Unconventional Recombination in the Mating Type Locus of Heterothallic Apple Canker Pathogen Valsa mali

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ABSTRACT  Sexual reproduction in filamentous ascomycetes is controlled by the mating type (MAT) locus, including two idiomorphs MAT1-1 and MAT1-2. Understanding the MAT locus can provide clues for unveiling the sexual development and virulence factors for fungal pathogens. The genus Valsa (Sordariomycetes, Diaporthales) contains many tree pathogens responsible for destructive canker diseases. The sexual stage of these ascomycetes is occasionally observed in nature, and no MAT locus has been reported to date. Here, we identified the MAT locus of the apple canker pathogen Valsa mali, which causes extensive damage, and even death, to trees. V. mali is heterothallic in that each isolate carries either the MAT1-1 or MAT1-2 idiomorph. However, the MAT structure is distinct from that of many other heterothallic fungi in the Sordariomycetes. Two flanking genes, COX13 and APN2, were coopted into the MAT locus, possibly by intrachromosomal rearrangement. After the acquisition of foreign genes, unequal recombination occurred between MAT1-1/2 idiomorphs, resulting in a reverse insertion in the MAT1-2 idiomorph. Evolutionary analysis showed that the three complete MAT1-1-2, COX13, and APN2 genes in this region diverged independently due to different selection pressure. Null hypothesis tests of a 1:1 MAT ratio of 86 V. mali isolates from four different provinces showed a relatively balanced distribution of the two idiomorphs in the fields. These results provide insights into the evolution of the mating systems in Sordariomycetes.

Sexual reproduction is important in pathogenic ascomycetes because new combinations of virulence alleles are created through outcrossing (McDonald and Linde 2002). Mating in filamentous ascomycetes is typically controlled by a single locus, termed the mating-type (MAT) locus, which includes two alleles called MAT1-1 and MAT1-2. These alleles have been termed “idiomorphs” due to their high sequence divergence (Metzenberg and Glass 1990). Filamentous ascomycetes generally exhibit self-incompatible (heterothallic) or self-compatible (homothallic) lifestyles. In heterothallic ascomycete fungi, the MAT locus carries either the MAT1-1 or the MAT 1-2 idiomorph, which contains at least a MAT1-1-1 α-domain gene or a MAT1-2-1 high-mobility-group (HMG) domain gene, respectively. In addition, several additional genes are also found in various ascomycetes, although the functions of these genes remain obscure (Dyer et al. 2016). These mating-type genes function not only in controlling sexual development, but also in regulating fungal secondary metabolites and hyphal morphology (Kück and Böhm 2013). Thus, understanding the MAT locus can provide insights into the sexual development and virulence factors in filamentous ascomycetes.

The genus Valsa (Sordariomycetes, Diaporthales) contains >500 species, including many aggressive tree pathogens responsible for canker diseases. Valsa cankers affect >70 species of trees worldwide, and often cause extensive damage to trees (Agrios 2005). V. mali Miyabe et Yamada [anamorph Cytospora sacculus (Schwein.) Gvrit.], causing destructive canker on apple and resulting in severe yield losses in eastern Asia (Lee et al. 2006; Li et al. 2013), infects mainly apple by conidia.
while ascospores are not common (Wang et al. 2014; Wang et al. 2016) (Figure 1). Of the 150 infected apple bark samples from different regions in China, only eight from dead trunks have ascostromata with ascospores (Wang 2007). In addition, the sexual reproduction of Valsa spp cannot be induced in the laboratory to date, and no MAT locus in Valsa has been reported. Thus, in this study, we identified and delineated the structure of the MAT locus of V. mali using comparative genomic approaches, and investigated the evolution of MAT genes and loci in V. mali and closely related species.

MATERIALS AND METHODS

Strains and culture conditions

All strains used in this study were deposited at the Laboratory of Integrated Management of Plant Diseases in College of Plant Protection, Northwest A&F University, Yangling, PR China. Cultures were grown on potato dextrose agar (PDA) medium with a layer of cellophane at 25°C.

Identification of the V. mali MAT locus

The mating type locus of V. mali strain 03-8 was identified by BLASTP searches against V. mali proteome (>1E-5) using protein sequences of MAT1-1-1 and MAT1-2-1 from the closely related species Cryphonectria parasitica as query sequences, which suggests that V. mali is heterothallic, and that strain 03-8 carries the MAT1-1 idiomorph. MAT1-2 candidate isolates were then identified by PCR using a VmMAT1-1-1 specific primer pair. To identify the MAT1-2 idiomorph, the genome of isolate SXLCl46 was sequenced using Illumina HiSeq technology. Filtered paired-end reads were assembled by ABysS v1.9.0 (Simpson et al. 2009), and gene models were predicted using MAKER v2.31.8 (Holt and Yandell 2011). Primer pairs of VmMAT1-1-1 (F: 5’-GAAAGGTCGGAAAGGCAAAG-3’ and R: 5’-AGGGTTCGGGCAGGCAAT-3’), and VmMAT1-2-1 (F: 5’-CAACATTGGCATTCAACTCA-3’ and R: 5’-GAAAGGTCGGGGCAAT-3’), were used for PCR detection of isolates from different geographic regions.

Evolutionary analyses of the MAT locus

Synteny of the MAT locus between MAT1-1 and MAT1-2 idiomorphs was analyzed using GATA (Nix and Eisen 2005). Protein sequences of mating type genes were aligned using MAFFT v7.245 (Katoh and Standley 2013), and poorly aligned regions were removed by trimAl v1.4 (Capella-Gutiérrez et al. 2009). Maximum likelihood trees were constructed by IQtree v1.3.11 (Nguyen et al. 2015), using the build-in best evolutionary model selection function. Branch supports were assessed with ultrafast bootstrap method (Minh et al. 2013) and SH-aLRT test (1000 replicates). Selection pressure on mating type genes were tested at the codon level using the ete evol tool in ETE package v3.0 (Huerta-Cepas et al. 2016). The coding sequence alignments of these genes were constructed by the ETE package using several build-in alignment tools, and CodeML and Slr analyses were then performed by the ete-evol program. Sites under selection were identified using the M2 and SLR models. The null hypothesis of a 1:1 MAT ratio of V. mali was tested using chi-square goodness-of-fit test using the online tool VassarStats (http://vassarstats.net/).

Transmission electron microscopy (TEM)

The perithecium, ascus, and ascospore of V. mali in the field were investigated by TEM. Ascostromata samples from the canker were processed for TEM as described by Ke et al. (2013). For TEM, ultrathin sections of specimens cut with a diamond knife were collected on copper grids. After contrasting with uranyl acetate and lead citrate, the grids were examined with a TEM 1230 (JEOL) at 80 kV.

Data availability

All strains used in this study are available upon request. The raw Illumina reads of isolate SXLCl46 have been deposited at the Sequence Read Archive (SRA) database of NCBI (SRP075864). The nucleotide sequence of MAT1-2 idiomorph has been deposited at the GenBank database (KX349090).

RESULTS AND DISCUSSION

Identification of MAT1-1 idiomorph

To identify the mating type locus in V. mali, protein sequences of core mating type genes MAT1-1-1 (GenBank: AAK83346) and MAT1-2-1 (AAK83343) of the closely related species C. parasitica were used to search against the genome of V. mali strain 03-8 (GCA_000818155.1) (Yin et al. 2015). By performing BLASTP searches, only MAT1-1-1 was
found in strain 03-8. The absence of the MAT1-2-1 HMG-box gene in the MAT locus of strain 03-8 suggests that V. mali is likely heterothallic. VmMAT1-1-1 (VM1G_08160) contains the typical α-domains, and adjacent genes include SLA2 (VM1G_08159), MAT1-1-2 (VM1G_08161), COX13 (VM1G_08162), APN2 (VM1G_08163), and MAT1-1-3 (VM1G_08164) (Figure 2). Intriguingly, COX13 and APN2 locate in the MAT locus, while the location of SLA2 and APN2 is fairly conserved, and often flanks the idiomorph among other mating type genes in many other filamentous ascomycetes (Dyer et al. 2016), such as C. parasitica (McGuire et al. 2001). Likewise, idiomorphs of two heterothallic species Coccidioides immitis (Fraser et al. 2007) and Uncinocarpus reesii (Mandel et al. 2007) also captured COX13 and APN2 into the MAT locus, while both these genes are adjacent to the MAT locus in closely related species. However, the role or influence of this kind of remodeling remains unknown.

As a heterothallic species, there must be MAT1-2 isolates of V. mali. However, MAT1-1 and MAT1-2 isolates of filamentous ascomycetes are morphologically indistinguishable for most of their life cycles (Debuchy et al. 2010). To identify MAT1-2 isolates, a specific primer pair for VmMAT1-1-1 (F: 5'-GAAAGGTCGGAAGGCAAAG-3' and R: 5'-AGAGTCGCGTCGGCAAT-3') was used to detect V. mali isolates. Isolate SXLC146, without a PCR product, was then used to identify the MAT1-2 idiomorph.

**Identification of the MAT1-2 idiomorph**

To determine the structure of the MAT1-2 idiomorph, the genome of isolate SXLC146 was sequenced de novo by Illumina HiSeq-PE150 platform. A total of 20,860,413 clean reads (5.2G, effective rate 96.46%) were subjected to assembly. Nucleotide sequences of genes

| Population      | Total Number | MAT1-1 | MAT1-2 | $\chi^2$, $P$ Value |
|-----------------|--------------|--------|--------|---------------------|
| Baoji, Shaanxi  | 10           | 4      | 6      | 0.4, $P = 0.7518$   |
| Yuncheng, Shanxi| 24           | 9      | 15     | 1.5, $P = 0.3078$   |
| Tianshui, Gansu | 13           | 6      | 7      | 0.08, $P = 1$       |
| Sanmenxia, Henan| 30           | 12     | 18     | 1.2, $P = 0.3594$   |
| All isolates    | 86           | 35     | 51     | 2.98, $P = 0.1055$  |
flanking the VmMAT1-1 idiomorph were used to perform BLASTN searches against genome assemblies of isolate SXLC146. Gene models of the scaffold that contains the MAT1-2 idiomorph were then predicted using MAKER v2.31.8 (Holt and Yandell 2011). VmMAT1-2-1 (GenBank: KX349090) contains the HMG-box, and adjacent genes includes SLA2, APN2, COX13, and MAT1-1-2 (Figure 2). Similar to the VmMAT1-1 idiomorph, the VmMAT1-2 idiomorph also captured APN2 and COX13 into the MAT locus. The MAT locus organization indicates that V. mali is heterothallic. Unexpectedly, a mating type gene MAT1-1-2 of the MAT1-1 idiomorph is present in the MAT1-2 idiomorph. MAT1-1-2 is ubiquitous in Sordariomycetes, and is required for fruit body development (Debuchy et al. 2010). However, the involvement of MAT1-1-2 (especially in the MAT1-2 idiomorph) in sexual development of V. mali remains unknown.

In order to test the null hypothesis of a 1:1 MAT ratio of V. mali, 86 isolates from four different provinces were detection by PCR using two pairs of specific primers targeting VmMAT1-1-1 and VmMAT1-2-1, respectively (Supplemental Material, Table S1). Both idiomorphs were present in the four provinces, and their MAT ratios did not deviate significantly from 1:1 (Table 1), which suggests a relatively balanced distribution of mating-type idiomorphs in the field.

Unconventional recombination of MAT locus in V. mali
Recombination at the MAT locus in ascomycetes is thought to be suppressed (Idnurm 2011). However, synteny analysis showed that the region carrying APN2, COX13, and MAT1-1-2 in the MAT1-2 idiomorph is a reverse insertion, probably acquired from the MAT1-1 idiomorph by recombination (Figure 2). Additionally, MAT1-1-2 contains many more sequence variations than APN2 and COX13. Protein sequence identity of these three genes are 100% (COX13), 92.13% (APN2), and 76.89% (MAT1-1-2), respectively. A similar event was also reported in the closely related species Diaporthe spp., the MAT1-2

![Figure 3](Image)

Phylogeny of mating type genes: (A) MAT1-1-1, (B) MAT1-2-1, (C) MAT1-1-3, (D) MAT1-1-2, (E) APN2, (F) COX13. Maximum likelihood phylogenetic trees were constructed from top 20 BLASTP hits in GenBank using IQtree. The scale bar represents substitutions per site.

![Figure 4](Image)

Evolutionary analysis of MAT1-1-2. The CodeML and Slr analyses were performed using the ete evol tool in ETE package. Site models M2 and SLR, and branch model fb, respectively, were used. Omega value of branch is represented in the node size and color. Small blue disk on the node of phylogenetic tree stands for low omega value.
idiomorph of which carries homologs of \textit{MAT1-1-2} (identity: 80.95\%) and \textit{MAT1-1-3} (54.62\%) in the same gene order and orientation as that in \textit{MAT1-1} (Kanematsu et al. 2007) (Figure 2). These unconserved “addi-
tional” mating type genes, caused by unequal recombination, are prob-
ably also functional, because they are transcriptionally active during
vegetative growth (Kanematsu et al. 2007). Nevertheless, future work is
required to determine the functions of these genes in sexual reproduction.

Unequal recombination at the \textit{MAT} locus has also been demon-
strated in many other ascomycetes, but genes involved in those events are
often fragments or truncated pseudogenes (Tsui et al. 2013). In \textit{V. mali}, unequal recombination resulted in the presence of three
compete genes in \textit{MAT1-2}. The types of \textit{MAT} structure in \textit{Valsa} spp.
(including \textit{V. malicola}, \textit{V. sordida}, and \textit{V. persoonii}, Z. Yin and
L. Huang, unpublished results) and \textit{Diaporthe} spp. are unconventional

\begin{figure}
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\includegraphics[width=\textwidth]{figure5}
\caption{Proposed evolution scenario of \textit{MAT} locus in the ancestor of \textit{Valsa mali}.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{Scanning electron micrographs and TEM graphs of the perithecium, ascus, and as-
cospore of \textit{V. mali}. (A) longitudinal section of the perithecium (\textit{Pe}) with a single cavity and beak (\textit{Pb}) under apple bark. (B) Trans-
verse section of perithecium (\textit{Pe}) showed a single orifice (\textit{Po}) on
the perithecial beak. (C) Clavate ascus (\textit{As}) in perithecium with as-
cus orifice (\textit{Ao}). (D) TEM graph of asci (\textit{As}) with ascospores (\textit{Asp}).
Bars, (A) 200 \textmu m; (B) 200 \textmu m; (C) 5 \textmu m; (D) 1 \textmu m.}
\end{figure}
and distinct from known heterothallic filamentous ascomycetes. In addition, another closely related heterothallic species, C. parasitica, which causes chestnut blight, has the typical MAT structure in heterothallic Sordariomycetes, carrying three genes, MAT1-1-1, MAT1-1-2, and MAT1-1-3 in the MAT1-1 idiomorph and only one gene, MAT1-2-1, in the MAT1-2 idiomorph (McGuire et al. 2001) (Figure 2). This finding suggests that this unconventional recombination occurred evolutionally from specific families in Diaporthales. Given that homothallic has likely evolved from heterothallic ascomycetes (Billiard et al. 2011; Dyer et al. 2016), the MAT structures of V. mali and Diaporthale spp. will provide clues for unveiling the evolutionary history of the mating systems in Sordariomycetes. However, to confirm this hypothesis, it is necessary to determine the MAT loci of additional species (especially homothallic) in Diaporthales.

**Evolutionary analyses of mating type genes**

The same MAT structure in V. mali and V. pyri, as well as the scenario in Diaporthale spp., indicates that recombination predates speciation (Figure 2). The three foreign genes (APN2, COX13, and MAT1-1-2) in the MAT1-2 idiomorph were unlikely to have been acquired independently, and probably diverged independently. We were thus interested in the phylogeny of these genes. For each mating type gene of V. mali, the protein sequences of the top 20 blast hits in the GenBank nr database were aligned, and subjected to maximum likelihood phylogenetic tree construction. The tree of the COX13 gene shows that this gene in the VmMAT1-2 idiomorph is more closely related to the homolog in VmMAT1-1 than to that in the MAT1-1 idiomorph of V. pyri (Figure 3), which suggests that COX13 was acquired before the divergence of Valsa species. Likewise, the MAT1-1-2 and MAT1-1-3 genes are present in both idiomorphs of the Diaporthale sp. group within G-type and W-type species, respectively. The VmCOX13 genes are highly conserved in both idiomorphs, and ancestral VmAPN2 and VmMAT1-1-2 genes are thus likely to have the same phylogenetic relationship as VmCOX13. However, the VmMAT1-1-1 gene in MAT1-1 is closely related to that in MAT1-1 of V. pyri, while VmAPN2 in MAT1-2 groups with that in MAT1-1 of V. pyri (Figure 3). This result suggests that the APN2, COX13, and MAT1-1-2 genes in the different idiomorphs diverged independently after acquisition.

Selection pressure analysis of the three acquired genes showed that MAT1-1-2 has been under purifying selection at interspecific level, indicating that this gene is preserved for proper function (Figure 4). Many sites of MAT1-1-2 under purifying selection are likely responsible for the dramatically divergent. In contrast, one site (60E) in the nuclease domain of APN2 is under positive selection, while the COX13 gene may go through neutral evolution without any site under positive or purifying selection. Thus, we can speculate that the different selection pressure of these three genes results in different levels of sequence divergence. Collectively, a possible scenario of the evolution of MAT loci in Valsa spp. is that ancestral MAT1-1 first coopted APN2 and COX13 into the MAT locus by intrachromosomal rearrangement, and the ancestral MAT1-2 then acquired MAT1-1-2, COX13 and APN2 by unequal recombination; finally these three genes diverged independently due to different selection pressure (Figure 5).

**Cryptic sexuality**

In nature, sexual reproduction of V. mali is occasionally observed, often on dead apple trunks in autumn. The matured ascostromata could be identified on apple barks by the exposed surface, which has many minute black papillae (Figure 1J). Each papilla has an opening of a long neck connected with a perithecium. A longitudinal section of the ascostroma shows that spherical or subglobose perithecia are partly immersed in bark (Figure 6A). When fully matured, the cavity of the perithecum is closely packed with eight asci, which are formed from the basal inner wall of the perithecium. The asci are clavate-oblong or clavate-fusiform, rounded or truncate at the apex, and subsessile (Figure 6C). Ascospores are produced in sausage-shaped ascii (Figure 6D) (Ideta 1909; Wang et al. 2011), and discharged into the air during wet weather (Agrios 2005).

Attempts to induce self-fertilization of V. mali in the laboratory failed. Mycelial plugs of two isolates with opposite mating types (03-8 and SXLC146) were placed at opposite sides of a sterile apple twig embedded on agar according to successful mating tests in C. parasitica (Marra and Milgroom 2001) and Diaporthale spp. (Kanematsu et al. 2000). However, selling of C. parasitica and Diaporthale spp. is still a rare event in the laboratory, in that only some cross assays succeed (Kanematsu et al. 2000; Marra and Milgroom 2001). In addition, mature perithecia were more likely to develop successfully in crosses between isolates derived from a single ascus naturally formed on host twigs, compared to a randomly selected ascus (Kanematsu et al. 2000). Therefore, it is necessary to test more isolates of V. mali, especially isolates from the same ascus.

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