A Genetic Marker Associated with the A1 Mating Type Locus in *Phytophthora infestans*

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Abstract: Sexual reproduction plays an important role in the biology and epidemiology of oomycete plant pathogens such as the heterothallic species *Phytophthora infestans*. Recent worldwide dispersal of A2 mating type strains of *P. infestans* resulted in increased virulence, gene transfer, and genetic variation, creating new challenges for disease management. To develop a genetic assay for mating type identification in *P. infestans*, we used the Amplified Fragment Length Polymorphism (AFLP) technique. The primer combination E+AT/M+CTA detected a fragment specific to A1 mating type (Mat-A1) of *P. infestans*. This fragment was cloned and sequenced, and a pair of primers (INF-1, INF-2) were designed and used to differentiate *P. infestans* Mat-A1 from Mat-A2 strains. The Mat-A1-specific fragment was detected using Southern blot analysis of PCR products amplified with primers INF-1 and INF-2 from genomic DNA of 14 *P. infestans* Mat-A1 strains, but not 13 *P. infestans* Mat-A2 strains or 8 other isolates representing several *Phytophthora* spp. Southern blot analysis of genomic DNAs of *P. infestans* isolates revealed a 1.6 kb restriction enzyme (EcoRI, BamHI, AvA I)-fragment only in Mat-A1 strains. The A1 mating type-specific primers amplified a unique band under stringent annealing temperatures of 63°C–64°C, suggesting that this PCR assay could be developed into a useful method for mating type determination of *P. infestans* in field material.

Key words: *Phytophthora infestans*, molecular marker, mating type A-1, AFLP

Late blight, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, is one of the most destructive diseases of cultivated potato and tomato [1]. Heterothallic species of the oomycetous genus *Phytophthora* exhibit two mating types, which are called A1 and A2 [3]. These two mating types are physiologically differentiated by their ability to produce hormones that are required to induce gametangia under normal conditions [20]. When isolates of the A1 and A2 sexual compatibility types (mating types) of heterothallic species of *Phytophthora* are cultured together, antheridia and oogonia are induced, which associate and may fuse to form an oospore [3, 33, 36]. Sexual compatibility is thought to be determined by the interaction between diffusible mating hormones and their receptors. Until the late 1970s, only one mating type (A1) of *P. infestans* was distributed worldwide, and management of the asexually reproducing pathogen was somewhat successful. However, severe late blight epidemics occurred in recent years after aggressive A2 mating type strains migrated to Europe and North America [8, 13, 21, 31, 35]. The occurrence of a sexual cycle has resulted in increased virulence, gene transfer, and genetic variation in populations of *P. infestans*, creating new challenges for late blight management.

The genetic basis for mating type in *Phytophthora* has been most thoroughly addressed in *Phytophthora infestans*, and its mating type was shown to be determined by a single locus, which displayed a pattern of non-Mendelian inheritance [15, 16]. This pattern was similar to that observed for genes linked to balanced lethal loci [27], as only two genotypes of loci linked to the mating-type determinant were observed in progeny, compared to the four that would be expected for diploids.

A genetic survey of isolates representative of the entire species indicated that this unusual segregation pattern was a general feature of the mating type locus of *P. infestans*, as opposed to being a phenomenon displayed only by selected or aberrant isolates [9]. The conservation throughout the
species of non-Mendelian segregation suggests that it may play an important role in the determination of mating type, possibly by eliminating potentially deleterious combinations of alleles or by suppressing mitotic crossing over.

Sexual reproduction plays an important role in the biology and epidemiology of *P. infestans*. Populations of *P. infestans* that contain A1 and A2 mating types carry the potential to complete their sexual life cycle. This results in the formation of oospores that enable the pathogen to survive for longer periods, as well as higher levels of virulence and high frequencies of resistance against the widely used fungicide metalaxyl, presumably because of increased gene flow [6, 22, 23, 37]. Therefore, a rapid genetic test to evaluate field populations of *P. infestans* for the frequency of A2 mating type has useful implications for disease management. Genetic studies of *P. infestans* and other oomycetes have been hampered by the lack of morphological and biochemical mutants but are benefiting from modern genetic tools [14, 17]. For example, the amplified fragment length polymorphism (AFLP) technique developed by [Vos et al. 39] is a powerful, reliable, stable, and rapid assay with application in genome mapping, DNA fingerprinting, and marker-assisted breeding [19, 25, 28, 30, 32]. AFLP has been successfully used in genetic marker discovery and genome mapping in *Phytophthora* [38].

Table 1. Isolates of *P. infestans* Mat-A1 and Mat-A2 used in this study.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Pathogen</th>
<th>Oospore formation</th>
<th>Mating type</th>
<th>Host</th>
<th>Location</th>
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<tbody>
<tr>
<td>Na1-2</td>
<td><em>Phytophthora infestans</em></td>
<td>A2</td>
<td>-</td>
<td><em>Solanum tuberosum</em> L.</td>
<td>Pyung Chang</td>
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<td><em>P. infestans</em></td>
<td>-</td>
<td>A2</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Na2-1</td>
<td><em>P. infestans</em></td>
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<td>A2</td>
<td>-</td>
<td>-</td>
</tr>
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<td>-</td>
<td>A2</td>
<td>-</td>
<td>Hoeng Gye</td>
</tr>
<tr>
<td>KAW-63</td>
<td><em>P. infestans</em></td>
<td>-</td>
<td>A2</td>
<td>-</td>
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<tr>
<td>RDA-2</td>
<td><em>P. infestans</em></td>
<td>-</td>
<td>A2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
<td>A2</td>
<td>Dae Gwan Yung</td>
<td>-</td>
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<tr>
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<td>-</td>
<td>A2</td>
<td>-</td>
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<tr>
<td>DGY3-2</td>
<td><em>P. infestans</em></td>
<td>-</td>
<td>A2</td>
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<tr>
<td>DGY3-4</td>
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<td>A2</td>
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<tr>
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<td>A2</td>
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<td>-</td>
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<td>A2</td>
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<td><em>P. infestans</em></td>
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<td>A1</td>
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<td>YY-9</td>
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<td>A1</td>
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<td>A1</td>
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<td>A1</td>
<td>-</td>
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<td>A1</td>
<td>-</td>
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<td>A1</td>
<td>-</td>
<td>-</td>
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<td><em>P. infestans</em></td>
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<td>A1</td>
<td>-</td>
<td>Wang San</td>
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<td>+</td>
<td>A1</td>
<td>-</td>
<td>-</td>
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<td><em>P. infestans</em></td>
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<td>A1</td>
<td>-</td>
<td>-</td>
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<td>WS6-16</td>
<td><em>P. infestans</em></td>
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<td>A1</td>
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<td>-</td>
</tr>
<tr>
<td>WS9-1</td>
<td><em>P. infestans</em></td>
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<td>A1</td>
<td>-</td>
<td>-</td>
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<td>N/A</td>
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<td>-</td>
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<td>N/A</td>
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<td>N/A</td>
<td><em>P. citricola</em></td>
<td>N/A</td>
<td>N/A</td>
<td>Zizyphus jujuba M.</td>
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<td><em>P. cryptogaia</em></td>
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<td>N/A</td>
<td>Brassica campestris subsp.</td>
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<td>N/A</td>
<td><em>P. capsici</em></td>
<td>N/A</td>
<td>N/A</td>
<td>Capsicum annuum L.</td>
<td>-</td>
</tr>
</tbody>
</table>

*Numbers in parenthesis indicate the lane numbers in Fig. 4.
**Numbers in parenthesis indicate the lane numbers in Fig. 5.
*The cross between A2 (Na1-2) and unknown isolate of *P. infestans*.
+ Oospores formation. - No oospores formation.
To improve management of the late blight disease, it is necessary to develop a diagnostic system that would evaluate the frequency of different mating types in latent infection of *P. infestans*. The aim of this study, therefore, was to use AFLP to identify and develop a genetic marker for mating type determination in *P. infestans*.

**Materials and Methods**

**Strains and Cultivating Condition**

Thirteen isolates of *P. infestans* Mat-A2, fourteen isolates of *P. infestans* Mat-A1 (Table 1), and eight isolates of *Phytophthora* species (Table 1 and Lanes 3–10 in Fig. 5) were used in this study. DGY and WS series of *P. infestans* isolates were kindly provided by Dr. B. S. Kim (Kangnung National University), and other sixteen isolates of *P. infestans* were isolated from infected plants. The *P. infestans* and other *Phytophthora* species isolates (Table 1) were cultured in a V8 juice medium (200 ml of V8 juice, 1 g of CaCO$_3$) in 1 l of distilled water for 10 days at 18–20°C [12].

**Mating-Type Assay**

Mating type was determined for twenty-six isolates of *P. infestans* by placing a plug of an unknown isolate on one side of a petri dish containing 20% clarified V8 medium, whereas the M+3 primers had the sequence 5’-CTGACGCA TGG TT AA-3’ (E+AT), in which AT is the selective nucleotide.

**Extraction of Genomic DNA and AFLP Analysis**

The genomic DNA of *P. infestans* was extracted by a modification of the method described by Goodwin *et al.* [11] for PCR and Southern blot analyses. Long-term stocks were maintained as an agar plug in 10% glycerol under liquid nitrogen [32]. The concentration of the RNase-modified genomic DNA was determined spectrophotometrically or by clariﬁed V8 medium with a plug of a known A2 isolate (Nal-2). The plates were incubated at 20°C for 14 days, after which formation of oospores was observed under a binocular microscope at low magnification (×20).

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 was started with a cycle of 30s at 94°C, 1 min at 60°C, and 1 min at 72°C, and 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 (*EcoRI*+2 and *MseI*+3; Bioneur, Korea). For the 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.

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suspensions ranging from $1.2 \times 10^6$ conidia/ml and DNA ranging from 10 ng to 10 pg were prepared by continual dilutions. The genomic DNAs were extracted from 
P. infestans-infected leaf tissues by a modification of the method described by Goodwin et al. [5, 11, 18].

**RESULTS AND DISCUSSION**

**Mating-Type Test**

Twenty-six South Korean isolates of 
P. infestans were paired with known A2 isolate, Na1-2, to determine their mating types. In pairings between A2-type Na1-2 and other isolates of 
P. infestans, oospores were formed only with those compatible isolates within the agar where the two isolates meet. An example of a compatible isolate combination (between Na1-2 and YY-8), which produced oospores, is shown in Fig. 1. In contrast, only mycelia and zoospores were observed on the edge of plates of incompatible combinations (Figs. 1A, 1C). Pairings of Na1-2 isolate with tester isolates of mating types A1 and A2 revealed that 46% (12/26) of the isolates were mating type A2 and 54% (14/26) were mating type A1 (Table 1; Fig. 1).

**Cloning and Sequencing of the 
P. infestans Mat-A1 Specific DNA Fragment**

The specific DNA fragment of 218 bp was gel-purified, ligated into the pGEM-T-Easy vector (Promega, U.S.A.), and transformed in 
E. coli (JM 109). Plasmid DNA was extracted and the sequence of 218 bp was determined using T-vector primers (T7 and SP6; Fig. 3). The sequence was used in BLASTN and BLASTX searches of the
nonredundant and dBEST databases of GenBank, as well as the *Phytophthora* sequences hosted in the *Phytophthora* Functional Genomics Database (PFGD, www.pfgd.org). No significant hits were detected, suggesting that the identified genetic marker is novel.

**A PCR Assay of Mating-Type in *P. infestans***

Based on the sequence of the 218 bp fragment, we designed a pair of primers and used them to differentiate *P. infestans* Mat-A1 from Mat-A2 by PCR amplification. The sequences of the forward primer was 5'-AAGCTTACTGGGACAGGGT-3' (INF-1) from 24 to 43 bp, and the G+C content of the primer was 50%. The reverse primer was 5'-GGCCTTTTCTGATTACAC-3' (INF-2) from 174 to 193 bp, and the G+C content was 45%. Using the forward and reverse primers, thirteen isolates of *P. infestans* Mat-A2 and fourteen isolates of *P. infestans* Mat-A1 were assayed by PCR with the primers INF-1 and INF-2. A single fragment of 170 bp was amplified from Mat-A1 isolates of *P. infestans*, but not from Mat-A2 isolates (Fig. 4A). These primers also failed to amplify genomic DNA of eight isolates of different *Phytophthora* spp. (Fig. 5A).

To ensure that the INF-1 and INF-2 primers failed to amplify genomic DNA from Mat-A2 strains, the amplified PCR products were separated in 0.8% agarose gel, blotted to nylon membrane, and hybridized with a probe (pINF) encoding the *P. infestans* Mat-A1-specific region amplified from the isolate YY-8. Hybridization signals were only detected in amplicons from the Mat-A1 isolates of *P. infestans* (Fig. 4B, lanes 1-13) and other *Phytophthora* spp. (Fig. 5B, lanes 3-10).

**Genomic Southern Blots Indicate that the Mat-A1-Specific DNA Fragment is a Single-Copy Sequence**

To estimate the INF marker copy number, we analyzed a genomic Southern blot of *P. infestans* isolates (Mat-A1: YY-8, W9-1, KC-C, and YY-19; Mat-A2: Na1-2, Na1-5, KAW-40, and DGY3-4). Extracted DNA was digested with three kinds of restriction enzymes, *BamH*II, *Aul*, and *EcoRI*, and hybridized with pINF as a probe. These restriction enzyme sites are not present in the INF marker clone (Fig. 6). This analysis identified an approximately 1.6 kb DNA fragment among the three restriction enzyme sites in the genomic DNA sequence of Mat-A1 (YY-8, KC-C, and
It also identified a fragment of about 1.4 kb in BamHI-digested WS9-1 isolate DNA. No signal was detected from isolates of *P. infestans* Mat-A2 (Fig. 6).

### Optimization of PCR Conditions

To optimize PCR amplification conditions using primers INF-1 and INF-2, we investigated annealing temperature and template DNA quantity, since they could influence the rate and specificity of the amplification [12, 20, 24]. The theoretical annealing temperature calculated from the primers was 52°C. However, temperatures between 63–64°C were found to be suitable for the observation of the PCR products of the A1 mating type of *P. infestans* on stained agarose. Furthermore, 10 ng–200 pg of the A1 mating type of *P. infestans* genomic DNA was established to be sufficient for a detectable production by PCR (Fig. 8B).

In this study, DNA obtained from the target *P. infestans* (Mat-A1)-infected leaf tissues was amplified by PCR with the primers INF-1 and INF-2. Consequently, a 170 bp amplified fragment was detected only in *P. infestans* Mat-A1-infected leaf tissues. Furthermore, the pair primers were not amplified in *P. infestans* Mat-A2-infected leaf tissues and control leaf tissues (Fig. 7). In addition, these primer sets were amplified at a conidial suspension of 1.2×10^6 conidia/ml (Figs. 8A, 8C).

Therefore, complementary use of the primer combination E+AT/M+CTA and the forward and reverse primers will make it possible to quickly and precisely diagnose the mating type of *P. infestans* isolates in field material of the late blight disease.

### Prospects for Late Blight Diagnostics

The primers and PCR assay described in this study should prove to be a useful tool to distinguish *P. infestans* Mat-A1

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**Fig. 5.** PCR profiles (A) and PCR-Southern hybridization profiles (B) of isolates of *P. infestans* Mat-A1 and other *Phytophthora* spp.

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**Fig. 6.** Genomic Southern blot analysis of INF genes in *P. infestans*.

An approximately 16 kb single band was shown only in the isolates of *P. infestans* Mat-A1, when the cloned fragment was used as a probe (pINF). Lane M, 1 kb DNA ladder; lanes 1–4, *P. infestans* Mat-A1: YY-8 (1), WS9-1 (2), KC-5 (3), and YY-19 (4); lanes 5–8, *P. infestans* Mat-A2: Nat-2 (3), Nat-5 (6), KAR-40 (7), and DGY3-4 (8).

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**Fig. 7.** Detection of the Mat-A1 marker in infected potato leaf (Dejima). Lane M, 1 kb DNA ladder; Lanes 1–5, *P. infestans* Mat-A1-infected potato leaf; lanes 5–10, *P. infestans* Mat-A2-infected potato leaf; lane C, Non-infected control potato plant leaf.

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**Fig. 8.** Determination of detection limit for conidial suspensions (A) and DNA quantities (B) by PCR.

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from *P. infestans* Mat-A2. This assay has significant practical applications, since Mat-A2 isolates are frequently associated with fungicide resistance and aggressiveness and should help implement disease management strategies [4, 7, 10, 24, 26, 29].

PCR technology can also be applied to detect pathogens *in vitro* and *in planta*, and can help in species identification [2, 9, 29, 34]. The described primers have useful potential to detect *P. infestans* among the micro flora that are isolated from leaves, stems, or tubers of potato and tomato plants. Also, further studies to test the inheritance patterns of DNA marker, linked to Mat-A1 in F1 progeny through crossing of Na1-2 (*P. infestans* Mat-A2) and YY-8 (*P. infestans* Mat-A1), will further establish the extent to which the discovered marker is linked to Mat-A1.

**Acknowledgment**

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**References**


