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FUNGAL DISEASES

Use of microsatellite marker Pi26 for rapid discrimination of five Japanese genotypes of *Phytophthora infestans*

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Abstract Microsatellite markers were tested to rapidly discriminate five Japanese genotypes (US-1, JP-1, JP-2, JP-3, and JP-4) of *Phytophthora infestans*. Collected from 1958 to 2007, 111 isolates of Japanese *P. infestans* were examined using a fluorescent-labeled primer and capillary electrophoresis. Microsatellite marker Pi26 generated specific products for each genotype without any differences in terms of isolation area or year for a particular genotype. The Pi26 marker is a powerful tool for obtaining information on the structure of Japanese populations of *P. infestans*.

Keywords Simple sequence repeats · Genotype identification · *Phytophthora infestans* · Potato late blight

Potato late blight is currently one of the most important diseases of potato in Japan. *Phytophthora infestans*, the pathogen of late blight in potatoes and tomatoes, has many different genotypes worldwide (Forbes et al. 1998). In Japan, five genotypes of *P. infestans*, defined as RG57 fingerprint types, have been reported in recent years (Gotoh et al. 2005). Isolates of genotypes US-1, JP-2, JP-3, and JP-4 are A1 mating types, and JP-1 is an A2 mating type. The proportion of these genotypes in Japanese isolates of *P. infestans* has rapidly changed in the last 10 years (Akino et al. 2009), and the genotypes differ in both pathogenicity and fungicide resistance (Kato and Naito 2001; Hirotomi et al. 2009). Thus, discriminating the genotypes of isolated *P. infestans* is important to determine the degree of danger they impose. The RG57 DNA fingerprinting method is required (Goodwin et al. 1992) to distinguish all genotypes of *P. infestans* in Japan (Gotoh et al. 2007). Although this method gives reliable information on genotypes, an easier and quicker method is needed because RG57 DNA fingerprinting is relatively difficult and time-consuming.

Akino et al. (2008) reported the N650ab marker for rapidly discriminating Japanese genotypes of *P. infestans*, but the practical performance of N650ab is limited because it cannot distinguish among JP-2, JP-3, and JP-4. Recently, however, new usable microsatellite (simple sequence repeat) markers that can discriminate several genotypes with high resolution have been reported for *P. infestans* (Knapova et al. 2002; Lees et al. 2006). In this study, we applied these markers to rapidly discriminate genotypes of Japanese isolates of *P. infestans*.

The tested isolates of *P. infestans* were derived from diseased leaves and tubers of potatoes collected from 1958 to 2007 in Japan. Five isolates of US-1 and 25 isolates of each of JP-1, JP-2, JP-3, and JP-4 were used in this study (Table 1). Methods for isolating and maintaining the isolates were described previously (Nishimura et al. 1999). The genotypes of these isolates were confirmed by RG57 DNA fingerprinting (Goodwin et al. 1992).

For PCR studies, genomic DNA of the tested isolates was extracted using the protocol described by Goodwin et al. (1992). DNA solutions were diluted to 10 ng/μl and used as templates of the PCR reactions. Preliminary tests were carried out using seven sets of PCR primers that can detect the loci *Pi02, Pi04, Pi26, Pi33, Pi56, Pi63*, and *Pi65* (Lees et al. 2006). These loci were selected because they are polymorphic in some European isolates and show good separation of bands in gel electrophoresis. For the preliminary tests, three isolates of each of Japanese genotypes

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Table 1 Genotypes of the tested isolates of *Phytophthora infestans* and results of the Pi26 marker detection system

Genotype	Origin	No. of isolates	Year	Mating type	Pi26 marker[d] (bp)					
					168	171	173	177	179	183
US-1	Hokkaido, Japan	4	1990–1993	A1	✔	✔				
	United States[c]	1	1994	A1	✔	✔				
US-1.4	Hokkaido	1	1958	A1	✔	✔				
JP-1	Hokkaido	14	1987–2007	A2		✔	✔	✔		
	Aomori	2	2007	A2		✔	✔	✔		
	Chiba	2	2006	A2		✔	✔	✔		
	Yamanashi	1	2007	A2		✔	✔	✔		
	Fukuoka	2	2003	A2		✔	✔	✔		
	Miyazaki	1	1999	A2		✔	✔	✔		
	Kagoshima	3	2002–2007	A2		✔	✔	✔		
JP-1.1	Hokkaido	2	1996–2002	A2		✔	✔	✔		
KR-1	Hokkaido	1	1993	A2		✔	✔	✔		
JP-2[a]	Hokkaido	21	1997–2007	A1				✔		
	Gunma	3	2000	A1				✔		
	Nagasaki	1	2002	A1				✔		
JP-2.1	Hokkaido	1	2001	A1				✔		
JP-2.2	Hokkaido	1	2001	A1				✔		
JP-3[a]	Hokkaido	21	1998–2007	A1			✔	✔		
	Ibaraki	1	2007	A1			✔	✔		
	Nagasaki	3	2002	A1			✔	✔		
JP-3.1	Hokkaido	1	2001	A1			✔	✔		
JP-4[b]	Hokkaido	20	2001–2007	A1				✔	✔	✔
	Ibaraki	4	2006–2007	A1				✔	✔	✔
	Miyazaki	1	2007	A1				✔	✔	✔

[a] Identified in Gotoh et al. (2005)

[b] Identified in Gotoh et al. (2007)

[c] American Type Culture Collections (ATCC208835)

[d] Size of the PCR products generated by Pi26F and Pi26R primers

(US-1, JP-1, JP-2, JP-3, and JP-4) were used. PCR conditions followed Lees et al. (2006); 10 µl of PCR solution was analyzed using 12% polyacrylamide gel electrophoresis. Electrophoresis was performed using the protocol of the AFLP Analysis System I (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Bands were detected by silver staining according to the manufacturer's protocol (DNA Silver Staining Kit; GE Healthcare Bioscience, Tokyo, Japan).

Primers for the loci *Pi02, Pi04, Pi33, Pi56, Pi63,* and *Pi65* generated the same pattern of bands in different genotypes (data not shown). We therefore concluded that these loci were not useful for the purpose of this study. However, primers for the *Pi26* locus (Pi26F: 5′-GCAG TAGCCGTAGTCCTCAG-3′; Pi26R: 5′-GTTCCAAATC GTCAACCAAC-3′) generated a different band pattern in all examined genotypes. These patterns consisted of one or two bands that could be distinguished on the polyacrylamide gels (data not shown). We therefore used the primers for the *Pi26* locus for further investigations.

One hundred and eleven isolates of Japanese *P. infestans* (Table 1) were tested for discrimination using primers for the *Pi26* locus. The PCR products generated using primer Pi26 for an English isolate of *P. infestans* (corresponding to "C9" in Lees et al. 2006) was used as a standard. The DNA samples were prepared as described earlier. For automated analysis, forward primers of the *Pi26* locus (Pi26F) were labeled at the 5′-end with fluorescent dye (Beckman Dye 2; Sigma-Genosys Co., Ltd., Tokyo, Japan). PCR reactions were done with three replications as described by Cooke (2008). PCR samples were loaded into the DNA sequencer (model CEQ 8000 Genetic Analysis System; Beckman

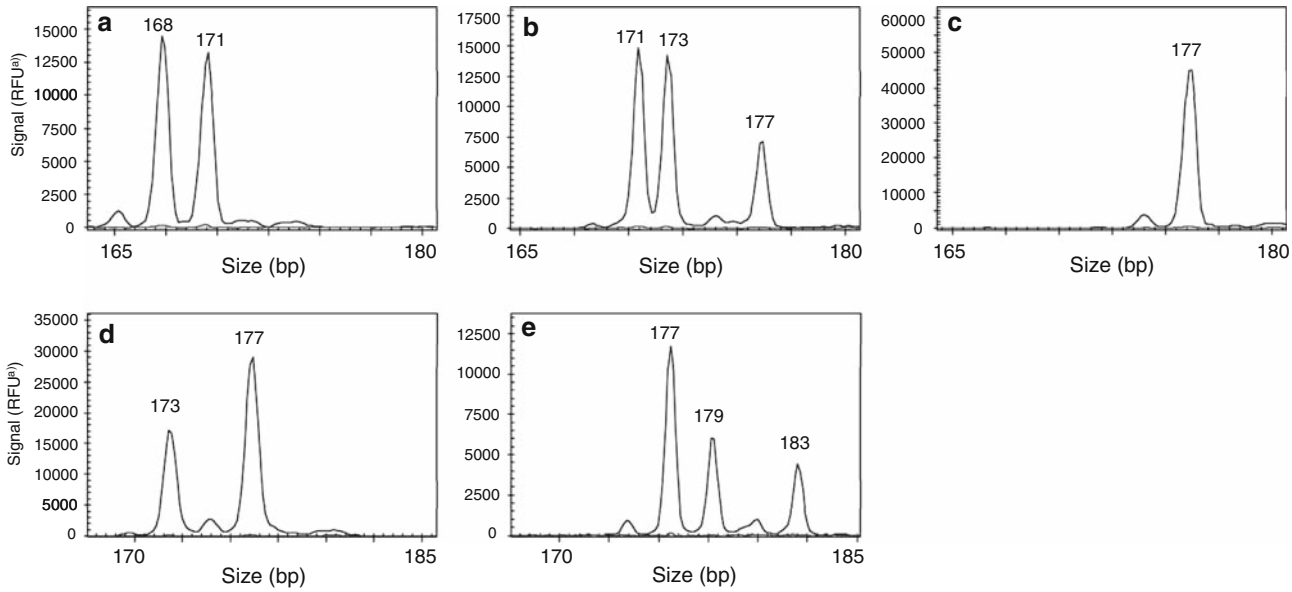

Fig. 1 Electropherograms of the fluoresceinated amplification products for locus *Pi26* in isolates of each genotype of *Phytophthora infestans* in Japan. **a** IB008S (US-1), **b** KG0701 (JP-1), **c** HK6 (JP-2), **d** Ho1-14 (JP-3), **e** IR0704 (JP-4). **a** Relative fluorescence units

Coulter, Tokyo, Japan) and run according to the manufacturer's instructions. Peak size data were analyzed using Fragment Analysis Software (Beckman Coulter) for estimating allele sizes. Consequently, the sizes of PCR products (base pairs, bp) gave specific patterns for each genotype (Fig. 1): 168/171 (US-1), 171/173/177 (JP-1), 177 (JP-2), 173/177 (JP-3), and 177/179/183 (JP-4). Despite differences in isolation site and year, these PCR products were the same in all tested isolates of each genotype in three replications (Table 1). Products of 168 bp in US-1 and 173 bp in JP-1/JP-3 found in the present study were not reported by Lees et al. (2006). Furthermore, the products of the minor genotypes (Gotoh et al. 2007) were identical to those of the original major genotypes (US-1.4 and US-1, JP-1.1 and JP-1, KR-1 and JP-1, JP-2.1 and JP-2, JP-2.2 and JP-2, JP-3.1 and JP-3). Although the Pi26 marker cannot distinguish these minor genotypes from the major ones, this poses no problem because the number of minor genotypes has been very low over the past 10 years.

Generally, PCR products can be analyzed using polyacrylamide gel electrophoresis (Baba et al. 2009). However, when analyzing many samples, discriminating bands close to each other was difficult in some cases (data not shown). Because it might be difficult to use polyacrylamide gel electrophoresis routinely for this purpose, we chose capillary electrophoresis for reliable discrimination in our study.

Most JP-1 isolates are highly aggressive in tomato and comprise the main pathogen of late blight of greenhouse tomatoes in Kanto district, Japan (Hirotomi et al. 2009). JP-4 isolates have spread from the eastern part of Hokkaido to other parts of Japan in recent years (Akino et al. 2009), and thus investigating all genotypes of *P. infestans* in Japan is needed. The Pi26 marker proved to be a powerful tool for obtaining information on the structure of Japanese populations of *P. infestans*.

The Pi26 marker can also be easily applied for analyzing the past isolates of *P. infestans*. However, identification techniques require constant improvement because populations of Japanese *P. infestans* have changed dramatically in recent years (Akino et al. 2009). Hence, the diversity of the populations of Japanese *P. infestans* should be estimated using several microsatellite markers to prepare marker sets for discrimination of new populations that may later arise. Developing a method for comprehensively estimating populations of Japanese *P. infestans* will be the subject of future investigations.

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