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FUNGAL DISEASES

N605ab, a specific molecular marker for *Phytophthora infestans*, distinguishes three genotypes in Japan

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Abstract Random amplified polymorphic DNA (RAPD) analysis using the OPG-06 primer generated specific patterns for Japanese genotypes US-1, JP-1, and a new A1 (JP-2, JP-3, and JP-4) of *Phytophthora infestans*. N605, a specific RAPD fragment, was cloned and sequenced. PCR primers BD1/BD2 were constructed based on the N605 sequence and were used to clarify the genotypes. PCR products using the BD1/BD2 primers (N605ab marker) easily distinguished the new A1 from US-1 and JP-1. This technique provides a simple and effective method for rapid genotype discrimination that can be used in ecological experiments and forecasts for the occurrence of late blight.

Keywords RAPD · Population genetics · Potato late blight

Phytophthora infestans is a plant pathogenic oomycete fungus that causes late blight on potato and tomato. It has two mating types, A1 and A2 (Brasier 1992), which form oospores by sexual reproduction. A single genotype, designated US-1, dominated *P. infestans* populations outside Mexico between 1840 and the mid-1970s (Goodwin et al. 1997). This situation changed in the early 1980s, when A2 mating type isolates appeared in Europe. Currently, A2 mating type isolates are present almost everywhere that potatoes are grown (Goodwin and Drenth 1997).

In Japan, three genotypes, named US-1, JP-1, and Japanese new A1 (Mosa et al. 1989; Koh et al. 1994; Kato and Naito 1997), are known. Japanese new A1 is divided into three large groups, new A1-A, -B, and -C (Kato and Naito 2001). New A1-A occurs in Europe, Russia, China, and Japan (Akino et al. 2004), while new A1-B has only been isolated in Japan (Akino et al. 2005). The A1-B may have been generated in Japan through sexual reproduction involving A1-A and JP-1 or a related genotype (Akino et al. 2005). The cultural characteristics (spore formation and metalaxyl resistance) and the pathogenicity against potato cultivars differ for these three genotypes (Kato and Naito 2001). More recently, A1-A and -B were defined as genotypes JP-2 and JP-3, respectively (Gotoh et al. 2005), and part of the A1-C isolates was defined as genotype JP-4 (Gotoh et al. 2007). Discriminating genotypes of *P. infestans* in the field is important for forecasting the occurrence of late blight. In this study, a PCR marker was created for easy discrimination of the three Japanese genotypes of *P. infestans*.

For random amplified polymorphic DNA (RAPD) analysis, isolates DN111 (US-1), TB201 (JP-1), and A8 (JP-3) were used. For the specificity test of the PCR marker, one isolate of JP-4, three isolates of Japanese US-1, and 10 isolates each of JP-1, JP-2, and JP-3 were chosen (Table 1). Of the list of tested isolates given in Akino et al. (2005), isolates HK2, HK6, 98H2 and TK0119 were not used. These isolates represent the constructs of the Japanese *P. infestans* genotypes. All isolates were maintained on rye agar medium supplemented with 2% (w/v) sucrose (RS medium) (Caten and Jinks 1968) at 18°C.

Genomic DNA of the tested isolates was extracted using the protocol described by Goodwin et al. (1992). RAPD

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Table 1 Genotypes of the tested isolates of *Phytophthora infestans* and the results of N605 marker detection

Genotype	Origin	No. of isolates	Mating type	N605ab marker[e] (bp)
US-1[a]	Hokkaido, Japan	2	A1	530
	United States (ATCC940501[d])	1		
JP-1[a]	Hokkaido	8	A2	580
	Nagasaki, Japan	1		
	Miyazaki, Japan	1		
JP-2[ab]	Hokkaido	8	A1	530 / 580
	Gunma, Japan	2		
JP-3[ab]	Hokkaido	10	A1	530 / 580
JP-4[c]	Hokkaido	1	A1	530 / 580

[a] Isolates tested in Akino et al. (2005)

[b] Described in Gotoh et al. (2005)

[c] Described in Gotoh et al. (2007) (isolate KM0102)

[d] American type culture collection

[e] Size of the PCR products generated by BD1 and BD2 primers

analysis of the isolates was performed using 220 10-mer primers (Operon RAPD 10 mer kit A–K; Operon Technologies, Alameda, CA, USA) and the protocol described by Monna et al. (1994). The amplification program (40 cycles) was as follows: denaturation at 94°C for 60 s, annealing at 35°C for 120 s, and extension at 72°C for 180 s. The OPG-06 primer represented the specific amplified products of the three genotypes. The approximately 610- and 650-bp bands were observed in the US-1 and JP-1 isolates, respectively, and both bands were represented in the JP-3 isolate (Fig. 1).

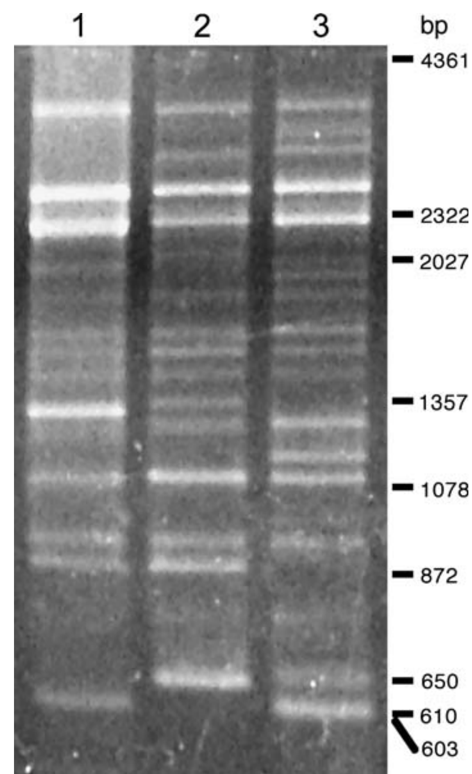

Fig. 1 Random amplified polymorphic DNA banding patterns in isolates of Japanese *Phytophthora infestans* genotypes using the OPG-06 primer. The molecular size is shown in the *right* panel. *1* DN111 (US-1), *2* TB201 (JP-1), and *3* A8 (JP-3)

The 610-bp band of the US-1 isolate (DN111) was purified (using GTG NuSieve agarose; FMC BioProducts, Rockland, ME, USA), cloned (using vector pBluescript II SK$^-$ and *Escherichia coli* XL1-Blue MRF'; Stratagene, La Jolla, CA, USA), and sequenced (Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit with 7-deaza dGTP; Amersham Pharmacia Biotech, Piscataway, NJ, USA; ALFexpress DNA Sequencer; Pharmacia Biotech, Uppsala, Sweden) according to the manufacturers' instructions. This DNA fragment (605 bp) was designated as N605 (GenBank accession no. AB331243). There were no similar sequences in the DNA database (FASTA/BLAST, DNA data bank of Japan).

PCR primers for genotype analysis (BD1, 5′-CGCTGGCAAAGCTAGAGC-3′; BD2, 5′-CATGTGGAAAAACAGGAC-3′) were constructed from the sequences inside the binding site of the OPG-06 primer in the N605 fragment. PCR analysis was performed using these primers on all tested isolates. The DNA extraction method was simplified as follows: 100 mg of mycelia, incubated in RS broth, were added to 500 µl of TE buffer in a 1.5-ml centrifuge tube, and sonicated for 10 s (UR-20P; Tomy Seiko, Tokyo, Japan) at 4°C. The suspensions were extracted once with equal volumes of phenol–chloroform–isoamylalcohol (25:24:1), and the DNA was precipitated with isopropanol. PCR was performed using Taq DNA polymerase (Takara Taq; Takara Biochemicals, Tokyo, Japan) according to the manufacturer's instructions. The amplification program (40 cycles) was as follows: denaturation at 94°C for 40 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s. Amplified products were analyzed using 1.5% agarose gel electrophoresis.

Bands of approximately 530- and 580-bp were observed in the US-1 and JP-1 isolates, respectively, and both bands were found in JP-2, JP-3, and JP-4 isolates. All the tested isolates gave the same results. These bands were designated N605ab marker (N605a: 530-bp band, N605b: 580-bp band). The expected length of N605a based on the sequence data (536 bp) agreed well with the electrophoretic data.

Additional bands were not observed in any of the genotypes. A part of the results are shown in Fig. 2.

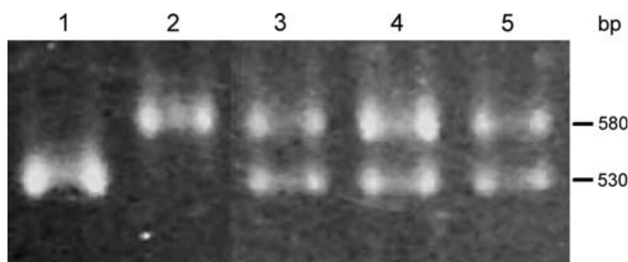

Fig. 2 PCR products of the primers BD1 and BD2 from the total DNA of Japanese isolates of *Phytophthora infestans*. The molecular size is shown in the *right* panel. Lanes *1* IB008s (US-1), *2* MY-1 (JP-1), *3* 98F1 (JP-2), *4* HO1-14 (JP-3), *5* KM0102 (JP-4)

Until now, it has been necessary to analyze allozyme (Goodwin et al. 1995) or DNA fingerprints (Goodwin et al. 1992) to distinguish genotypes of *P. infestans*. Although some novel molecular markers were reported for *P. infestans* (Knapova et al. 2002; Lees et al. 2006), it was necessary to develop marker suites for analyses of Japanese isolates. From the results of this study, N605ab marker provides a simple and effective method for rapid genotype discrimination. This marker easily distinguished the new A1 from the US-1 and JP-1. At present, only a few US-1 and JP-1 isolates occur in Hokkaido, and the new A1 genotypes (JP-2 and JP-3) are the main constituent elements of *P. infestans* (unpublished data). However, it is likely that the JP-1 and new A1 genotypes exist together in other parts of Japan (Akino et al. 2005). Although the isolates of US-1 and JP-1 are sensitive to the fungicide metalaxyl, many JP-2 and JP-3 isolates are highly resistant to it (Gotoh et al. 2005). PCR analysis of the isolates using N605ab marker will be useful to examine the existence of both mating type, A1 (JP-2, 3, 4) and A2 (JP-1). This tool will be helpful to judge the possibility of sexual reproduction and the occurrence of metalaxyl-resistant isolates in specific areas. Additionally, this primer set has great promise for discriminating isolates in various types of ecological experiments (e.g., for rapid discrimination of isolates recovered from mixed infections in plants). However, it is still necessary to analyze allozyme or DNA fingerprints to discriminate among JP-2, JP-3, and JP-4, which are closely related to each other (Akino et al. 2005; Gotoh et al. 2007). Developing a method for the rapid discrimination of these genotypes will be the subject of future investigations.

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