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RESEARCH ARTICLE

Mating type loci analysis indicates that *Villosiclava virens*, the casual agent of false smut disease of rice, is a homothallic fungus

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ABSTRACT

Villosiclava virens (anamorph: *Ustilaginoidea virens*) was isolated from infected rice plants, where it caused false smut. Similar to other ascomycetes, the sexual compatibility of *V. virens* is controlled by the mating type (*MAT*) locus. In this study, we applied a PCR-based approach to screen mating type genes in the single asexual spore isolate. Our results showed that *V. virens* possesses the two master genes required for mating (*MAT1-1-1* and *MAT1-2-1*), suggesting that this fungus is likely to be homothallic. Mating type primer pairs were developed to genotype the single ascospore isolates from different places in China. These analyses provided further evidence that *V. virens* is a homothallic species and suggest novel genetic mechanisms of sexual reproduction in *V. virens*.
Key words: *Ustilaginoidea virens*, asexual spores, homothallic, *MAT*, multiplex-PCR.

INTRODUCTION

Villosiclava virens (Nakata) E. Tanaka & C. Tanaka is the causative agent of the important false smut of rice (*Oryza sativa* L.) and maize (*Zea mays* L.). The anamorphic state of the causal fungus was called *Ustilaginoidea virens* (Cooke) Takah. (Takahashi, 1896). The teleomorphic state of this fungus is allied with the family Clavicipitaceae and named *Claviceps virens* M. Sakurai ex Nakata (Sakurai, 1934). However, phylogenetic analyses have demonstrated that *Ustilaginoideae* species are not allied with the genus *Claviceps* (White et al., 2000; Bischoff et al., 2004). Therefore, a new genus *Villosiclava* was proposed and *V. virens* has been used as the name for the rice false smut fungus (Tanaka et al., 2008). The pathogen can infect roots at the young seedling stage and rice coleoptiles and staminal filaments at the earlier bolting stage (Tang et al., 2013). The typical symptom of rice false smut is the formation of greenish spore balls in spikelets, which are covered by an abundance of chlamydospores. The spore balls are initially yellow and turn greenish black with maturity, which is when they contaminate the rice grains. Ustiloxins are an additional concern for humans and livestock (Koiso et al., 1994). *Villosiclava virens* can form conidia and ascospores (Fu et al., 2012), which are often the source of infection. The sclerotia of *V. virens*, which often form on the surface of mature spore balls, may germinate and produce sexually reproductive stages of the fungus (Singh & Dubey, 1984). Sexually reproduction results in genetic variation and contributes to evolution.

The sexual cycle of the pathogen plays an important role in disease epidemiology (Hayden et al., 2003). Mating type genes are the master loci that govern sexual reproduction and development in fungi (Metin et al., 2010; Butler, 2010), whereas sexual identity in plants and animals is known to be governed by genes located on the sex chromosomes (Fraser & Heitman, 2005; Marais & Galtier, 2003). Fungi can reproduce by selfing or outcrossing. In heterothallic ascomycetes such as *Tuber melanosporum* Vittad. (Rubini et al., 2011), mating requires a partner of the opposite mating type. In different strains, the genes of opposite mating types are dissimilar sequences: one encodes an alpha-box-domain protein (*MAT1-1*), and the other encodes a high mobility group (HMG) protein (*MAT1-2*) (Turgeon & Yoder, 2000). In contrast to heterothallic species, in most homothallic ascomycetes a single strain contains the genes for both transcription factors, *MAT1-1* and *MAT1-2*. This genetic organization confers self-fertile mating ability (Debuchy & Turgeon, 2006; Yun et al., 2000). These genes in homothallic fungi are usually linked, but in some case they are not, such as in *Aspergillus nidulans* (Eidam) G. Winter (Paoletti et al., 2007).

Similar to other ascomycetes, sexual reproduction in *V. virens* is controlled by the *Mat* loci. In previous studies, Yokoyama et al. (2006) suggested that *V. virens* (*Claviceps oryzae-sativae* in his work) was an heterothallic fungus using a PCR-based assay. Here, we reexamined the *V. virens* mating type genes in single asexual-spore (chlamydospore) isolates and ascospores isolates collected from China and determined the mating type genotype using multiplex PCR. This multiplex PCR approach can be applied to investigate

the distribution of mating types (Dyer et al., 2001). In addition, we analyzed the distribution of mating type genes in several isolates from the natural population.

MATERIAL AND METHODS

Fungal isolates and culture conditions

Isolates derived from conidia and ascospores used in this study are listed in Tables 1 and 2, respectively.

Isolation from conidia

These isolates were collected in southwestern and eastern China in 2011 and were obtained from naturally infected rice spikelets showing typical false smut symptoms. The yellowish rice kernels, which were covered with a mass of *V. virens* chlamydospores, were surface sterilized with 75% ethanol for 2 min and subsequently rinsed three times with sterile water. The treated specimens were resuspended into a chlamydospore suspension and diluted to 10^3 ml^{-1}. An aliquot (150 µl) of the spore suspension was poured onto potato dextrose agar (PDA) solid medium containing 100 µg/ml chloramphenicol. The plate was incubated at 28ºC in the dark. When visible colonies appeared, the colonies were transferred individually onto fresh PDA plates and incubated at 28ºC in the dark. Each isolate was individually maintained at 4°C on a separate 9-cm Petri dishes containing PDA solid medium.

Isolation from ascospores

Sclerotia were collected in the rice field and were buried in moist sterile sand at 25°C under light. The single ascospore was isolated as previously described (Singh & Dubey, 1984). The isolates were maintained at 4°C as described above.

DNA isolation and ITS-PCR

Villosiclava virens mycelia were inoculated into 50 ml potato dextrose liquid and kept on an incubator-shaker at 150 rpm. The mycelia were harvested by filtration and ground to a powder in the presence of liquid nitrogen. DNA was isolated as reported by Murray and Thompson (1980), resuspended in deionized water, and stored at -20°C.

Specific internal transcribed spacer (ITS) primers were used to verify the identity of *V. virens* isolates. The polymerase chain reaction (PCR) mix (50 µl) contained 2 U polymerase (Takara), 5µl of 10× Taq buffer, 400 mM of dNTP's, 2 µmol of DNA template and primers US1-5 and US3-3 (Table 3) (Zhou et al., 2003). PCR was performed using the following conditions: 96°C for 1 min, followed by 30 cycles of denaturing at 96°C for 20 s, annealing at 58°C for 30 s and extension 72°C for 30 s, followed by a final extension for 7 min at 72°C. PCR-amplified products were resolved by standard agarose gel electrophoresis and detected by Gelview (BioTeke) staining.

TABLE 1 - Isolates of conidia used in this study.

Isolates	Locality	Longitude	Latitude	Strain number[a]	*MAT1-1-1*	*MAT1-2-1*
Uvsf	Shifang	104.16	31.19	8	+	+
Uvmy	Mianyang	104.42	31.30	10	+	+
Uvdz	Dazhou	107.29	31.14	6	+	+
Uvmz	mianzhu	104.19	31.32	12	+	+
Uvnj	Neijiang	105.02	29.36	9	+	+
Uvzg	Zigong	104.46	29.23	7	+	+
Uvfa	Fuan	119.39	27.06	6	+	+
Uvlz	Luzhou	105.24	28.54	12	+	+
Uvsn	Shuining	105.33	30.31	9	+	+
Uvkm	Kunming	102.42	25.04	14	+	+
Uvcd	Chengdu	104.04	30.40	26	+	+
Uvhf	Hefei	117.17	31.52	12	+	+
Uvnc	Nanchong	106.04	30.49	10	+	+
Uvfz	Fuzhou	119.18	26.05	16	+	+
Uvya	Yaan	102.59	29.59	10	+	+
Uvql	Qionglai	103.34	30.26	7	+	+
Uvhz	Hangzhou	120.10	30.16	10	+	+
Uvxc	Xichang	102.16	27.54	14	+	+
Uvcz	Chongzhou	103.40	30.39	8	+	+
NBRC31672	Japan			1	+	+

[a]A total of 206 isolates collected in 19 Chinese regions.
[+]Detected.

TABLE 2 - Analysis of the mating type in single ascospores isolates.

Isolates	Locality	Longitude	Latitude	Strain number[a]	*MAT1-1-1*	*MAT1-2-1*
Uvascd	Chengdu	104.04	30.40	8	+	+
Uvasql	Qionglai	103.34	30.26	6	+	+
Uvasya	Yaan	102.59	29.59	5	+	+
Uvnc	Nanchong	106.04	30.49	7	+	+

[a]A total of 26 isolates.
[+]Detected.

TABLE 3 - Primers used in this study.

Genes	Primers	Sequences
ITS	US1-5	5'-CCG GAG GAT ACA ACC AAA AAA ACT CT-3'
	US3-3	5'-GCT CCA AGT GCG AGG ATA ACT GAA T-3'
MAT1-1-1	MAT1 F1	5'-GAA ACT CYA ACT CAA ACR AAG TCG-3'
	MAT1 R1	5'-GKA AAC YTT GGC TAT CAR CGC CC-3'
	MAT1 F2	5'-GAA ACT CCA ACT CAA ACG AAG TCG-3'
	MAT1 R2	5'-GTA AAC TTT GGC TAT CAA CGC CC-3'
MAT1-2-1	MAT2 F1	5'-GGA GCR ACA TAA TAC CGT YAA AGA-3'
	MAT2 R1	5'-GGR GTG TTT TWC TAA GAR GGC CT-3'
	MAT2 F2	5'-GGA GCG ACA TAA TAC CGT CAA AGA-3'
	MAT2 R2	5'-GGG GTG TTT TTC TAA GAG GGC CT-3'

PCR amplification of mating type genes and DNA sequencing

The primers for the *MAT1* genes were designed based on the high similarity to amino acid sequences of the alpha box and HMG box in *Clavicipitaceae*. We constructed two sets of primers (MAT1-F1/MAT1-R1, MAT2-F1/MAT2-R1; Table 3) to amplify the relatively conserved *MAT1-1* and *MAT1-2* genes in *V. virens*.

PCR reactions were carried out in a 25 µl volume containing 25 ng DNA template, 20 mM Tris-HCl pH 8.4, 1.5 Mm $MgCl_2$, 200 mM dNTP's, 25 pmol of each primer, and 1 U Taq polymerase (Takara). Amplification conditions were 95°C for 1 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 56°C for 1 min and extension at 72°C for 40 s, followed by a final extension for 7 min at 72°C. The PCR amplicons were resolved, and their sizes were determined on a 1.2 % agarose gel run in 0.5× TBE.

The amplicons of the *MAT1-1* and *MAT1-2* genes were excised from the gel and purified using a Gel Extraction kit (Axygen). The purified fragments were cloned into the pEASY-T1 vector (TransGene) according to the manufacturer's instructions. Sequences were obtained using Illumina GA technology, and analysis was carried out using the National Center for Biotechnology Information (NCBI) BLAST program.

Analysis of mating type gene distribution in natural populations of *Villosiclava virens* in China

We designed two sets of specific primers (MAT1-F2/MAT1-R2, MAT2-F2/MAT2-R2; Table 3) to amplify the *MAT* genes in *V. virens* isolated from naturally infected rice spikelets showing typical false smut symptom by multiplex PCR. The PCR reaction mixture (20 µl) contained 10× Taq buffer, 4 mM $MgCl_2$, 0.4 mM each dNTP, 0.2 µM of each of the four primers and 1 U of Taq DNA polymerase (Takara). PCR products were amplified according to the following conditions: initial denaturation at 95°C for 1 min, follow by 40 cycles of denaturing at 95°C for 30 s, annealing at 56°C for 40 s and extension at 72°C for 40 s, and a final elongation step for 7 min at 72°C.

RESULTS

Isolation and characterization of *Villosiclava virens*

Chlamydospores of *V. virens* germinated easily on PDA medium. Colonies appeared as white dots at 5 days after inoculation. As the fungus grew, the colony gradually resembled white bread (Figure 1). A single colony of *V. virens* was transferred and maintained in a fresh culture. In this study, we obtained 206 conidia isolates from southwestern and eastern China (Table 1) and 26 ascospore isolates (Table 2).

The identity of the pathogen was further verified by ITS-PCR. The specific primers of *V. virens* amplified a 380-bp product (Figure 2) from all the isolates specific to the false smut pathogen.

Identification of *MAT1-1* and *MAT1-2*

After DNA extraction from asexual single spore isolates, their mating type genes were identified by PCR amplification. The results showed that *V. virens* possesses two types of mating type loci, *MAT1-1-1* and *MAT1-2-1*. The genomic sequences of the *MAT1* genes have been deposited in GenBank (accession numbers KC920891 and KC920892). In the *MAT1-2-1* gene sequences, comparison of KC920892 with AB124632 (which was already deposited in GenBank) indicated 89% identity. The length of the mating type genes *MAT1-1-1* and *MAT1-2-1* were 256 bp and 222 bp, respectively (Figure 3).

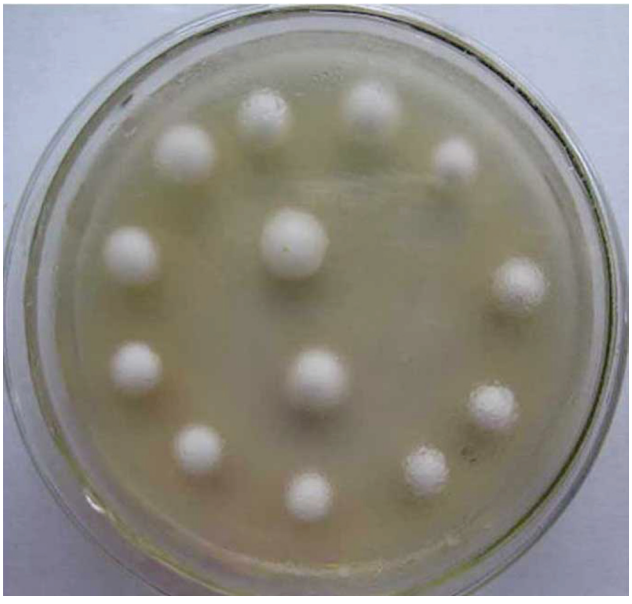

FIGURE 1 - Colony morphology of asexual spores (chlamydospores) germination (5 days).

Mating type gene distribution in natural populations

The presence and distribution of mating type genes collected from natural populations of the fungus were estimated using a multiplex PCR method. As shown in Table 1, *V. virens* isolated from each local population contained both *MAT1-1* and *MAT1-2* genes.

***V. virens* resulting from selfing**

To confirm our hypothesis that *V. virens* is derived from selfing and that it is a homothallic fungus, we performed multiplex PCR using two sets specific primers (MAT1-F2/MAT1-R2 and MAT2-F2/MAT2-R2) to screen mating type genes in ascospore isolates. The results showed that the ascospore isolates exhibited both *MAT1-1-1* and *MAT1-2-1* genes (Figure 4). These results suggested that *V. virens* is self-fertile and does not need the participation of a partner.

DISCUSSION

In this study, the ITS from all isolates tested, including the reference strain for *V. virens*, showed 99.9 to 100% sequence similarity, confirming that these isolates are genetically closely-related.

Fungal mating type genes play an important role in regulating sexuality, virulence and survival. Sun et al. (2013) have reported that the genetic diversity of *U. virens* was high in some areas of China. In this study, we used asexual spores to amplify the mating type genes of *V. virens* and found that a single asexual spore isolate carries both *MAT1-1-1* and *MAT1-2-1* genes. This

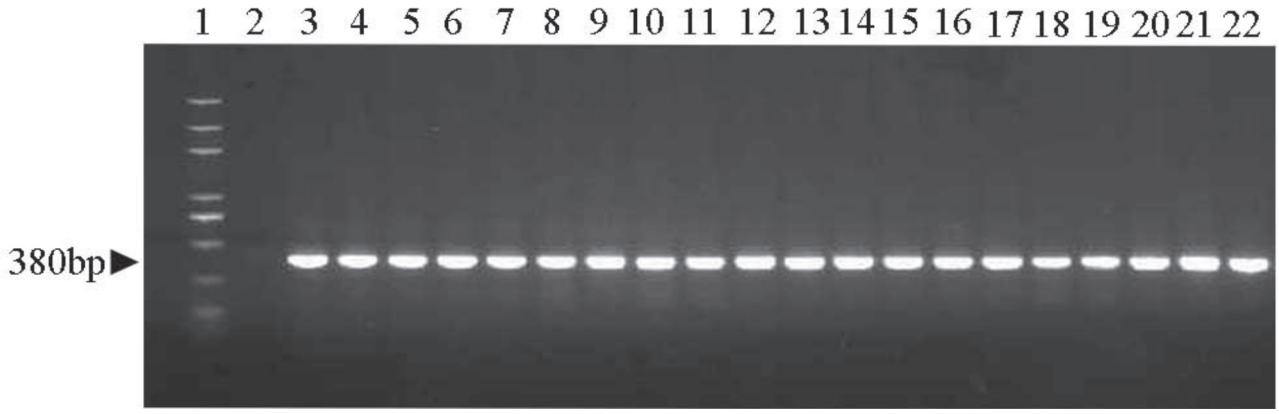

FIGURE 2 - Gelview-stained agarose gel showing representative results of PCR amplification of the ITS regions of *V. virens* isolates. Lane 1, DNA marker (Trans2K, TransGen); lane 2, negative control; lane 3, positive control; lanes 4-22, isolates Uvsf2, Uvmy5, Uvdz1, Uvmz10, Uvnj9, Uvzg7, Uvfa1, Uvlz9, Uvsn1, Uvkm1, Uvcd1, Uvhf10, Uvnc1, Uvfz12, Uvya10, Uvql6, Uvhz9, Uvxc12, Uvcz8.

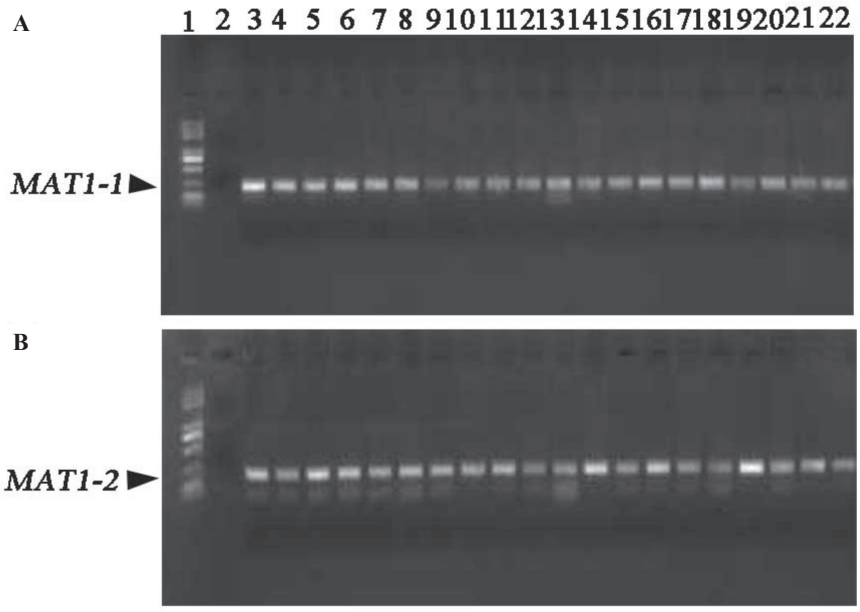

FIGURE 3 - Goldview-stained agarose gel showing representative results of PCR amplification of mating-type gene fragments in *V. virens* isolates. **A.** Fragment of *MAT1-1-1* gene (256 bp) (accession number KC920891); **B.** Fragment of *MAT1-2-1* (222 bp) (accession number KC920892). Lane 1, DNA marker (Trans2K, TransGen); lane 2, negative control; lane 3 positive control; lanes 4-22, isolates Uvsf2, Uvmy5, Uvdz1, Uvmz10, Uvnj9, Uvzg7, Uvfa1, Uvlz9, Uvsn1, Uvkm1, Uvcd1, Uvhf10, Uvnc1, Uvfz12, Uvya10, Uvql6, Uvhz9, Uvxc12, Uvcz8.

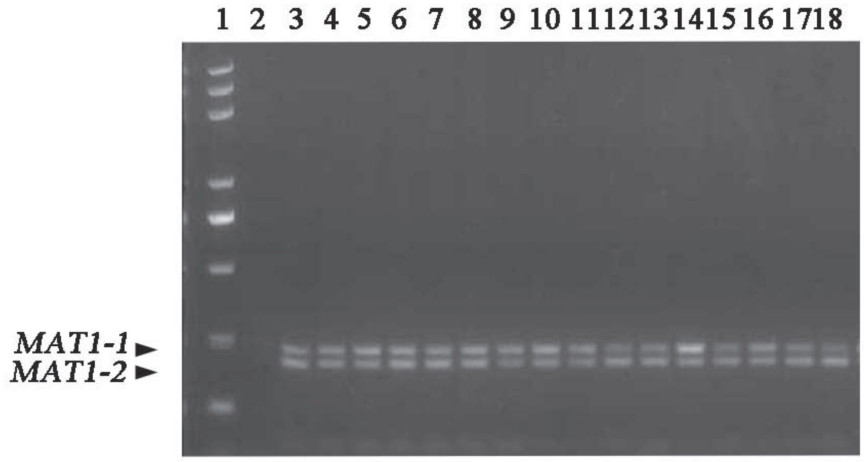

FIGURE 4 - Multiplex PCR amplification of mating-type genes from representative ascospore isolates. Lane 1, DNA marker (Trans2K, TransGen); lane 2, negative control; lanes 3-18, isolates Uvascd1, Uvascd2, Uvascd6, Uvascd8, Uvasql2, Uvasql3, Uvasql5, Uvasql6, Uvasya1, Uvasya2, Uvasya3, Uvasya5, Uvasnc2, Uvasnc3, Uvasnc5, Uvasnc6.

result differs from those reported by Yokoyama et al. (2006), who found only *MAT1-2* gene in *U. virens*. This difference may be due to primer design and PCR conditions. Mating type genes have previously been described in several heterothallic and homothallic filamentous fungi as master regulators of sexual compatibility and sexual reproduction (Casselton, 2002; Kronstad 2007; Paoletti et al., 2007). These genes in homothallic fungi are usually linked. An exception is self-compatible *Neurospora africana* L.H. Huang & Backus, which contains only MAT-A-1 present in the genome (Glass & Smith, 1994). Homothallic fungi such as *A. nidulans* are also cross-fertile (Pontecorvo et al., 1953). Pontecorvo et al. (1953) propose the term "relative heterothallism" to explain the ability of a homothallic species to cross-fertilize. Several studies suggest that sexual identity in homothallic systems might be regulated by differential expression of mating type genes or selective epigenetic silencing one of two *MAT* genes (Raju & Perkins, 2000; Pöggeler, 2002; Scazzocchio, 2006). Further research is necessary to determine whether or not the *MAT* genes in the genome of *V. virens* are linked.

In this study, we developed a simple and reliable multiplex PCR-based marker for determining the distribution of mating type genes in *V. virens*, and our results showed that all isolates displays both *MAT* genes. Furthermore, the multiplex PCR codominant marker was used to screen mating type genes in ascospore isolates, and the results showed that the ascospore isolates exhibited both *MAT1-1-1* and *MAT1-2-1* genes. The results provide further evidence that *V. virens* is a homothallic fungus (Yun et al., 2000; Debuchy & Turgeon, 2006). The

multiplex PCR was also used to ascertain the heterothallism in the ascomycete *Tuber melanosporum* (Rubini et al., 2011). Our studies suggest a novel reproductive mechanism of *V. virens*. Determination of the mating type of field isolates may provide important insights into the genetic basis and molecular mechanism of reproduction in *V. virens*.

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