Mating Type Locus-Specific Polymerase Chain Reaction Markers for Differentiation of *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata*, the Causal Agents of Barley Net Blotch

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ABSTRACT

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Fourteen single nucleotide polymorphisms (SNPs) were identified at the mating type (*MAT*) loci of *Pyrenophora teres* f. *teres* (*Ptt*), which causes net form (NF) net blotch, and *P. teres* f. *maculata* (*Ptm*), which causes spot form (SF) net blotch of barley. *MAT*-specific SNP primers were developed for polymerase chain reaction (PCR) and the two forms were differentiated by distinct PCR products: *PttMAT1-1* (1,143 bp) and *PttMAT1-2* (1,421 bp) for NF *MAT1-1* and *MAT1-2* isolates; *PtmMAT1-1* (194 bp) and *PtmMAT1-2* (939 bp) for SF *MAT1-1* and *MAT1-2* isolates,

respectively. Specificity was validated using 37 NF and 17 SF isolates collected from different geographic regions. Both *MAT1-1* and *MAT1-2* SNP primers retained respective specificity when used in duplex PCR. No cross-reactions were observed with DNA from *P. graminea*, *P. tritici-repentis*, or other ascomycetes, or barley. Single or mixed infections of the two different forms were also differentiated. This study provides the first evidence that the limited SNPs at the *MAT* locus are sufficient for distinguishing closely related heterothallic ascomycetes at subspecies levels, thus allowing pathogenicity and mating type characteristics of the fungus to be determined simultaneously. Methods presented will facilitate pathogen detection, disease management, and epidemiological studies.

Additional keywords: mating-type genes, molecular diagnostics.

Pyrenophora teres Drechsler (anamorph: *Drechslera teres* [Sacc.] Shoem.) is the causal agent of barley net blotch, one of the most important barley diseases in North America and worldwide. Direct crop yield losses due to the disease typically range from 10 to 40% in cool and wet growing regions where the disease is usually prevalent and may reach even higher in regions where highly susceptible cultivars are grown and conditions favoring the disease are present. Infection by *P. teres* also has negative effects on malting and feed quality, thus leading to significant losses to the barley industry and farmers (24,26,27,41,45).

The fungus is a heterothallic ascomycete and occurs in the field as two forms: *P. teres* f. *teres* Smedeg, which causes the net form (NF) of net blotch and *P. teres* f. *maculata* Smedeg, which causes the spot form (SF) of net blotch. Both NF and SF isolates have two opposite mating types (*MAT1-1* or *MAT1-2*) that are controlled by a single locus, the mating type (*MAT*) locus (12,48,49,53). Sexual recombination occurs naturally among NF or SF isolates. The ratio of *MAT1-1*:*MAT1-2* isolates has been found to be 1:1 for both forms in the field (34,39,40). Hybridization between NF and SF isolates is rare or absent in the field but can be induced under laboratory conditions (9,10,19,34,35,42). Because NF and SF isolates appear to be genetically well-isolated, pro-

posals have been made to change the taxonomic status of these two forms (34,36) but have not been officially accepted.

P. teres f. *teres* and *P. teres* f. *maculata* are morphologically very similar. Differentiation of these two forms in culture cannot be easily achieved using routine laboratory procedures. Pathogenicity assays using differential barley lines are generally required for correct identification. These assays are time-consuming and often have limitations. For example, it is not feasible to evaluate certain isolates that are avirulent or have low virulence. Molecular markers derived from randomly amplified polymorphic DNA (RAPD) (9,47) and amplified fragment length polymorphism (AFLP) (22,23,39,40,52) have been developed to overcome the inability to discriminate the two forms using traditional markers, such as those based on internal transcribed spacer (ITS) sequences (32,46). However, the application of these random DNA fragment-based markers is limited due to the high intra-specific variability associated with the *P. teres* genome (17,28,33). Cross reaction to the closely related barley stripe fungus *P. graminea* S. Ito & Kurib (3,22,44) is also another major concern. Differentiation of *P. teres* from *P. graminea* is important in European countries where the presence of *P. graminea* in seed samples may result in rejection of otherwise acceptable seed lots because the seed infection threshold is lower for *P. graminea* than for *P. teres* (18).

Recently, two isolates of *P. teres* f. *maculata* have been identified from diseased barley at different locations in North Dakota (25), where NF isolates have been most prevalent as in other parts of the northern Great Plains of the United States. The occurrence of SF isolates in these areas poses a new challenge to barley disease management and breeding programs, because NF and SF isolates differ in their tolerance to commercial fungicides (9,27,38), and thus may require different control strategies. Fur-

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thermore, the resistance to these two different forms of net blotch is known to be conditioned by independent host genes involving different breeding programs (1,6,8,13–15,21,24,29,51).

The main goal of this research was to develop conventional polymerase chain reaction (PCR) markers for differentiation of *P. teres* f. *teres* and *P. teres* f. *maculata*. Because the *MAT* locus has been widely used for species phylogeny and population genetics studies on phytopathogenic ascomycetes (4,7,16,31,34,36,50), we hypothesized that the same locus could be targeted directly for subspecies differentiation. The objectives of this study were (i) to isolate the full-length *MAT* idiomorph from *P. teres* NF and SF isolates collected from different geographic regions worldwide, (ii) to identify single nucleotide polymorphisms (SNPs) strictly conserved at the *MAT* locus, and (iii) to develop form-specific SNP primers that can be used for conventional PCR assays to rapidly and accurately differentiate the two forms of the net blotch pathogen.

MATERIALS AND METHODS

Fungal isolates and culture conditions. Thirty-seven NF and 17 SF isolates collected from different geographic regions (Supplemental Table 1) were used for genomic DNA extractions. The NF and SF isolates from North Dakota were collected from Fargo and Langdon from 2004 to 2008 (25). The eight NF and 13 SF isolates from Australia were collected from New South Wales, Queensland, South Australia, Victoria, and Western Australia during the years 1977 to 2008. All NF and SF isolates were purified by single spore culture and tested on differential barley lines to confirm the net or spot form phenotype. Isolates were recovered from stocks as described previously (1) and grown in the dark for 7 days before being used for genomic DNA extractions. Isolates of *P. tritici-repentis* (Died) Drechsler (1C-BFP), *Stagonospora nodorum* (Berk.) E. Castell. & Germano (Sn4 and SN15) and other ascomycetes (Supplemental Table 1) were also included in genomic DNA extractions to be used for controls. DNA samples of *P. graminea* isolates were provided by A. F. Justesen of University of Aarhus, Denmark, J. E. Thomas of the National Institute of Agricultural Botany, United Kingdom, and M. Burrow of Montana State University.

Fungal and plant DNA extractions. Fungal mycelia were scraped from the surface of V8-agar plates and transferred into 2-ml Lysing Matrix D tubes containing 1.4-mm ceramic spheres (QBiogene, Morgan Irvine, CA). The samples were frozen in liquid nitrogen for 2 to 5 min immediately before homogenization using a Mini-Bead-beater-16 Cell Disrupter (BioSpec Products, Bartlesville, OK). The homogenized material was then suspended in 1 ml of isolation buffer (200 mM Tris-HCl, pH 8.0, 25 mM EDTA, pH 8.0, 250 mM NaCl, and 0.5% sodium dodecyl sulfate [SDS]), followed by phenol/chloroform extraction and ethanol precipitation. For extraction of total genomic DNA from barley, infected leaves (≈ 50 mg) were cut into small segments (≈ 1.0 cm) before homogenization in the Lysing Matrix D tubes. The homogenized leaf tissues were then suspended in 1 ml of extraction solution (2% cetyl trimethyl ammonium bromide, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, and 1.4 M NaCl) and incubated at 65°C for 20 min followed by chloroform extraction and ethanol precipitation. All DNA samples were finally resuspended in TE buffer (pH 8.0) and the concentration was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE).

Genomic DNA clones. PCR primers Pt-MATF and Pt-MATR (Supplemental Table 2) were designed based on the common flanking regions of the *P. teres* *MAT1-1* (GenBank accession no. AY950585), *MAT1-2* (GenBank accession no. AY950586) and *P. graminea* *MAT1-1* (GenBank accession no. DQ823079), and *MAT1-2* (GenBank accession no. DQ823080) idiomorphs (34,36) and used for PCR to predetermine the mating types of the NF and

SF isolates collected from different geographic regions (Supplemental Table 1). The PCR-amplified putative *MAT1-1* or *MAT1-2* products were cloned into a pCR2.1 vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Cloned products were sequenced at the Cornell University Life Sciences Core Laboratories Center. Sequencing was initiated with vector-specific primers (M13F and M13R) and continued with primers designed based on newly sequenced regions. The identity of the cloned *MAT* sequences was verified by BLAST searches (2) against the GenBank nonredundant nucleotide database (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Sequence analysis and design of *MAT*-specific primers. The nucleotide sequences of *P. teres* f. *teres* (Ptt) and *P. teres* f. *maculata* (Ptm) *MAT1-1* or *MAT1-2* idiomorphs were assembled and aligned using the SeqMan and MegAlign programs from Lasergene 8.1 software (DNASTAR Inc., Madison, WI) to identify SNPs that were conserved in all *MAT1-1* or *MAT1-2* sequences. SNP primers were designed by including a form-specific SNP at the 3' end of the oligonucleotides (Fig. 1). Four pairs of primers were selected for specific amplification of internal fragments of the corresponding *MAT1-1* or *MAT1-2* idiomorphs from each type of isolate. The name and oligonucleotide sequences of these primers and the size of the expected PCR products are given in Supplemental Table 2. Two pairs of control primers (Supplemental Table 2) were also designed. Primers PtGPD1F and PtGPD1R were designed for the *P. teres* glyceraldehyde-3-phosphate dehydrogenase gene (GenBank accession no. EF513234). Primers HvACT1F and HvACT1R were designed for the barley actin gene (GenBank accession no. AK252278). All primers had a calculated melting temperature (T_m) of 60 to 65°C and were predicted to be free of dimers or strong secondary structure.

Determination of PCR specificity and sensitivity. Genomic DNA of NF isolates LDNH07Pt-4 (*MAT1-1*, GenBank accession no. HM121990) and LDNH04Pt-1 (*MAT1-2*, GenBank accession no. HM121997), and SF isolates HRS07033 (*MAT1-1*, GenBank accession no. HM121994) and SNB247 (*MAT1-2*, GenBank accession no. HM122006) were used as template standards. DNA concentrations were equalized and a series of 10-fold dilutions (ranging from 100 to 0.001 ng/ μ l) were made for individual samples. Mixed samples were prepared by combining equal volumes of two or four different templates in the same tube. Genomic DNA of *P. graminea*, *P. tritici-repentis*, and other ascomycetes (Supplemental Table 1) were included in the tests as controls to check for possible cross reactions. Plasmid DNA of the corresponding PttMAT or PtmMAT idiomorphs cloned from the four NF/SF isolates were also used as controls. Duplex PCR assays were performed by including the NF (or SF)-specific *MAT1-1* and *MAT1-2* primer sets in the same PCR reaction. To monitor the quality and relative quantity of genomic DNA samples, primers PtGPD1F and PtGPD1R were included in all diagnostic PCRs (Figs. 2, 3, and 4). Primers HvACT1F and HvACT1R were included in PCR amplifications when genomic DNA extracted from infected or healthy barley tissues was used (Fig. 4). For blind tests, the primers and the template genomic DNA used for PCR were labeled with a random number individually and the identities of the primers and the template were not revealed until the final PCR results were obtained.

PCR conditions. For cloning of *MAT* idiomorphs from the *P. teres* genome, PCR was performed in a 50- μ l volume containing 1 to 10 ng of fungal genomic DNA, 0.5 μ M each primer, 0.2 mM each dNTP, 2 mM MgSO₄, 1 $\times$ High Fidelity PCR Buffer, and 1 U of High Fidelity Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA). PCR was started with an initial preheat for 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 30 s, and extension at 68°C for 3 min, with a final extension at 68°C for 10 min. PCR products were verified on a 0.7% agarose gel in TAE buffer. For differentiation tests, PCR was performed in a 25- μ l volume containing 1 μ l of fungal

genomic DNA (0.1 to 10 ng), 0.2 μ M of each primer, and 12.5 μ l of GoTaq Green Master Mix (Promega, Madison, WI) using the following parameters: an initial preheat for 5 min at 95°C, followed by 30 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The annealing temperature was increased to 59°C with 34 cycles for PCR when the total genomic DNA of infected barley leaves was used. PCR products (10 μ l per sample) were verified on a 1% agarose gel in TAE buffer.

Plant inoculations. The susceptible barley line Hector was planted in the greenhouse and allowed to grow to the two-leaf stage. Inoculations were done as described previously (13). Plants were inoculated separately with conidia of NF isolates 15A (*MAT1-1*) and 0-1 (*MAT1-2*), SF isolates SNB05064 (*MAT1-1*), and SG1 (*MAT1-2*) (Supplemental Table 1), or with a mixture that contained conidia of all four isolates in equal amounts. Symptoms were evident approximately 5 days after inoculations, at which time the infected leaves with typical net or spot form lesions (on plants inoculated with a single isolate) or both forms of lesions (on plants inoculated with mixed populations) were collected for genomic DNA extractions.

RESULTS

Mating type assays. The common primer set (Pt-MATF and Pt-MATR) amplified a $\approx$ 2.0-kb fragment from 24 NF and 8 SF isolates and a $\approx$ 2.2-kb fragment from 13 NF and 9 SF isolates (Supplemental Table 1). DNA cloning and sequencing confirmed

that the 2.0- and 2.2-kb fragments contained the *MAT1-1* and the *MAT1-2* idiomorphs, respectively (see below). The ratio of *MAT1-1*:*MAT1-2* isolates was nearly 1:1 among both NF and SF isolates collected from Australia (Supplemental Table 1). A majority (16 out of 22) of NF isolates collected in North Dakota were *MAT1-1*. This was probably due to the fact that these isolates were collected in different years in small numbers, and thus this distribution of mating types may not be statistically meaningful. The same primer pair also amplified a 2.2-kb fragment from isolates of *P. graminea* but not from *P. tritici-repentis* or other more distantly related ascomycetes (Supplemental Table 1), suggesting that the *MAT* flanking regions can be easily targeted to determine the mating types of both *P. teres* and *P. graminea* isolates. Interestingly, all nine *P. graminea* isolates (six from Denmark, one from the United Kingdom, and two from the United States) were all *MAT1-2* types (Supplemental Table 1).

Isolation of *MAT* idiomorphs. The PCR-amplified 2.0-kb *MAT1-1* fragment was cloned from three NF isolates (LDNH07Pt-4, FuPt0105, and HRS08194) and four SF isolates (HRS07033, SNB222, SNB258, and Den2.6). The 2.2-kb *MAT1-2* fragment was cloned from five NF isolates (LDNH04Pt-1, JP0101, BrPteris, NB077, and SNB172i) and six SF isolates (FGOB06Pt-8, LDNH08Pt-4, SNB0131i, SNB175, SNB247, and SNB167i) (Supplemental Table 1). DNA sequencing confirmed that the 2.0-kb fragments all contained the full-length *MAT1-1* idiomorph, which was 1,993 bp in all three NF *MAT1-1* isolates and one SF *MAT1-1* isolate (Den2.6), and 1,933 bp in three SF *MAT1-1* isolates (HRS07033, SNB222, and SNB258). The shorter *MAT1-1*

Fig. 1. Schematic diagrams showing single nucleotide polymorphisms (SNPs) identified at the **A**, *MAT1-1* and **B**, *MAT1-2* loci of *Pyrenophora teres* f. *teres* (Ptt) and *P. teres* f. *maculata* (Ptm). Open boxes indicate the *MAT* idiomorphs. Shaded arrows inside indicate open reading frames (ORFs). Checkered rectangles on the ends indicate flanking regions that are identical between the two *MAT* loci. Vertical bars indicate the positions of SNPs (asterisks indicate SNPs located outside of the ORFs). Dotted areas indicate introns located in the *MAT* ORFs. Boxes drawn with dotted lines indicate the conserved DNA binding domains. Triangle in **A** indicates the position of a 60-bp deletion in some *PtmMAT1-1* sequences. Arrows indicate relative positions of the *MAT* primers (black for *P. teres* f. *teres*, gray for *P. teres* f. *maculata* and open-ended for common primers). Nucleotides and positions of each SNP are given below along with *P. graminea* (Pg) *MAT1-1* (GenBank accession no. DQ823079) and *MAT1-2* (GenBank accession no. DQ823080) sequences. SNPs included at the 3' end of the corresponding PttMAT or PtmMAT primers shown above are underlined. Complete nucleotide sequence alignments are provided in Supplemental Figure 1.

sequence in the three SF isolates was due to a 60-bp deletion located in the *MAT1-1-1* gene open reading frame (ORF) that was also found in other SF isolates (34). The 2.2-kb fragments all contained the full-length *MAT1-2* idiomorph which was 2,149 bp in all NF and SF isolates. The organization of *PttMAT* and *PtmMAT* idiomorphs (Fig. 1) was the same as that described previously for the *P. teres* *MAT* locus (36). Nucleotide sequence alignments indicated that the full-length sequences of *MAT1-1* or *MAT1-2* idiomorphs were highly similar (99.6 and 99.5% identity, respectively) between NF and SF isolates (Supplemental Figure 1). This was consistent with previous analysis of the partial sequences of *PttMAT* and *PtmMAT* idiomorphs (34).

BLAST searches against the NCBI nonredundant nucleotide database suggested that the full-length *PttMAT1-1* (or *PtmMAT1-1*)

and *PttMAT1-2* (or *PtmMAT1-2*) sequences were nearly identical (99% identity) to the *MAT1-1* (GenBank accession no. DQ823079) and the *MAT1-2* idiomorph (GenBank accession no. DQ823080) of *P. graminea*, respectively, and had a lower identity (98%) to the *MAT1-1* (isolate 15A, GenBank accession no. AY950585) and the *MAT1-2* idiomorph (isolate 0-1, GenBank accession no. AY950586) of *P. teres* f. *teres* (36). A close examination revealed that the lower identities were due to a number of mismatches, most of which were found in the 5' regions outside of the *MAT* ORF, specifically positions 537 to 589 in the *MAT1-1* idiomorph of 15A and positions 737 to 778 in the *MAT1-2* idiomorph of 0-1 (Supplemental Figure 2). These mismatches were not used for SNP analysis because they were likely due to the poor quality of the original sequences used for assembling. No

Fig. 2. Agarose gel images showing specificity and sensitivity of polymerase chain reaction (PCR) amplifications using the *MAT*-locus specific primers. **A**, Size-differentiable PCR products (arrows) amplified with single nucleotide polymorphisms primers designed to the *MAT1-1* or *MAT1-2* idiomorphs of *Pyrenophora teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*). Genomic DNA templates used for PCR are shown at the top (isolates are given in parentheses). Lanes 1 to 4, single samples (1 for *MAT1-1*, 2 for *MAT1-2*); lanes 5 and 6, two forms with the same mating-type (mixture of equal amounts of DNA); lane 7, all four types of isolates (mixture of equal amounts of DNA); lane 8, *P. graminea* (isolate *Pg*-Alexis, control); and lane 9, *P. tritici-repentis* (isolate *Ptt*-1C-BFP, control). **B**, PCR products amplified from serial dilutions of genomic DNA (100 to 0.001 ng/μl). Primers used for PCR and sizes of the products are the same as those of parallel panels shown in **A**. Numbers on the right indicate the template DNA (corresponding to the template DNA in lanes 1 to 4 in **A**). M, 1-kb DNA ladder.

full length *PtmMAT* idiomorph sequences were found in database searches, although partial or full length ORF sequences of *MAT1-1-1* (1,133 or 1,393 bp) and *MAT1-2-1* (1,058 bp) have been previously determined for several SF isolates (3,34). The 18 full-length *MAT* idiomorph sequences cloned in this study have been deposited into the GenBank database under accession numbers HM121990 to HM122007 (Supplemental Table 1).

Identification of SNPs at the *MAT* locus. Nucleotide sequence alignments revealed eight SNPs in the *MAT1-1* idiomorphs and six SNPs in the *MAT1-2* idiomorphs (Fig. 1). All these SNPs were found to be conserved in NF or SF isolates regardless of the geographic regions (Supplemental Figure 1). In addition to the eight *MAT* ORF-associated SNPs that have been described previously (34), six new SNPs were identified in the 5' regions outside of the *MAT* ORF: two in the *MAT1-1* (positions 90 and 116) (Fig. 1A) and four in the *MAT1-2* idiomorphs (positions 123, 158, 431, and 1,022) (Fig. 1B). In comparison with the *P. graminea* *MAT1-1* and *MAT1-2* sequences mentioned above, more than half of the new SNPs were found to be specific to *PttMAT* or *PtmMAT* idiomorphs: position 116T (*PttMAT*-specific) in *MAT1-1* (Fig. 1A), and positions 123A (*PtmMAT*-specific), 431T (*PttMAT*-specific), and 1022A (*PtmMAT*-specific) in the *MAT1-2* sequences (Fig. 1B). These four new SNPs were selected for designing *PttMAT* and *PtmMAT*-specific primers as shown in Fig. 1. The *MAT* idiomorphs of *P. graminea* were identical to *PtmMAT* at most SNP positions (six out of eight in *MAT1-1* and four out of six in *MAT1-2*) (Fig. 1). There were also a few non-form-specific SNPs found in the *MAT1-2* idiomorphs. Two NF isolates (LDNH04Pt-1 from the United States and JPt0101 from Japan) had an "A" at positions 918 and 1,437 instead of "T" and three SF isolates (SNB0131i, SNB167i and SNB247, all from Australia) had an "A" at position 446 instead of a "G" as in other NF and SF isolates (Supplemental Figure 1B). These SNPs may

represent different haplotypes within the NF or SF *MAT1-2* populations.

Specificity and sensitivity of the *MAT*-specific primers. The four primer pairs for *PttMAT* or *PtmMAT*-specific SNPs (Supplemental Table 2; Fig. 1) were first tested in PCR reactions using the genomic DNA extracted from representative NF and SF isolates from which the corresponding *MAT* idiomorphs were cloned and sequenced (Supplemental Table 1). In all cases, each primer pair amplified a single PCR product with the expected size, regardless of the number of DNA samples added to the reaction mixtures. For example, the primer pair Ptt-MAT1F1/Ptt-MAT1R1 designed to the *PttMAT1-1* locus amplified a single 1,143-bp fragment from DNA of a single NF *MAT1-1* isolate (LDNH07Pt-4) (Fig. 2A, panel 2 from the top, lane 1) and did not give any products from NF *MAT1-2* (LDNH04Pt-1), SF *MAT1-1* (HRS07033), SF *MAT1-2* (SNB247) (lanes 2 to 4) or the NF *MAT1-2* mixed with the SF *MAT1-2* isolate (lane 6). The same primer pair also amplified a single 1,143-bp fragment from DNA templates containing both the NF and SF *MAT1-1* isolates (Fig. 2A, lane 5) or all four types of isolates mixed together (lane 7). Similarly, the primer pair Ptm-MAT1F1/Ptm-MAT1R1 designed to the *PtmMAT1-1* locus amplified a single 194-bp fragment from DNA of the SF *MAT1-1* isolate (Fig. 2A, panel 4, lane 3), or the SF *MAT1-1* isolate mixed with the NF *MAT1-1* isolate (lane 5), or all four types of isolates mixed together (lane 7) but gave no products from DNA containing the NF *MAT1-1* (lane 1) or *MAT1-2* (lane 2) isolate only or both the NF and SF *MAT1-2* isolates (lane 6). The duplex PCRs that included both *PttMAT1-1* and *PttMAT1-2* or *PtmMAT1-1* and *PtmMAT1-2*-specific primers amplified a single fragment with the expected size from DNA containing a single isolate (Fig. 2A, lanes 1 and 2 or 3 and 4) or both the NF and SF isolates with the same mating type (lanes 5 and 6), and gave two products with the expected size from DNA

Fig. 3. Agarose gel images showing polymerase chain reaction (PCR)-amplified products from 28 isolates of *Pyrenophora teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*) in a blind test. Primers used: **A**, *PtGPD1* (control); **B**, *PttMAT1-1/MAT1-2* (duplex); and **C**, *PtmMAT1-1/MAT1-2* (duplex). Names of isolates are given at the top. *P. teres* f. *maculata* isolates are underlined. Lane 29 was loaded with PCR products amplified from *P. tritici-repentis* isolate Ptr-1C-BFP (included as control). M, 1-kb DNA ladder.

containing all four types of isolates (1,143 and 1,421 bp for *PttMAT1-1* and *PttMAT1-2*; 194 and 939 bp for *PtmMAT1-1* and *PtmMAT1-2*) (bottom two panels, lane 7). This indicated that the duplex PCR retained the same specificity as observed in the uniplex PCRs and can be used more conveniently for differentiation of a large number of isolates (described below). The same amplification specificities were also observed when the genomic DNA templates were replaced with plasmid DNA from *E. coli* clones carrying the corresponding full-length *MAT* idiomorph sequences (data not shown).

No cross reactions were found to genomic DNA of *P. graminea*, *P. tritici-repentis*, or other related ascomycetous species (Supplemental Table 1) in PCR amplifications using the *PttMAT* or *PtmMAT*-specific primers (Fig. 2A, panels 2 to 7, lanes 8 and 9). Primer pair PtGPD1F/PtGPD1R amplified a 586-bp fragment from all isolates included in the same PCR experiment (Fig. 2A, top panel, lanes 1 to 9), confirming that the negative results observed were not due to the quality of genomic DNA templates used in PCR reactions.

The minimum amount of genomic template required for differentiation of NF and SF isolates from fungal cultures appeared to be 0.1 to 1 ng, depending on the primer sets used for PCR reactions. As shown in Figure 2B, all primer pairs were able to amplify the expected products from template genomic DNA with the amount ranging from 100 to 0.1 ng and gave readily visible signals (Fig. 2B, all panels, lanes 1 to 4) except for one of the duplex PCRs (*PttMAT1-1* plus *PttMAT1-2*) that produced no products at 0.1 ng (Fig. 2B, panel 2 from the bottom, lane 4). No PCR products were obtained when any of the genomic DNA standards were replaced with the same amount of standards of other types that did not contain the corresponding *MAT* idiomorph sequences. Some nonspecific amplifications were observed only when a high concentration of template DNA (>1 µg) or a high number of cycles (>35) were used in PCRs (data not shown).

The specificity of the four SNP primer pairs was further validated by double blind tests using all 37 NF and 17 SF isolates in our collection (Supplemental Table 1). In all uniplex PCR reactions, the *PttMAT*- or *PtmMAT*-specific primer pair amplified a product of expected size from the “matching” isolate only (results are shown in Supplemental Table 1). Duplex PCR provided the same results as those obtained from uniplex PCR amplifications (Fig. 3). For example, the *PttMAT1-1/MAT1-2* duplex PCR amplified a 1,143-bp fragment from the NF *MAT1-1* isolate 15A (Fig. 3, panel B, lane 3) and a 1,421-bp fragment from the NF *MAT1-2* isolate 0-1 (lane 28). In contrast, the *PtmMAT1-1/MAT1-2* duplex PCR, which produced a 194-bp product for the SF *MAT1-1* isolate Den2.6 (Fig. 3, panel C, lane 6) and a 939-bp product for the SF *MAT1-2* isolate LDNH08Pt-4 (lane 5), did not give visible products from either of these two NF isolates (Fig. 3, panel C, lanes 3 and 28). The example also indicated that the *PttMAT*-specific SNPs were conserved in the *MAT* idiomorphs of isolates 15A and 0-1, both of which, as mentioned above, had apparent mismatches in the corresponding published nucleotide sequences (36) when compared with the *PttMAT* sequences cloned in this study (Supplemental Figure 2).

No discrepancies were found between the results of the uniplex and duplex PCR reactions for any isolates. The *PtmMAT1-1/MAT1-2* duplex PCR amplified a weak product from some NF *MAT1-1* isolates (Fig. 3, panel C, lanes 12, 20, 26, and 27), but this nonspecific product can be easily excluded by the presence of a strong 1,143 bp *PttMAT1-1*-specific fragment in the *PttMAT1-1/MAT1-2* duplex PCR reaction (Fig. 3, panel B, the same lanes).

Differentiation of NF and SF isolates from infected barley tissues. Two representative NF isolates (15A, *MAT1-1* and 0-1, *MAT1-2*) and two representative SF isolates (SNB05064, *MAT1-1* and SG1, *MAT1-2*) (Supplemental Table 1) inoculated (individually or mixed together) onto susceptible barley plants all induced typical net or spot form disease symptoms 5 days after

inoculation. The net form symptoms were characterized by elongated lesions with dark brown blotches, and longitudinal and transverse striations with a net-like appearance, while the spot form symptoms were characterized by dark-brown, elliptical lesions surrounded by a distinct chlorotic zone. Plants coinoculated with all four isolates produced both net and spot form symptoms on the same leaf (Fig. 4A). PCR amplifications using individual *MAT* primer pairs produced a single product of the expected size from barley leaves infected with a matching isolate only, e.g., the *PttMAT1-1*-specific primers amplified a 1,143-bp fragment from the total genomic DNA extracted from a barley leaf inoculated with the NF *MAT1-1* isolate 15A, but not from those inoculated with the other three isolates (Fig. 4B, panel 3, lanes 1 to 4). Each primer pair produced only the expected single product from leaves coinoculated with all four types of isolates (Fig. 4B, lane 5). As mentioned above, PCR amplification using the *PtmMAT1-1* primers gave a weak 194-bp product from 15A (Fig. 4B, panel 2 from bottom, lane 1) that could be easily distinguished from the true positive product (1,143 bp) observed in the amplification with the *PttMAT1-1* primers (panel 3, lane 1). The same amplification specificity was observed when barley leaves were inoculated with each type of NF or SF isolate along with other barley-infecting or noninfecting pathogens (e.g., *Cochliobolus sativus* [S. Ito & Kurib.] Drechsler ex Dastur, *P. tritici-repentis* and *S. nodorum*). The same specificity was also observed with different inoculum levels, or where naturally infected barley leaves collected in the field were used for PCR assays (data not shown).

The host DNA-specific primer pair HvACT1F/HvACT1R designed for the barley actin gene (*HvAct1*) amplified 1,634-bp fragments with similar signal intensities from all leaf samples (Fig. 4B, top panel, lanes 1 to 6), whereas the fungal-specific primer pair PtGPD1F/PtGPD1R designed for the *P. teres* *GPD1* gene (*PtGPD1*) gave 586-bp products of varying signal intensities from the inoculated leaves only (Fig. 4B, panel 2 from the top, lanes 1 to 5). This variation in signal intensity was due to variation in the mycelial mass sampled as a result of variation in the number of lesions present on collected leaves, whereas a uniform amount of barley leaves (≈50 mg per sample) was used for genomic DNA extraction. Non-specific products were not evident from the uninoculated barley in any PCR reaction using individual *MAT* primer pairs (Fig. 4B, panels 2 to 6, lane 6), suggesting that all *PttMAT* and *PtmMAT* primers were specific to the fungal pathogen only.

DISCUSSION

The *MAT* genes are the master regulators of fungal sexual reproduction. The organization of the *MAT* locus as a single genetic element with two alternative forms (idiomorphs) in the fungal genome is conserved in all heterothallic ascomycetes (12, 48,49,53). Sequence information obtained from the *MAT* locus has been widely used for species phylogeny and population genetics studies on phytopathogenic ascomycetes (4,7,16,31,50) including several *Pyrenophora* species (3,34,36). A *MAT*-based mating type assay has become a useful tool for studies on heterothallic ascomycete pathogens. These studies include genetic analyses in which isolates with correct mating types must be identified for the purpose of population development as well as for disease management, e.g., monitoring the coexistence of opposite mating types in the same field. Mating type monitoring may help to determine the risk of sexual spores serving as primary inoculums, leading to a choice of effective control strategies. In contrast, *MAT*-associated SNPs present in nearly identical *MAT* idiomorphs of closely related taxa have not been investigated seriously. The application of these SNPs to DNA sequencing-independent fungal pathogen detection and differentiation has not been reported.

The data presented in this study provide the first evidence that the limited SNPs at the *MAT* locus are sufficient for developing PCR markers to distinguish closely related taxa like *P. teres* f. *teres* and *P. teres* f. *maculata* without the need for DNA sequencing as is required for phylogenetic analysis. This simple diagnostic tool allows us to determine the pathogenicity and mating type characteristics of the isolates in question simultaneously. Such twofold usefulness cannot be found in other random DNA fragment-derived molecular markers (RAPD or AFLP) developed in previous studies (5,9,22,23,39,40,47,52).

The high specificity of *MAT* SNP-based PCR markers is somewhat beyond our expectation because each SNP primer contains only a single base pair difference at the 3' end. This kind of design does not always guarantee high specificity in SNP primer applications (20). The observed high specificity was probably due to the fact that the *MAT* sequences themselves are simple (no repeats), and unique (no apparent similarity to other genes in the fungal or plant genomes) and the two *MAT* idiomorphs (*MAT1-1* and *MAT1-2*) consist of completely dissimilar sequences (no cross-reactions to the opposite *MAT* genes). These characteristics make the PCR amplifications reliable and reproducible without the need for extensive optimization of reaction conditions. The presence of weak nonspecific products for the *PtmMAT1-1* primers was probably due to the fact that the 3' ends of both forward and reverse primers were designed to a SNP with a C to T mismatch that usually reduces specificity (20,30). This pair of primers was chosen solely to prevent potential cross-reactions to *P. graminea*, which is the most closely related species to *P. teres* in the genus (3). It is the only primer pair that can

distinguish both NF and SF isolates from those of *P. graminea*, based on a comparison of *PtmMAT1-1* with *PgMAT1-1* sequences (GenBank accession no. DQ823079). We tested different *PtmMAT1-1*-specific primer sets that contained SNPs at other positions (e.g., positions 90, T/A, and positions 1009, A/G) and were able to get a result just as clean as those obtained using the other three *MAT* primer pairs. If the presence of *P. graminea* *MAT1-1* isolates is not a major concern as discussed below, the primer pairs that give higher specificity can be used for PCR amplifications to eliminate the weak cross-reaction observed.

The conservation of the *MAT*-associated SNPs among isolates collected from diverse geographic regions is striking. Although we started with only 18 isolates (3 to 5 for each type of isolate) to obtain the full-length *MAT* idiomorph sequences, the PCR primers designed for the identified SNPs based on the cloned *MAT* sequences worked perfectly in the blind tests that included more than 50 isolates collected from different continents including Asia, Europe, Australia, and South and North America. This suggests that the SNPs included in each *MAT*-specific primer may have been fixed in natural populations, although we cannot exclude the presence of other *MAT* haplotypes that may escape detection since the number of isolates used in this study is still limited. Our success in differentiating the two forms of *P. teres* may be due to the *MAT* genes themselves being subjected to natural selection (31,48), thus having the potential to mark the "boundaries," not only for fungal species as proposed previously (53), but in some situations, also for the differentiation of subspecies. On the other hand, the investigation of whether the *MAT* locus of *P. teres* is tightly linked to certain pathogenicity or

Fig. 4. Differentiation of *Pyrenophora teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*) from infected barley. **A**, Photographs showing typical net (top two leaves) and spot form (third and fourth leaves) of net blotch symptoms on infected barley leaves. Plants were inoculated with *P. teres* f. *teres* (15A, *MAT1-1* and 0-1, *MAT1-2*) and *P. teres* f. *maculata* (SNB05064, *MAT1-1*, SG1, *MAT1-2*) isolates individually (top four leaves) or mixed together with equal amounts of inocula (bottom). Photograph was taken 5 days after inoculation. **B**, Agarose gels showing polymerase chain reaction-amplified products (arrows) from total genomic DNA of infected barley leaves shown in **A**. Names of isolates are given at the top. Primers are indicated on the left. Uninoc, uninoculated barley. M, 1-kb DNA ladder.

virulence genes controlling the net or spot form phenotypes is needed. Campbell et al. (9) have demonstrated that most AFLP/RAPD marker-defined recombinant progeny of crosses between NF and SF isolates induced an intermediate disease symptom type on differential barley lines and that no independent segregation of the typical net or spot form phenotypes was observed. However, the possible physical linkage between the *MAT* locus and form-specific virulence factors cannot be fully excluded since the segregation of *PttMAT* and *PtmMAT* genes among these recombinant progeny obtained in laboratory conditions has not been documented. Comparative genomic analyses should be conducted to address this question once the genome sequencing projects for the two forms have been completed.

The differentiation of *P. teres* f. *teres* and *P. teres* f. *maculata* using RAPD or AFLP-based markers is often hindered by cross reactions to *P. graminea*, which is a major concern in certain European countries (18). The *MAT*-specific PCR primers developed in this study do not amplify any fragments from genomic DNA of *P. graminea* isolates collected from different geographic regions, including Europe and North America. To our surprise, all these isolates are of the same mating type (*MAT1-2*), including the six isolates that were collected from different locations in Denmark (18). In a related study, partial *MAT* sequences were amplified from three isolates of *P. graminea* and all were found to be *MAT1-2* (3). We noticed that there are only two *P. graminea* *MAT1-1* sequences in the GenBank database (accession numbers DQ823079 and DQ823062) and both are nearly identical (>99.5% identity) to *PtmMAT1-1*. It is unknown if these two isolates could actually be different haplotypes of *P. teres* f. *maculata*. Even though these *P. graminea* *MAT1-1* isolates do occur in the field, the *PttMAT1-1*- and *PtmMAT1-1*-specific PCR primers are not expected to amplify these *PgMAT1-1* sequences because both the forward and the reverse primers in these two *MAT1-1* primer sets have mismatches at the 3' end with the corresponding positions of the *PgMAT1-1* sequences. This prediction can be justified by the fact that the *PttMAT1-2*- and *PtmMAT1-2*-specific primers, which consistently gave a negative result from *P. graminea* *MAT1-2* isolates, were designed using the same "3' end mismatch" strategy.

It is not known if the *MAT1-1* isolates of *P. graminea* occur rarely in nature or do not exist at all. The lack of *MAT1-1* type isolates in a heterothallic ascomycete has been reported for the oat Victoria blight fungus *Cochliobolus victoriae* R.R. Nelson, which consists of only the *MAT1-2* isolates in natural populations (11). It has been hypothesized that *C. victoriae* was derived from a single *MAT1-2* female sterile strain of a closely related species, the corn pathogen *C. carbonum* R.R. Nelson that had acquired oat-specific pathogenicity genes through horizontal transfer (11). It has been shown that the *MAT1-2* sequences of *C. victoriae* and *C. carbonum* are also nearly identical (99.6% identity) (48). A similar scenario may be true for *P. graminea*, which is considered a distinct species due to its unique disease symptoms and pathogenicity but is known to be able to mate with certain isolates of *P. teres* f. *maculata* (10,42,43).

All *PttMAT*- or *PtmMAT*-associated SNPs identified in previous studies were based on the analysis of the *MAT* gene ORF (≈1 kb) sequences because of the lack of full-length *PttMAT* and *PtmMAT* idiomorph sequences. Based on these *MAT* SNPs, one cannot differentiate SF isolates from *P. graminea*. In this study, we identified six SNPs in the 5' regions outside of the *MAT* gene ORF by analyzing 18 full-length *MAT* idiomorph sequences from both NF and SF isolates. The two *PtmMAT1-2*-specific SNPs (positions 123 and 1,022) are especially important since they are the only two sites that can be used to distinguish NF from SF isolates without concern about interference from *P. graminea*.

The finding of conserved SNPs outside the *MAT* ORF regions also raised the question of whether these sequences (0.6 to 0.9 kb) may contain any *cis* elements involved in the regulation of *MAT*

genes. The subtle differences between these sequences may determine specificity of recognition by key regulatory proteins that control the mating between two opposite mating types of the same form, but not different forms, leading to natural genetic isolation as observed (35,37). Since both the *MAT1-1-1* and *MAT1-2-1* proteins differ by only two amino acids between NF and SF isolates, it would be interesting to test whether the sequence difference between these potential *cis* elements affects the mating behavior of these two genetically isolated forms. The fact that the number of SNPs in the 5' outside region (four) is more than that in the *MAT1-2-1* ORF (only two) also suggests the necessity to include this region for *MAT*-based phylogenetic analysis.

Technically, the methods developed in this study use conventional PCR procedures which are less costly and more accessible to laboratory and field researchers than more sophisticated methods such as TaqMan probe-based real-time PCR procedures adapted for AFLP-based markers (23). As shown, the methods presented here are also highly sensitive. To differentiate each type of isolate when grown in culture, only 0.1 to 1 ng of unpurified genomic DNA is needed for PCR amplification. This amount of genomic DNA can be easily obtained from fungal mycelia or spores using routine DNA extraction procedures. For differentiation of the two different forms from infected barley plants, about 200 ng of total genomic DNA is sufficient for detection. This amount of DNA can be easily obtained from small amounts of infected leaf tissue (50 to 100 mg). Multiple isolates infecting barley leaves can be differentiated at the same time using the same amount of DNA template. The minimum amount of fungal genomic DNA in the infected tissue detectable by our procedures has not been determined, but we were able to amplify the expected PCR products using leaf tissues (<50 mg) that contain less than 30 fully developed lesions. Quantitative real-time PCR can be performed (23) if any quantification becomes necessary prior to the differentiation of NF and SF isolates.

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