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Genetic DNA Marker for A2 mating type in *Phytophthora infestans*

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The *Phytophthora infestans* requires two mating types for sexual reproduction. Amplified fragment length polymorphism (AFLP) was used to specifically detect different mating types of *P. infestans*. The AFLP primers E+AA (5'-GACTGCGTACCAATTCAA-3') and M+CAA (5'-GATGAGTCCTGAG-TAAC AA-3') detected a fragment that is specific in the A2 mating type of *P. infestans*. This fragment was cloned and sequenced. Based on the sequence data, PHYB-1 and PHYB-2 primer were designed to detect the A2 mating type of *P. infestans*. A single 347 bp segment was observed in the A2 mating type of *P. infestans*, but not in the A1 mating type of *P. infestans* or other *Phytophthora* spp. Identification of mating type was performed with phenotype (sexual reproduction) and genotype (CAPs marker) methods. Two factors, the annealing temperature and template DNA quantity, were investigated to determine the optimal conditions. Using mating type-specific primers, a unique band was obtained within annealing temperatures of 57°C-62°C and DNA levels of 10pg-100 ng (data not shown).

Key words: *Phytophthora infestans*, annealing temperature, A2 mating type, AFLP, PCR

The late blight disease of potatoes is found in nearly all areas of the world where potatoes are grown. It is most destructive, however, in areas with frequent cool, moist weather. Late blight is also very destructive to tomatoes and some other members of the family Solanaceae. Late blight may kill the foliage and stems of potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill) plants at any time during the growing season (Fry, 1992; Goodwin, 1992a). Pathogens of the late blight of potatoes, *P. infestans*, exhibit two mating types which are called A1 and A2 (Gallegly, 1958; Brasier, 1992). These two mating types are physiologically differentiated by their ability to produce and respond to specific sexual compatibility substances.

The genetic mechanism for differentiating the A1 and A2 types in *P. infestans* has been the subject of much speculation, particularly since oomycetes are diploid and therefore mating-type alleles potentially operate in a heterozygous condition. This is quite unlike the common situation in ascomycetes and basidiomycetes, where the mating type is expressed in a haploid life age (Kües and Casselton, 1992). Until the late 1980s, only one mating type (A1) was present in countries outside Mexico. In the last few years, however, both mating types (A1, A2) have become widely distributed in most countries. Strains of the new mating type (A2) are much more aggressive than those of the old one (A1) and quickly replace them. When the two mating types grow adjacently, the female hypha grows through the young antheridium and develops into a globose oogonium above the antheridium. The antheridium then fertilizes the oogonium, which develops into a thick-walled and hardy oospore. Oospores germinate by means of a germ tube that produces a sporangium, although at times the germ tube grows directly into the mycelium.

For a long time genetic studies on *P. infestans* and other oomycetes were hampered (Judelson, 1996) by the lack of morphological and biochemical mutants which have been very useful in unravelling the genetics of true fungi. Instead, isozyme markers were used, but again this approach faced limitations because very few polymorphisms were found. In *P. infestans*, only 3 of 50 tested enzymes appeared to be polymorphic (Tooley *et al.*, 1985). Recently, a new technique called amplified fragment length polymorphism (AFLP) was developed by Vos *et al.* (1995). AFLP is a powerful, reliable, stable and rapid assay with potential application in genome mapping, DNA fingerprinting and marker assisted breeding (Old and Primrose, 1994; Rusell *et al.*, 1997; Reineke and Karlovsky, 2000). The aim of this study was to develop a DNA marker linked to the determinants of the A2 mating type in *P. infestans* with AFLP analysis.

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Materials and Methods

Fungal strains and genomic DNA isolations

The isolates used in this study including *P. infestans* were either isolated from infected plants or were provided by other researchers. Mating types were determined by growing strains adjacent to known A1 mating type (Na1-5, Na2-1) and the A2 mating type (YY-8, YY-9, YY-12) isolates and microscopically checking for oospores after 7-14 days. Afterward, the mating types were tested by using the CAPs marker W16 (Judelson, 1995).

The *P. infestans* isolates were cultured in a rye medium (60 g of rye grain and 20 g of dextrose) in 1 *l* of distilled water for 10 days at 18°C (Griffith, 1995), whereas the other *Phytophthora* species were incubated in V8 juice (200 ml of V8 juice, 5 g of $CaCO_3$) and a PDB (potato dextrose broth, 20 g PDB and dextrose each) medium for 10 days at 24°C in darkness. The bacterial isolates were grown in a Luria Bertani broth at 27°C overnight (Sambrook, 1989). The total genomic DNA of each isolate was extracted by a modification of the method described by Goodwin *et al.* (1992a).

AFLP analysis

The AFLP method developed by Vos *et al.* (1995) was used with some modifications. Genomic DNA (1 μg) was digested with the restriction endonucleases *Eco*RI and *Mse*I. Double-stranded adapters were then ligated to the ends of the restriction fragments followed by ethanol precipitation and resuspension in 40 μl of distilled water.

The sequence of the adapter fitting the *Eco*RI site was
5'-CTCGTAGACTGCGTACC
→ CTGACGCATGGTTAA-5'.

The sequence of the adapter fitting the *Mse*I site was
5'-GACGATGAGTCCTGAG
→ TACTCAGGACTCAT-5'.

Pre-amplification PCR was performed using standard adapter primers containing no selective nucleotides, followed by selective amplification using similar primers with two or three selective bases. Pre-amplification PCR started with a cycle of 30s at 94°C, 1 min at 60°C, and 1 min at 72°C and was followed by 20 cycles. After the pre-amplification, the reaction mixture was diluted to 200 μl with distilled water. For the selective amplification of a limited number of DNA restriction fragments, the secondary template DNA was amplified with primers containing two or three selective 3' nucleotides (*Eco*RI+2 and *Mse*I+3 primers; Bioneer, Korea). For selective amplification, the following PCR profile was used: the first cycle with 30s at 94°C, 30s at 65°C, and 1 min at 72°C followed by 9 cycles with a stepwise lowering of annealing temperature by 1°C in each cycle and 29 cycles with an annealing temperature of 56°C.

The purpose of this first selective PCR amplification was to reduce the complexity of the template fragments and to generate large quantities of template suitable for the second PCR amplification with radioactively labeled selective primers. The primers used in the amplification step are E+2 and M+3 primers that correspond to the *Eco*RI and *Mse*I ends of the primary template, respectively. "+2" indicates that the primer contains two selective nucleotides. The sequence of the E+2 primer was 5' -GACTGCGTTACCAATTCAA (E+AA), in which AA is the selective nucleotide, whereas the M+3 primers had the sequence 5'-GATGAGTCCTGAGTAACAA (M+CAA).

Amplification products were separated using standard 6% denatured polyacylamide gel-electrophoresis and detected using autoradiographic procedures.

Cloning and sequencing of specific DNA fragments

Selected amplification products were cloned into pGEM® -T Easy Vector (Promega, USA) and transferred into competent *E. coli* according to the supplier's instructions. Plasmid DNA was extracted by the alkaline lysis method and harvested by centrifugation at 15,000 rpm for 20 min at 4°C after incubation on ice for 20 min with the PEG solution (20% of polyethyleneglycol, 2.5 M of NaCl). A specific DNA fragment was sequenced using a silver Sequence™ DNA sequencing system (Promega, USA). In order to design proper primers, the DNA fragment was analysed using Primer3 Output (Primer3. cgi v 0.6) and the primer pairs were designed as PHYB-1 forward primer (5'-GATCGGATT AGTCAGACGAG-3') and PHYB-2 reverse primer(5'-GCGTCTGCAAGGCGCATTTT-3').

Southern blot hybridization

The specific fragment was electrophoretically separated on 0.8% agarose TBE gel (0.089M Tris, 0.089M Boric acid, 0.02M EDTA). After the gels were stained with ethidium bromide, they were transferred to a nylon membrane (Hybond™-N^+, Amersham, England). This membrane was prehybridized and hybridized in a hybridization incubator. The cloned DNA fragment was prepared as a probe for Southern blot analysis by random-primed labeling. A total of 100 μl of the PCR labeling reaction mixture containing 10 ng of template DNA, 0.5 uM of each primer (M13F, M13R), 1 × PCR buffer, 5 units of *Taq* polymerase (Dynazyme, USA), 0.1 mM of dGTP, dCTP, dATP, 0.09 mM dTTP, and 1 mM digoxigenin dUTP (Boehringer Mannhein, Germany) was prepared and reacted in a program consisting of 30 sec at 94°C, 30 sec at 62°C, 30 sec at 72°C for 30 cycles, and 8 min at 72°C. The color detection was processed using a DIG nucleic acid detection kit and all procedures were carried out using the supplied instruction manual (Boehringer Mannhein, Germany).

Idetification of mating type by using CAPs marker

For CAPs analysis, the reaction mixtures consisted of 10 ng of genomic DNA, 0.5 uM of each primer (W16-1,

W16-2), 200 uM dNTP, 1 unit *Taq* polymerase (Biotools, Spain), 1 × buffer. For the CAPs marker amplification, the following PCR profile was used: 30s at 94°C, 30s at 55°C, and 30s at 72°C for 30 cycles. DNA amplified using the W16-1 (5'-AACACGCACAAGGCATATAAATGTA-3') and W16-2 (5'-GCGTAATGTAGCGTAACAGCTCTC-3')

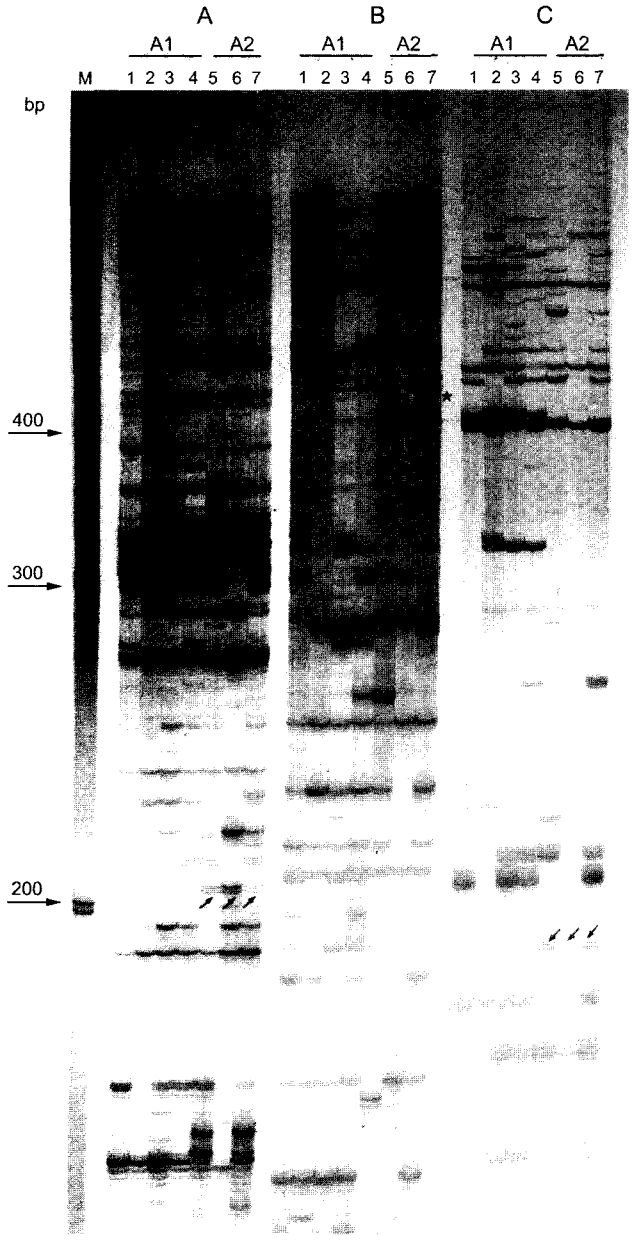

Fig. 1. Section of autoradiograph showing AFLP DNA fingerprints of *P. infestans*. The AFLP DNA fingerprints were generated with different primer combinations, A, E+AA/M+CTC; B, E+AA/M+CAA; and C, E+TA/M+CTT. Arrows indicate genomic DNA fragment derived from the A2 mating type in *P. infestans*. AFLP DNA marker linked to the A2 mating type in *P. infestans* was indicated with a star in B. Lane M, 1 kb DNA ladder; lanes 1-4, *P. infestans* Mat A1: YY-8 (1), YY-9 (2), YY-12(3), and YY-27 (4); lanes 5-7, *P. infestans* Mat A2: Na1-5 (5), Na2-1 (6), and Na1-2 (7).

primer was purified by ethanol precipitation, cleaved with *Hae*III restriction enzyme with 4-bp recognition sites, resolved by agarose gel electrophoresis, and visualized by staining with ethidium bromide (Judelson, 1995).

Results

Selection of AFLP primers for detection of A2 mating type specific DNA fragment of P. infestans

The genomic DNAs were extracted from the four strains of *P. infestans* Mat-A1 and three strains of *P. infestans* Mat-A2. Different isolates of *P. infestans* species were used for AFLP. A total of forty primer combinations were screened to identify the A2 mating type of *P. infestans* species. The A2 mating type in *P. infestans* was detected in three of the forty combinations (fig. 1; arrow). However, two DNA fragments of E+AA/M+CTC and E+TA/M+CTT combinations were not linked to the A2 mating type (data not shown). From among the primer combinations, we selected the specific bands generated with E+AA (5'-GACTGCGTACCAATTCAA-3') and M+CAA (5'-GATGAGTCCTGAGTAACAA-3') combination only. This primer set was amplified as a 402 bp fragment which is specific to the A2 mating type of *P. infestans* (Fig. 1; star).

Cloning and sequencing of the P. infestans Mat A2-specific DNA fragment

The specific DNA fragment of 402 bp was isolated and ligated into a T-vector, followed by transformation in *E. coli*. Plasmid DNA was extracted from transformants, and the target product was harvested. Next, the whole sequences of 402 bp were determined using T-vector primers (T7 and SP6). Primer pairs PHYB-1 (5'-GATCGGATTAGT-CAGACG AG-3') and PHYB-2 (5'-GCGTCTGCAAGGCG-CATTTT-3') were designed from the sequence of the selected clone (Fig. 2).

Southern blot hybridization

The genomic DNAs of the 11 strains of *P. infestans* Mat-

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1   GACTGCGTAC CAATTCAACC TCGATCGGAT TAGTCAGACG AGACATATCG
51  ATTGATCCGA TTCAATCCAT TCCCCAAGCC TAATAGGAAC TGTCCGTCCA
101 CAGAGACCGG TAGAAAACCG GTATAGCGTG CATGAGTGGT TTGTTCCTGC
151 GAGATGGATG CGGGGCGACT GAGGCTCGAA TGATGGTTCC GGACGCTTTC
201 GGCCGCTGCG TGGTTAGCGC TCTCATTAGG AGACAGCTCT TCAACGACAG
251 CGCCGGGCTG CTGAGACACC GATCAAGCGG GACGCGCGCC TACAAAAGGA
301 GCGTCTGAAA CGCGCGAAGA AGCCAGCGGA GGAGACTCCA GCCGCGTGAA
351 AAATGCGCCT TGCAGACGCT TCCGACCGCT GCGTTGTTAC TCAGGACTCA
401 TC
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Fig. 2. Nucleotide sequences of *P. infestans* Mat A2 specific DNA fragment. Numbers on the left refer to the nucleotide sequences. Underlined sequences are the E+AA and M+CAA primers that are used in PCR. Sequences in shadel boxes are forward (PHYB-1) and reverse (PHYB-2) primers for the detection of a specific DNA fragment in *P. infestans* Mat A2.

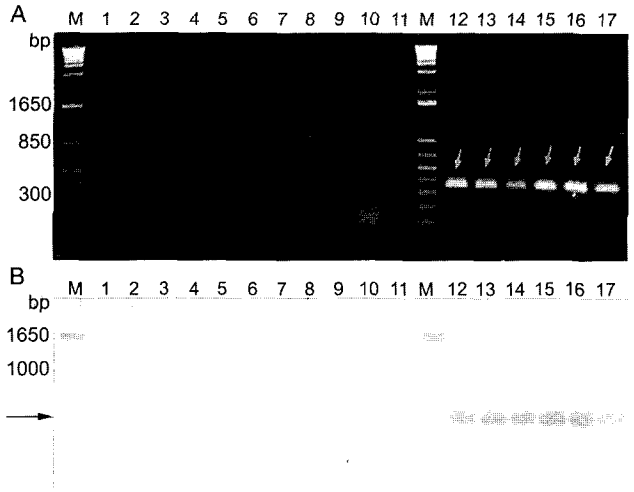

Fig. 3. Analysis of strains of *P. infestans* Mat A1 and *P. infestans* Mat A2 (A, PCR profiles using pair primers, PHYB-1 and PHYB-2; B, Southern blot profiles using cloned fragment as a probe). DNA marker linked to *P. infestans* Mat A2 is indicated with arrows. Lane M, 1 kb DNA ladder; lanes 1-11, strains of *P. infestans* Mat A1; lanes 12-17, strains of *P. infestans* Mat A2. Strains of *P. infestans* are listed in Table 1.

A1 and 6 strains of *P. infestans* Mat-A2 were amplified by PCR with the primers PHYB-1 and PHYB-2. The amplified PCR products were separated in 0.8% of agarose gel (Fig. 3A). A probe encoding the *P. infestans* Mat-A2

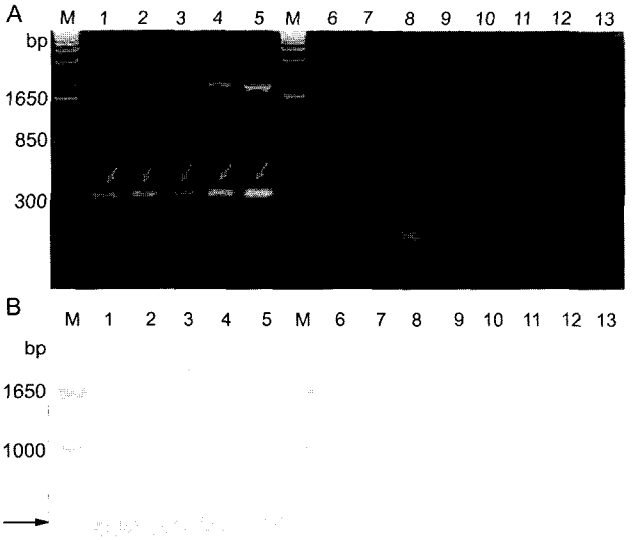

Fig. 4. Analysis of strains of *Phytophthora infestans* Mat A2 (lanes 1-5) and *P. infestans* spp. (lanes 6-13). A, PCR profiles using pair primer, PHYB-1 and PHYB-2; B, Southern blot profiles using the cloned fragment as a probe. DNA marker linked to *P. infestans* Mat A2 is indicated as arrows. Lane M, 1 kb DNA ladder; lanes 1-5: *P. infestans* Mat A2 isolates Na1-2, Na1-5, Na2-1, KAW-40, and KAW-63; lane 6: *P. megasperma*; lane 7: *P. sojae*; lane 8: *P. cactorum*; lane 9: *P. cinnamomi*; lane 10: *P. citricola*; lane 11: *P. cryptogea*; lane 12: *P. capsici*; and lane 13: *P. capsici*.

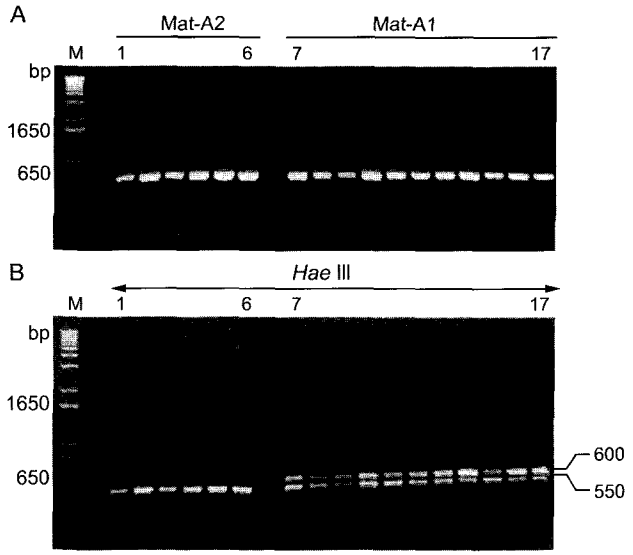

Fig. 5. Ethidium bromide-stained gel of cleaved amplified polymorphic sequences (CAPS) within W16 fragments resulting from single digestion with *Hae*III (B). The W16 fragments were amplified by polymerase chain reaction (PCR) using primers w16-1/w16-2 (A). Lane M, 1 kb DNA ladder; lanes 1-6, *P. infestans* Mat-A2: Na1-2 (1), Na1-5 (2), Na2-1 (3), KAW-40 (4), KAW-63 (5), 20B02 (6); lanes 7-17, *P. infestans* Mat A: YY-8 (7), YY-9 (8), YY-11 (9), YY-12 (10), YY-15 (11), YY-27 (12), SS-1 (13), DD-8 (14), KAW-31 (15), NW-7 (16), WS7-13 (17).

region of the isolate Na1-5 was hybridized with the PCR products made by the PHYB-1 and PHYB-2 primers. In a Southern hybridization, the same results as with the pair primers were observed (Fig. 3B). A single signal was detected on the 347 bp fragments from all of the strains of *P. infestans* Mat-A2 but not from *P. infestans* Mat-A1 (Fig. 3). Strains of other *Phytophthora* spp. were not amplified by the PHYB-1 and PHYB-2 primers either (Fig. 4).

CAPs analysis by using W16 primers

In order to identify the mating type in *P. infestans*, the genomic DNA of the 17 strains of *P. infestans* were amplified by PCR with the primers W16-1 and W16-2. The amplified PCR products were detected on the 600 bp fragments from all of the strains of *P. infestans* (Fig 5A). The amplified product was digested with *Hae*III restriction enzyme, revealing a 600 bp band linked to the A1 mating type and a 550 bp band linked to the A2 mating type (Fig. 5B; Judelson, 1995). These CAPs polymorphisms were able to precisely differentiate A2 mating type (lanes 1-6) and A1 mating type (lanes 7-17).

Discussion

Various DNA-based diagnostic methods have been proved to be highly sensitive and species-specific tools. These techniques are very powerful in detecting and

determining the taxonomy of fungi. PCR-based techniques are particulary valuable when compared with conventional methods, because they require only small quantities of DNA and little handling time. *P. infestans*, an important pathogen of potato and tomato plants, has a mating system that is representative of most heterothallic oomycetes (Bishop, 1940; Papa *et al.*, 1967; Gallegly, 1968; Michelmore, 1982; Shaw, 1991; Brasier, 1992). Two mating types exist, A1 and A2, which represent compatibility types rather than dimorphic sexual forms. Each is distinguished by its production of a specific pheromone and its response to the pheromone of the opposite type (Ko, 1988). These compounds induce the diploid, vegetative mycelia to differentiate both male and female gametangia (antheridia and oogonia), in which meiosis occurs. Gametangial fusion results in diploid oospores with one viable zygotic nucleus, which develop into diploid vegetative mycelia of the A1 or A2 types.

This study has illuminated key aspects of the genetics of mating types in *P. infestans*, by using a DNA marker linked to the A2 mating type in *P. infestans*. Markers linked to A2 mating types are genetically and physically linked to one region (Judelson, 1995). A DNA marker linked to the A2 mating type in *P. infestans* was detected by a powerful DNA fingerprinting technique called AFLP (Vos *et al.*, 1995). The specific primers, PHYB-1 and PHYB-2, designed for the amplification of the DNA marker linked to the A2 mating type locus in *P. infestans*, amplified the expected 347 bp PCR fragment only in the 6 strains of *P. infestans* Mat-A2 and not in the 11 strains of *P. infestans* Mat-A1 (Fig. 3). Also, we tested if these specific primers amplified from other *Phytophthora* spp. (Fig. 4). In this experiment, about 347 bp of the fragment in the PCR products that are amplified by the PHYB-1 (5'-GATCGGATTAGTCAGACGAG-3') and PHYB-2 (5'-GCGTCTGCAAGGCGCATTTT-3') was detected only in *P. infestans* Mat-A2 but not in other *Phytophthora* spp. The 347 bp specific fragment was within the suggested optimal range for the amplification by PCR.

Afterward, in order to test the mating type with the other genetic marker in *P. infestans*, we used the CAPs marker that designed by Judelson, the result revealed that amplified fragment (600 bp band) was detected from all of the strains in *P. infestans* and when reactions were digested with *Hae*, the polymorphism between A2 mating type and A1 mating type strains in *P. infestans* were observed to be different. The 550 bp fragment was observed to be all of the strains and the 600 bp fragment was only observed in the A1 mating type strains in *P. infestans* (Fig. 5).

Annealing temperature and template DNA quantity were also investigated, since they could influence the rate and specificity of the amplification (Innis, 1990; McPherson, 1992; Paabo, 1990). The theoretical annealing temperature calculated from the primers was 57°C. However, temperatures between 57°C-62°C were found to be suitable for the observation of the PCR products of the A2 mating type of *P. infestans* on stained agaroses. Futhermore, 10 ng-100 ng of A2 mating type of *P. infestans* genomic DNA was established to be sufficient for a detectable production by PCR (data not shown). The application of PCR technology is effective in detecting a certain fungus both *in vitro* and *in planta*, and can help us to study the taxonomy of an orgaism. Therefore, futher study on the *in planta* detection of A2 mating type of *P. infestans* in infected tissues or tubers and at different developmental stages of the host *in vivo* and *in vitro* is needed. Also, further studies to test the inheritance patterns of the DNA marker linked to Mat A2 in F1 progeny through crossing of Na1-2 (*P. infestans* Mat-A2) and YY-8 (*P. infestans* Mat-A1) are necessary.

Table 1. Fungal isolates used for Mat A2-specific detection of *P. infestans* in *S. tuberosum* L

Isolate	Pathogen	Mating Type
YY-8 (1)*	*Phytophthora infestans*	A1
YY-9 (2)	*P. infestans*	A1
YY-11 (3)	*P. infestans*	A1
YY-12 (4)	*P. infestans*	A1
YY-15 (5)	*P. infestans*	A1
YY-27 (6)	*P. infestans*	A1
SS-1 (7)	*P. infestans*	A1
DD-8 (8)	*P. infestans*	A1
KAW-31 (9)	*P. infestans*	A1
NW-7 (10)	*P. infestans*	A1
WS7-1 (11)	*P. infestans*	A1
Na1-2 (12)	*P. infestans*	A2
Na1-5 (13)	*P. infestans*	A2
Na2-1 (14)	*P. infestans*	A2
KAW-40(15)	*P. infestans*	A2
KAW-63(16)	*P. infestans*	A2
20B02 (17)	*P. infestans*	A2

*Numbers in parentheses indicate the lane number in Figure 3.

References

Bishop, H. 1940. A study of sexuality in *Sapromyces reinschii*. *Mycologia* 32, 505-529.

Brasier, C.M. 1992. Evolutionary biology of *Phytophthora*. Part I: genetic system, sexuality and the generation of variation. *Ann. Rev. Phytophthol.* 30, 153-177.

Fabritius, A.L. and H.S. Judelson. 1997. Mating-type loci segregate aberrantly in *Phytophthora infestans* but normally in *Phytophthora parasitica*: implications for models of mating-type determination. *Curr. Genet.* 32, 60-65.

Fry, W.E., S.B Goodwin, J.M. Matuazak, L.J. Spielman, and M.G. Milgroom. 1992. Population genetic and international migrations of *Phytophthora infestans*. *Ann. Rev. Phytopathol.* 30, 107-129.

Gallegly, M.E. and J. Galindo. 1958. Mating types and oospores of *Phytophthora infestans* in nature. *Phytopathol* 48, 274-277.

Gallegly, M.E. 1968. Genetics of pathogenicity of *Phytophthora*

infestans. *Ann. Rev. Phytopathol.* 6, 375-396.

Goodwin, S.B., J.L. Spielman, J.M. Matuszak, S.N. Bergeron, and W.E. Fry. 1992. Clonal diversity and genetic differentiation of *Phytophthora infestans* population in Northern and Central Mexico. *Phytopathol.* 82, 955-961.

Goodwin, S.B., A. Drenth, and W.E. Fry. 1992. Cloning and genetic analysis of two highly polymorphic moderately repetitive nuclear DNAs from *Phytophthora infestans*. *Curr. Genet.* 22, 107-155.

Griffith, G.W., R. Snell, and D.S. Shaw. 1995. Late blight (*Phytophthora infestans*) on tomato in the tropics. *Mycologist* 9, 87-89.

Innis, M.A. and D.H. Gelfand. 1990. Optimization of PCRs; In *PCR Protocols. A Guide to Methods and Applications*, Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (eds.), pp. 3-12, *Academic Press*, Inc., Harcourt Brace Jovanovich, NY, U.S.A.

Judelson, H.S. 1996. Recent advances in the genetics of oomycete plant pathogens. *Mol. Plant-Microbe Interact.* 9, 443-449.

Judelson, H.S. 1996. Physical and genetic variability at the mating type locus of the oomycete, *Phytophthora infestans*. *Genetics*. 144, 1005-1013.

Judelson, H.S., L.J. Spielman, and R.C. Shattock. 1995. Genetic mapping and non-Mendelian segregation of mating-tpye loci in the oomycete, *Phytophthora infestans*. *Genetics*. 141, 503-512.

Ko, W.H. 1988. Hormonal heterothallism and homothallism in *Phytophthora*. *Ann. Rev. Phytopathol.* 26, 57-73.

Kües, U. and L.A. Casselton. 1992. Fungal mating-type genes - regulators of sexual development. *Mycol. Res.* 96, 993-1006.

McPherson, M.J., R.J. Oliver, and S.J. Gurr. 1992. The polymerase chain reaction; in *Molecular Plant Pathology. A Practical Approach*, Gurr, S.J., McPherson, M.J. and Bowles, D.J. (eds.), Vol. 1, pp. 123-145, IRL Press, NY, U.S.A.

Michelmore, R.W. and E.R. Sansome. 1982. Cytological studies of heterothallism and secondary homothallism in *Bremia lactucae*. *Trans. Br. Mycol. Soc.* 79, 291-297.

Old, R.W. and S.B. Primrose. 1994. Polymerase chain reaction; in Principles of Gene Manipulation, pp. 178-190, Blackwell Science, Cambridge, MA. USA.

Paabo, S. 1990. Amplifying ancient DNA; in *PCR Protocols. A Guide to Methods and Applications*, Innis, M. A., Gelfand, D. H., Sninsky, J.J. and White, T.J. (eds.), p. 159-166, Academic Press, Inc., Harcourt Brace Jovanovich, NY, U.S.A.

Papa, K.E., W.A. Campbell, and F.F. Hendrix. 1967. Sexuality in *Pythium sylvaticum* heterothallism. *Mycologia* 59, 589-595.

Rusell, J.R., J.D. Fuller, M. Macaulay, B.G. Hatx, A. Jahoor, W. Powell, and R. Waugh. 1997. Direct comparison of levels of genetic variation among barely accessions detected by RFLPs, AFLPs, SSRs and RAPDs. *Theor. Appl. Genet.* 95, 714-722.

Reineke, A. and P. Karlovsky. 2000. Simplified AFLP protocol: replacement of primer labeling by the incorporation of alpha-labeled nucleotides during PCR. *Biotechniques* 28, 622-623.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor, NY, U.S.A.

Shaw, D.S. 1991. Genetics. In: *Phytophthora infestans*. D.S. Ingram and D.H. Williams. Eds. Vol. 7. Pp. 131-170. Academic Press, New York.

Tooley, P.W., W.E. Fry, and M.J. Villarreal Gonzalez. 1985. Isoenzyme characterization of sexual and asexual *Phytophthora infestans* population. *J. Hered.* 76, 431-435.

Van de Lee, T., I.D. Witte, A. Drenth, C. Alfonso, and F. Govers. 1997. AFLP Linkage Map of the Oomycete *Phytophthora infestans*. *Fungal Genet. and Biol.* 21, 278-291.

Vos, P., R. Hoger, M. Bleeker, M. Reijans, T. Van de Lee, M. Hornes, A. Fijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau, 1995. AFLP: A new technique for DNA fingerprinting. *Nucl. Acid Res.* 23, 4407-4414.