCB830.2	ZJ1_49	CTTCATCACTAGGCATGCGG	AACGAATCAGTGCATGGACC	485	118258	118742
>LCB975.2	ZJ1_56	GGTACCGGTTCTTCACGTTC	GAGTGAACCCAGACCTGACA	1450	138164	136715
>LCB1188.2	ZJ1_61	TTCACATCACGTTGCACAGG	CCTCCACCGCGTTATCAGTA	452	806998	807400

2016; Kim et al., 2016; Lail et al., 2017). Fresh clubroot galls were collected and washed for isolation of *P. brassicae* genomic DNA.

***P. brassicae* DNA isolation.** DNA was isolated following the method of Laila et al. (2017). Galls from all isolates were cleaned in running tap water and the outer surface was peeled off. The cleaned galls were then surface-sterilized with 70% (*v/v*) ethanol for 60 s. Approximately 100 mg of gall tissue was collected in a 1.5 ml Eppendorf tube. Individual gall samples were homogenized in 1.5 ml cetrimethyl ammonium bromide (CTAB) (20 g CTAB, 12.11 g Tris, 81.8 g NaCl, and 7.4 g·l^{-1} disodium-ethylene diamine tetraacetic acid (EDTA); pH 8.0) solution with 30 µl proteinase K (20 mg·l^{-1}, GeneScan) and 30 µl DNaseI (Sigma, St. Louis, MO, USA). The homogenized galls were then incubated at 60°C for 2 h. DNaseI eliminates *Brassica rapa* host DNA as much as possible (Buhariwalla and Mithen, 1995; Buhariwalla et al., 1995; Manzanares-Dauleux et al., 2001) as it is believed that DNA of *P. brassicae* become integrated with host DNA during infection process (Bryngelsson et al., 1988). The homogenized suspensions were then centrifuged at 10,000 × *g* for 5 min. SFG1 Buffer (400 µl) and RNase A (4 µl, Omega Bio-Tek) were mixed with the supernatant in a 1.5 ml tube and vortexed at a maximum speed. The EZNA SP Fungal DNA Miniprep Kit (Omega Bio-Tek, Doraville, GA, USA) was used to extract *P. brassicae* DNA, following the manufacturer's protocol. DNA was stored at −20°C after its quantity and quality were assessed with a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Extraction of DNA from host control plant. A Japanese cabbage variety (Akimeki) that is resistant to clubroot disease was used as a control to assess the specificity of *P. brassicae* host genomic DNA. Host genomic DNA was extracted with the Qiagen DNeasy Minikit following the manufacturer's instructions (Qiagen, Hilden, Germany).

PCR amplification and agarose gel electrophoresis. The Phusion High-Fidelity DNA PCR kit (New England Biolabs, Inc., Ipswich, MA, USA) was used to amplify genomic DNA of 11 *P. brassicae* isolates. Each 50 µl PCR reaction included 1 µl gDNA, 2 µl of each primer, 0.6 µl Phusion DNA Polymerase, 10 µl Phusion HF Buffer, 4 µL dNTPs, and 29.4 µl sterile distilled water (see Table 2 for primer sequences). Reactions were pre-incubated for 5 min at 95°C followed by 35 cycles of amplification at 95°C for 30 s, 58°C for 30 s and 72°C for 45 s in an Eppendorf thermal cycler (Eppendorf AG, Hamburg, Germany). The

melting of the mixture was carried out at 72°C for 5 min. PCR products were visualized on a 1.2% agarose gel with 1 × TBE buffer.

Isolate-specific marker selection. Race-specific markers were selected based on the presence and absence of amplified bands. When a particular primer pair amplified only one isolate and host DNA, the amplified region was considered an isolate-specific marker.

Results and Discussion

Sequence comparison. By comparing genomic DNA sequences, we have found several regions that vary among *P. brassicae* isolates. There e3 and ZJ-1 isolates had 59.2% and 59.0% GC content, respectively. Notable variation in %GC content was also evident in four Korean *P. brassicae* isolates, ranging from 56.0-59.0 (Fig. 1). We used the e3
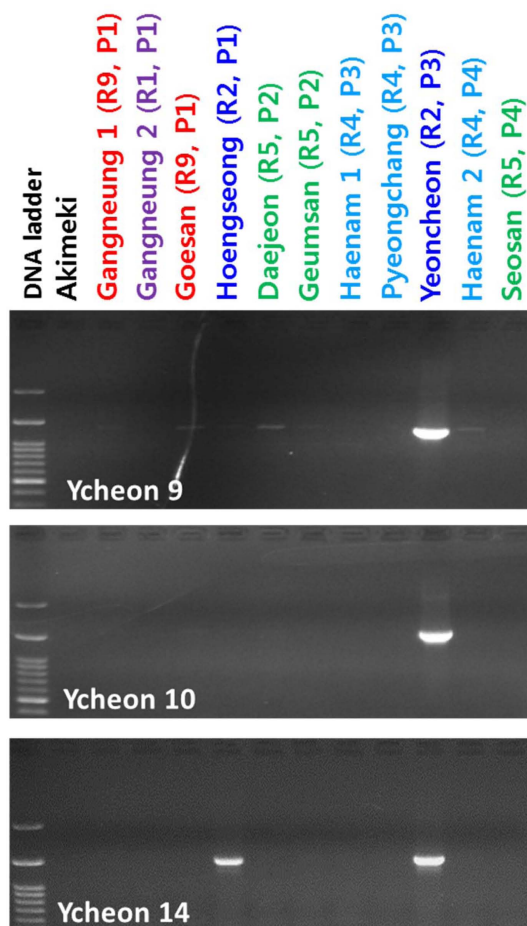
Fig. 2. Three markers specific to the Yeocheon (Ycheon 9 and Ycheon 10), Yeocheon and Hoengseong (Ycheon14) isolates of race 2. Akimeki is a control host. R, race; P, pathotype.

isolate as a reference genome for the purpose of genome comparisons.

Yeoncheon-isolate specific marker. Out of 117 designed markers, 20 primers were e3-specific, 61 primers were ZJ1-specific, two primers were Daejeon-specific, 17 primers were Yeoncheon-specific, six primers were Haenam2-specific, and 11 primers were Gangneung1-specific. Only 12 markers amplified genomic DNA of either a single isolate or two isolates of the same race. The remaining 107 marker sets were non-specific (Supplementary Fig. 1). Two Yeoncheon-specific primers, Ycheon9 and Ycheon10, produced only a single amplicon (Fig. 2), indicating that these sequences characterized amplified region (SCAR) markers might enable specific detection of the Yeoncheon isolate. The primer pair Ycheon14 amplified the targeted region in both the Yeoncheon and Hoengseong isolates (Fig. 2). Since both of these isolates belong to race 2, this marker may be broadly useful in detecting race 2 *P. brassicae* isolates.
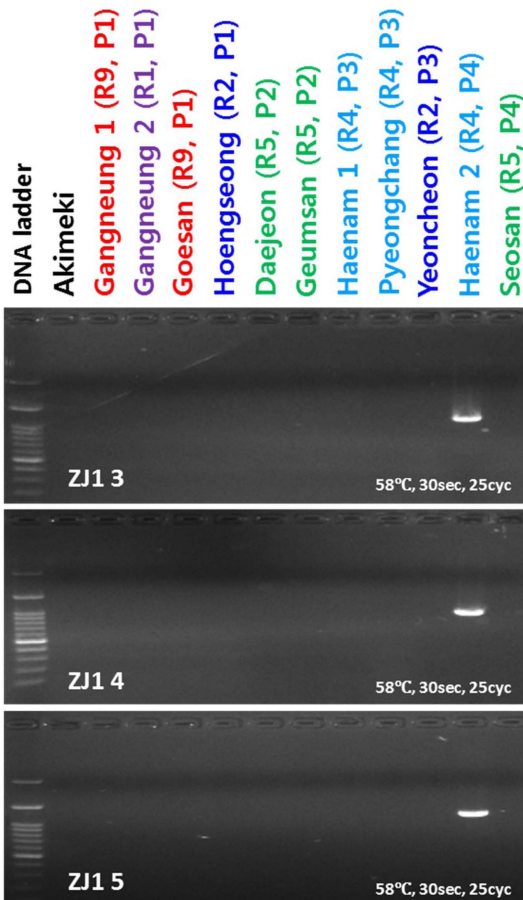
Fig. 3. Three markers showing specificity to the Haenam2 isolate of race 4 and pathotype 4. Akimeki, a Japanese Chinese cabbage cultivar, was a control host. R, race; P, pathotype.

The difference in isolate specificity of markers Ycheon9 and Ycheon10 within race 2 might be due to sequence variation between the isolates Yeoncheon and Hoengseong.

Haenam2-isolate specific markers. Three primer pairs that were designed based on the genomic sequence of the Chinese isolate ZJI (ZJI 3, ZJI 4 and ZJI 5) produced a single amplicon from the Haenam2 isolate (Fig. 3). These three SCAR markers failed to amplify the target region in the other 10 isolates and the control host plant (Fig. 3). It is possible that these markers also amplify the Chinese *P. brassicae* isolate ZJI, but we did not test this possibility.
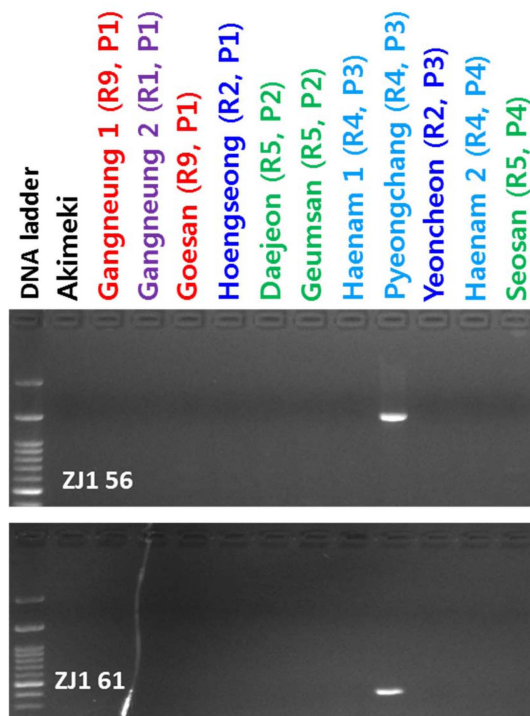
Hoengseong-isolate specific markers. Four primer pairs (ZJI 35, ZJI 40, ZJI 41 and ZJI 49) amplified the target region in the Hoengseong isolate only (Fig. 4), indicating that these markers can specifically detect this isolate. Amplification of the Hoengseong isolate with ZJI-specific SCAR markers indicates that the specific primer sequence used is absent in the other *P. brassicae* isolates examined in this study.

Pyeongchang-isolate specific markers. Two other markers (ZJ1 56 and ZJ1 61) amplified the target region in the Pyeongchang-isolate only (Fig. 5). The amplicons were of expected size. These two SCAR markers can be utilized in identifying the Korean *P. brassicae* isolate Pyeongchang. This result further indicated that ZJ1 isolate has genome similarity with only Pyeongchang-isolate at the primer binding site.

Race- and isolate-specific markers and their potential applications. In this study, we developed 12 SCAR markers that identify particular Korean isolates of the clubroot pathogen *P. brassicae*. Eleven of these markers were specific to four single isolates. Importantly, two Yeoncheon isolate-specific markers perfectly amplified only Yeoncheon isolate of Korea. These markers have the potential to specifically identify Yeoncheon-like isolates from Korean fields. Similarly, the other nine SCAR markers were specific to the Haenam2, Hoengseong, and Pyeongchang isolates. Because three Haenam2-specific markers, four Hoengseong-specific markers, and two Pyeongchang-specific markers were designed based on the genome sequence of the Chinese isolate ZJ1, we predict that these markers may also amplify this Chinese isolate, but we did not test this possibility. We therefore recommended that these markers be tested on all *P. brassicae* isolates worldwide to further test their specificity and potentially establish them as universal markers. One of the pre-existing limitation at the
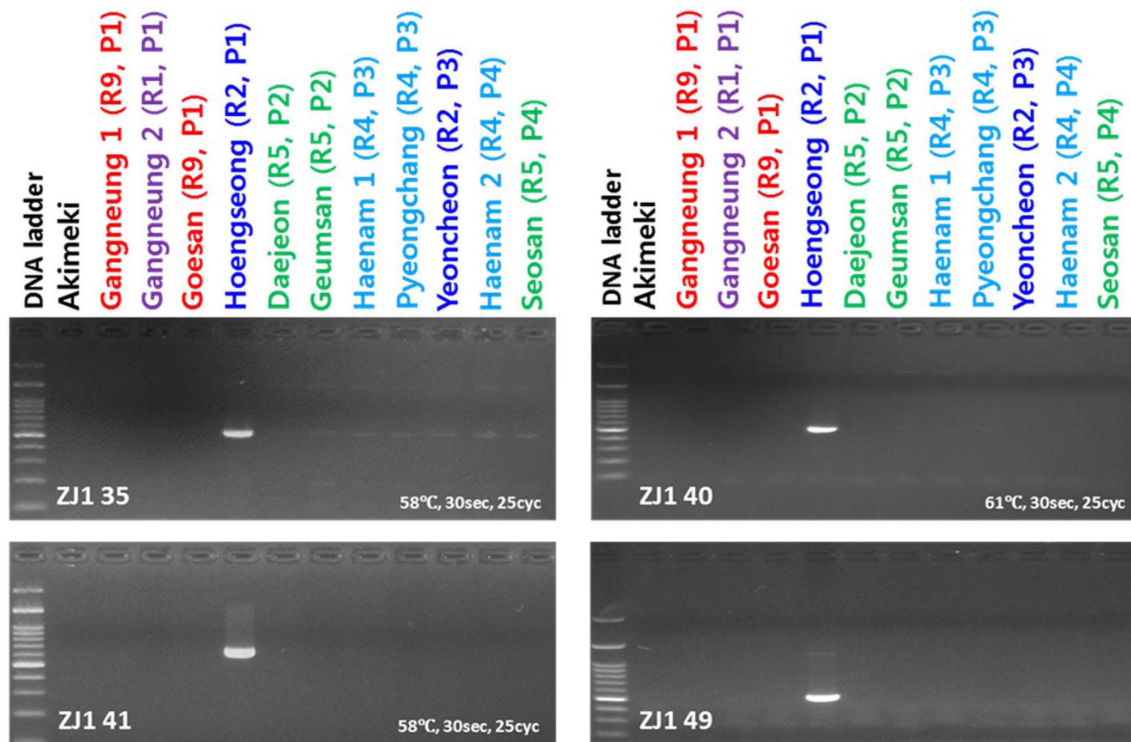
Fig. 4. Four markers showing specificity to the Hoengseong isolate of race 2 and pathotype 1. Akimeki, a Japanese Chinese cabbage cultivar, was a control host. R, race; P, pathotype.

commencement of this study was lack of any race-specific and isolate-specific markers that can detect a particular *P. brassicae* isolate. The newly developed marker can efficiently detect four Korean *P. brassicae* and possibly one Chinese isolate based on genomic DNA sequence variation. Efforts are needed to develop markers to enable detect other Korean *P. brassicae* isolates.

Author contributions

I-SN, J-IP and SKN conceived and designed the study. SKN conducted the *in silico* analysis. RL and JY prepared fungal spores. RL isolated *P. brasicae* DNA. JY and HTK conducted PCR amplification. AHKR wrote the manuscript. AHKR, SKN and JY created the tables and figures. All authors read and approved the final draft of the manuscript.

Acknowledgments

Fig. 5. Four markers showing specificity to the Pyeongchang isolate of race 4 and pathotype 3. Akimeki, a Japanese Chinese cabbage cultivar, was a control host. R, race; P, pathotype.

Conflict of interest

No conflicts of interest exist.

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