Chapter 4
Genomics of *Tuber melanosporum*: New Knowledge Concerning Reproductive Biology, Symbiosis, and Aroma Production

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4.1 Introduction

The symbiotic ascomycetes belonging to the genus *Tuber* produce hypogeous fruit bodies, known as truffles, highly priced and praised for their distinctive aroma and taste by gourmets. Although the life cycle of symbiotic ascomycetes remains to be fully elucidated, the establishment of methodologies to inoculate host plants and the growing market for these fungi have contributed to the growth of large-scale truffle cultivation programs over the last several decades. These programs were first developed in Europe, where highly valued *Tuber* spp. are endemic, then later established in other countries worldwide (Hall et al. 2007).

Molecular investigations of *Tuber* spp. were initiated more than a decade ago, with the primary goals of reliably identifying morphologically similar truffle species throughout their entire life cycle and preventing economic fraud (Henrion et al. 1994; Paolocci et al. 1995). The wealth of molecular-based knowledge amassed, especially in the last few years, has made it possible to tackle key biological questions regarding truffle reproductive biology and ecology. This research mainly yielded the following developments: recent progress on methodologies for dissecting and genotyping each of the structures that these fungi develop throughout their complex life cycle (i.e., single spore and single mycorrhizal root tip), high-throughput technologies for transcriptomic and ecological studies, and, most importantly, the recent release of the *Tuber melanosporum* Vittad. genome. This species produces the most highly valued black truffle, known as the Périgord black truffle. The availability of the *T. melanosporum* genome has made this species the model fungus not only among *Tuber* spp. but among all symbiotic ascomycetes. Thus, the characterization of the genomic organization and genes of this species that are committed to symbiosis

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provides mycologists a more comprehensive understanding of the similarities and differences between symbiotic ascomycetes and basidiomycetes. Moving from the comparison of these two fungal types, this chapter will highlight the genetic and environmental determinants of the aroma of this truffle species. Finally, recent findings on the genetic diversity and reproductive biology of *T. melanosporum* will be discussed, along with aspects relevant to the cultivation and marketing of this fungus and its importance for understanding the biology of other *Tuber* spp. of economic interest.

4.2 Basic Features of the *T. melanosporum* Genome

With 125 megabases, the *T. melanosporum* genome is the largest fungal genome sequenced thus far and is approximately four times greater in size than the genomes of other ascomycetes (Martin et al. 2010a; Galagan et al. 2005). Its unusually large size has not been attributed to genome duplications, which characterize the genomes of other ascomycetes, such as *Saccharomyces cerevisiae* Meyen ex E. C. Hansen and other yeast species (Wolfe and Shields 1997), and fungi belonging to other lineages, such as *Rhizopus oryzae* Went & Prins. Geerl. (Ma et al. 2009). Evidence indicating large-scale dispersed segmental duplications was also not observed (Martin et al. 2010a). Instead, the large genome size likely resulted from the proliferation of transposable elements (TEs), which account for about 58 % of the genome. Automatic annotation of the TEs in the *T. melanosporum* genome indicated that these elements are primarily retrotransposons, with an abundance of Gypsy/Ty3-like elements (29.5 % of the assembled genome) followed by long interspersed elements (5.6 % of the genome). The major wave of TE expansion likely occurred approximately 2–3 million years ago. In turn, TE proliferation may have facilitated deep rearrangements of the genome, resulting in a low level of gene synteny with other ascomycetes (Martin et al. 2010a).

Despite the large genome size, the number of predicted genes is surprisingly small (about 7,500) and falls within the lower range of the gene numbers observed among sequenced filamentous fungi (Martin et al. 2010a; Martin 2011). Moreover, protein-coding genes and TEs are not randomly distributed, with blocks of relatively high gene density and a scarcity of TEs separated by large genomic regions with low gene content and a high abundance of TEs. Compared to other filamentous ascomycetes, analyses of the *T. melanosporum* genome revealed a high number of simple sequence repeats (SSRs) in addition to TEs. In fact, searching for mono-, di-, tri-, tetra-, penta-, and hexanucleotide repeats led to the identification of more than 22,000 SSRs (Murat et al. 2011). The majority of these SSRs are located in noncoding DNA, although a small number of SSRs are located within coding sequences and UTR regions. Interestingly, the gene ontology classification revealed that some of the genes carrying SSR motifs in their sequences are genes involved in regulatory networks, nucleotide binding, signaling pathways, symbiosis, and host interactions. One interesting challenge will be to determine whether changes in the SSR length and position affect the role and function of genes important for the fungal life cycle, such as those controlling symbiosis.

Another distinctive trait of the *T. melanosporum* genome is the small number of multigene families and the low level of sequence similarity of paralogous genes compared to other ascomycetes. Furthermore, the level of similarity between *T. melanosporum* predicted proteins and those of other ascomycetes is not very high (approximately 45 %), which is in full agreement with the phylogenetic reconstructions placing Pezizomycetes as the earliest diverging lineage within the Pezizomycotina (James et al. 2006).

4.3 Analysis of the *T. melanosporum* Genome Provides New Insight into the Mechanisms of Mycorrhizal Symbiosis

The availability of the complete genomes of the basidiomycete *Laccaria bicolor* (Maire) P. D. Orton (Martin et al. 2008) and *T. melanosporum* (Martin et al. 2010a) gives mycologists the opportunity to compare the genetic networks of two mycorrhizal fungi belonging to different phyla and to compare these fungi to others characterized by different lifestyles, such as saprotrophs and plant pathogens.

The *T. melanosporum* and *L. bicolor* genomes share some common features. For example, the large reduction in the number of genes encoding plant cell wall (PCW) degradation enzymes, compared to saprophytic and pathogenic fungi, appears to be specific to the mycorrhizal fungi. This significant reduction in the number of PCW-degrading enzymes suggests that mycorrhizal fungi have developed a host colonization strategy that prevents the elicitation of the pathogen-triggered response in their hosts (Martin et al. 2010a; Plett and Martin 2011). As a result, however, mycorrhizal fungi rely almost entirely on their host plants for carbon.

Of the few upregulated genes in the ectomycorrhizas of these two symbiotic fungi, the majority of them encode membrane transporter proteins essential for nutrient exchange between the fungus and the host plant. The genomes of these two mycorrhizal fungi also show the expansion of particular gene families, including the tyrosine kinase family. Kinases are proteins involved in many cellular processes, such as cellular differentiation and proliferation, and their expansion in the genomes of both mycorrhizal species suggests that kinases may have a key role in mycorrhiza development (Plett and Martin 2011).

In addition to these common features, there are also many differences in the expression patterns of symbiosis-related genes in *L. bicolor* and *T. melanosporum*, suggesting a divergent evolution of the symbiotic lifestyles of symbiotic basidiomycetes and ascomycetes. First, transcriptomic analyses have shown that only a small number of orthologous genes are upregulated in both *T. melanosporum* and *L. bicolor*. Moreover, no PCW-degrading enzymes are expressed during the symbiotic stage in *L. bicolor*, although some of these enzymes are produced in the hyphae growing in the soil (Martin et al. 2008). In *T. melanosporum*, on the other hand, some PCW-degrading enzymes are expressed during the colonization of the host roots (Martin et al. 2010a; Plett and Martin 2011). Overall, these data

suggest that the mycelia of *L. bicolor* have a higher saprotrophic capability than those of *T. melanosporum*. Along these same lines, a distinguishing feature of *T. melanosporum*, compared to other mycorrhizal fungi, is the presence of a gene that encodes invertase, an enzyme that hydrolyzes sucrose (Ceccaroli et al. 2011). In contrast to the saprotrophic and pathogenic fungi, almost all mycorrhizal species lack invertase genes and are thus unable to hydrolyze the sucrose synthesized by their hosts. Instead, mycorrhizal fungi depend on the activity of the host-derived invertase to utilize their major carbon source. This appears to be a mechanism by which host plants control the spread of their symbiotic partners but one that *T. melanosporum* may be able to avoid.

Another striking difference between the sequenced symbiotic fungi involves the presence and expression of mycorrhiza-induced small secreted proteins (MiSSPs). In *L. bicolor*, many MiSSPs have been identified, and some of these (10) resemble the effector proteins of pathogenic fungi (Martin et al. 2010a; Plett and Martin 2011; Veneault-Fourrey and Martin 2011). Recently, Plett et al. (2011) demonstrated that one of these *L. bicolor* proteins, MiSSP7, is an indispensable effector for the establishment of symbiosis. An effector protein, named SP7, has also been reported in the mycorrhizal fungus *Glomus intraradices* N. C. Schenck & G. S. Sm. (Kloppholz et al. 2011), suggesting that mutualistic and pathogenic fungi may share common host manipulation mechanisms (Plett et al. 2011; Koeck et al. 2011). Although the *T. melanosporum* genome contains several genes encoding small secreted proteins, none of these genes appear to be upregulated in the ectomycorrhizas. Whether *T. melanosporum* establishes symbiosis with its hosts using an effector-mediated mechanism is therefore a crucial question with both basic and applied relevance that will need to be addressed in the near future.

Overall, whole genome and high-throughput transcriptome analyses depict a scenario in which the mechanisms for the establishment and maintenance of symbiotic mutualism have diverged evolutionarily between basidiomycetes and ascomycetes.

4.4 Genes for the Production of Truffle Aroma

The widespread appreciation and gastronomic status of truffles reside in their distinctive aroma. The chemical compounds responsible for the truffle aroma vary among the different *Tuber* spp. both in quality and quantity. Among the volatile organic compounds (VOCs) identified in *Tuber* fruiting bodies, those derived from sulfur metabolism (S-VOCs) are the most important. Many studies have demonstrated that sulfur compounds such as bis(methylthio)methane are characteristic of the white truffle *T. magnatum* Pico, whereas dimethylsulfide and dimethyldisulfide are the main components of the black truffle aroma (for a recent review, see Splivallo et al. 2011). The release of chemicals by these species allows signaling information to be exchanged with the surrounding environment to ensure the completion of the fungal life cycle (Pacioni et al. 2007). For example, spore dispersal in these hypogeous fungi depends on mycophagists that are attracted by the volatiles emitted from

fruit bodies. Dimethylsulfide from *T. melanosporum* is regarded as the main attractant of pigs (Splivallo et al. 2011), and notably, truffle hunters originally used pigs to locate truffles.

Although many VOCs have been identified in the fruiting bodies of different *Tuber* spp., the ability of truffles to produce all of the volatile components responsible for their aroma has been questioned. Indeed, a variety of microorganisms, such as bacteria, yeasts, and filamentous fungi, have been reported to grow within truffle fruiting bodies (Barbieri et al. 2007; Buzzini et al. 2005; Pacioni et al. 2007). Pure cultures of the yeast strains isolated from *T. magnatum* and *T. melanosporum* fruiting bodies produce the VOCs characteristic of their hosts (Buzzini et al. 2005). Furthermore, chemical analyses on the mycelia of different truffle species grown in vitro found that some of the compounds present in the corresponding fruiting bodies were absent (Tirillini et al. 2000; Splivallo et al. 2007), suggesting either an exogenous origin of the truffle VOCs or a tightly controlled, stage-specific emission of these compounds.

The release of the *T. melanosporum* genome has allowed mycologists to address this particular question. Enzymes and putative metabolic pathways involved in the biosynthesis of volatile compounds have been identified. *In silico* analyses have shown that the genes required to synthesize most of the key components of the black truffle aroma are present in the *T. melanosporum* genome. More specifically, genome analysis has revealed the presence of 126 genes related to sulfur assimilation and S-amino acids interconversion and metabolism such as the genes coding for cystathionine β- and γ-lyases, thought to be involved in the production of S-VOCs (Martin et al. 2010a).

Furthermore, the genes for the enzymes of the Ehrlich pathway, likely responsible for the synthesis of other components of truffle aroma (i.e., fusel alcohols 2-methylbutanal and 3-methylbutanal) and a complete set of genes involved in the biosynthesis of isoprenoids, have been identified (for more details, see Martin et al. 2010a; Splivallo et al. 2011). Significantly, the expression of most of these genes is upregulated in the fruiting body compared to the mycorrhizas and free-living mycelium; thus, the emission of the most important components of the truffle aroma appears to depend on stage-specific gene regulation (Martin et al. 2010a; Splivallo et al. 2011). Notwithstanding, a synergic action between truffle species and their associated microorganisms that qualitatively and quantitatively shapes the arrays of chemicals that give rise to particular truffle bouquets cannot be ruled out. More chemical and genetic studies are thus required to determine the relative contributions of each partner to the aroma of truffles. A similarly intriguing and challenging question is one of the most vexing in *T. melanosporum* research: whether the changes in the aromatic properties of truffles of different geographical origin are solely due to the different pedoclimatic conditions (Bertault et al. 1998). Significant differences in the proportion of VOCs among *T. magnatum* truffles of different origin have been reported (Gioacchini et al. 2008). Chemical and population genetics analyses along with studies aimed at investigating genetic polymorphisms and the expression profiles of candidate genes for the production of the aromatic compounds in *T. melanosporum* specimens of different origin should provide meaningful insights to address this question.

4.5 *T. melanosporum* Population Genetics: Approaches, Results, and Implications

The assessment of the extent and distribution of the intraspecific genetic variability among and within natural truffle populations helps determine the history, evolution, and the present status of the population genetic structure of *Tuber* spp. Population genetics studies on *T. magnatum* and *T. melanosporum* have provided preliminary evidence on the reproductive biology of *Tuber* spp. (see below). Furthermore, population genetics analyses are of interest for tracing the geographical origins of truffles. This is particularly relevant because, historically, the market value of truffles has depended on both the species and the geographical origin of the fruit bodies. Thus, the ability to identify the geographic origin of truffles is important for associations of truffle harvesters and local governments, which aim to promote the economic and social development of rural and marginal areas. It may also prevent the erosion of local biodiversity by encouraging the use of autochthonous fungal genotypes for the artificial inoculation of host plants to be transplanted to naturally producing truffle areas.

Early studies using molecular markers suggested a very limited genetic diversity in *T. melanosporum* populations and led to the conclusion that *T. melanosporum* experienced a population bottleneck during the last glaciation (Bertault et al. 1998, 2001). Later studies, however, revealed significant genetic differences among *T. melanosporum* populations in France using polymorphisms within the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) (Murat et al. 2004). Similarly, based on amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSR), and ITS markers, *T. melanosporum* populations have been found to exhibit extensive genetic variability, with the southernmost populations showing the highest levels of genetic diversity (Riccioni et al. 2008). These findings support the hypothesis that the postglacial *T. melanosporum* expansion followed a northward pattern from refugia located in the Italian and, possibly, the Iberian Peninsula.

The successful discrimination of truffle populations according to their origin depends heavily on sampling strategies and on the number of polymorphic markers used to screen the specimens (Rubini et al. 2007). The use of a few polymorphic SSRs was sufficient to identify a phylogeographic structure in natural populations of *T. magnatum* (Rubini et al. 2004, 2005). Beyond the importance of SSRs for studying the organization of the truffle genome and their putative role in modulating gene expression, SSRs are one of the most suitable markers for population genetic analyses. In this regard, 135 SSRs mined from the *T. melanosporum* genome were recently used to evaluate the degree of polymorphism among fruiting bodies of different geographical origins; approximately 44 % of the SSRs were polymorphic, with up to 10 or more alleles in some cases, a number much higher than those found for previously characterized SSR loci (Murat et al. 2011). Thus, these new SSRs may be highly informative for identifying the provenance of *T. melanosporum* individuals.

4.6 Information from Genomic Analyses on the Reproductive Biology of *T. melanosporum*

4.6.1 *The Life Cycle and Reproductive Biology of* T. melanosporum

Karyological studies have suggested the prevalence of a dikaryotic phase in the truffle life cycle (Lanfranco et al. 1995). As a result, when DNA isolated from *T. melanosporum* fruiting bodies was amplified using codominant markers (SSR), the lack of heterozygosis in this supposedly dikaryotic structure was interpreted as a clear indicator of selfing (Bertault et al. 1998, 2001). However, a study that employed SSR markers in *T. magnatum* provided the first evidence that outcrossing can occur in *Tuber* spp. (Rubini et al. 2005). The absence of significant linkage disequilibrium among the SSR loci of truffles from the same or proximal populations indicated the occurrence of extensive gene flow between individuals, a finding in conflict with the thesis that truffles strictly self-fertilize. Definitive evidence of outcrossing in both species was later provided by the use of codominant markers to genotype the DNA isolated from the gleba and pooled spores of single *T. magnatum* and *T. melanosporum* fruiting bodies (Paolocci et al. 2006; Riccioni et al. 2008). More specifically, the spores of a few truffles exhibited two alleles at some SSR loci, while the corresponding gleba always had a single allele per locus. In light of these genetic data, it has been postulated that the gleba of truffles is made up of haploid hyphae of uniparental (maternal) origin and that the occurrence of outcrossing is either possible or mandatory (Rubini et al. 2007; Riccioni et al. 2008). Not all of the truffles analyzed displayed additional alleles of paternal origin in the pool of spores, and it has been reported that homothallic (self-fertile) ascomycetes may also have some rate of outcrossing (Kronstad 2007). The dilemma regarding *T. melanosporum*'s reproductive mode has been only recently solved by studying the structure and organization of the mating type genes in its genome. On the basis of all this information, it was possible to define a reliable model of the *Tuber* spp. life cycle (Fig. 4.1).

4.6.2 *The Mating Type Locus of* T. melanosporum

Sexual reproduction in fungi is controlled by small regions of the genome known as the mating type (*MAT*) loci (for recent reviews, see Debuchy et al. 2010; Casselton and Feldbrügge 2010). Molecular analyses have revealed that filamentous ascomycetes (Pezizomycotina) have a single *MAT* locus with two master regulators of sexual reproduction: the *MAT* gene *MAT1-1-1*, which encodes an α-box domain protein, and the *MAT1-2-1* gene, which encodes a high-mobility group (HMG) domain protein (Butler 2007; Debuchy et al. 2010).

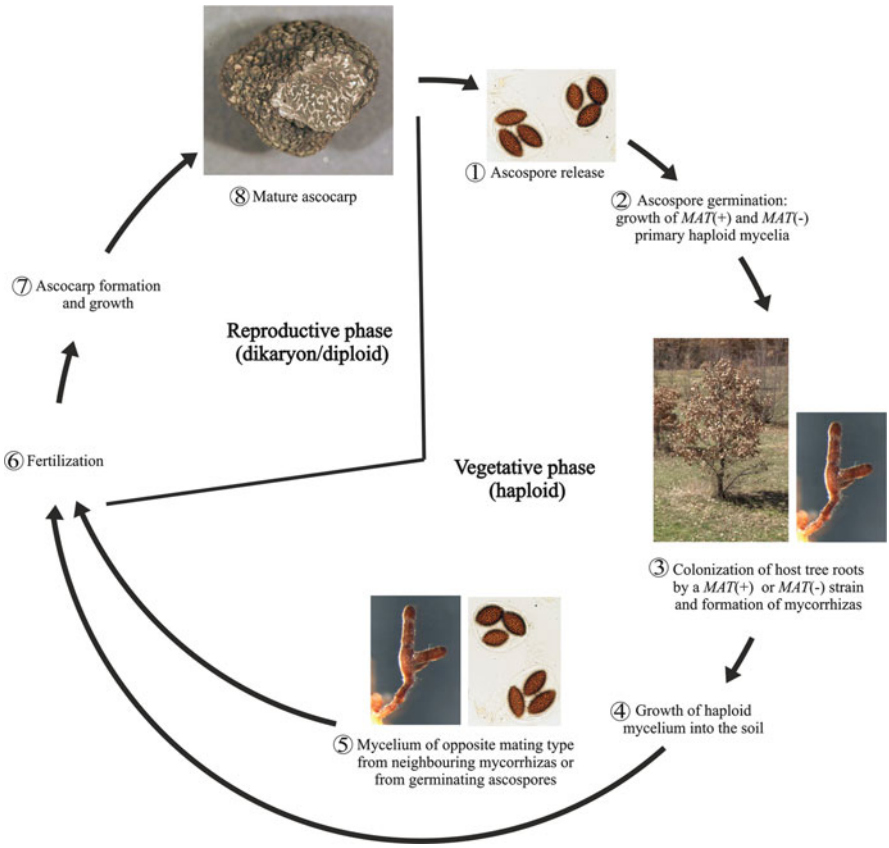

Fig. 4.1 Schematic representation of *Tuber* spp. life cycle. The ascospores released in the soil from mature ascocarps (1) germinate and produce *MAT*(+) or *MAT*(−) primary haploid mycelia (2). The primary mycelia colonize host tree roots and the ectomycorrhizas are formed (3); under a given tree, competition among different strains may result in the formation of mycorrhizas that share the same mating type. In presence of favorable climatic conditions, *MAT*(+) and/or *MAT*(−) mycelia originating from mycorrhizas grow in the soil (4); contact with mycelia of opposite mating type (5), which may originate from either ascospores or mycorrhizas from neighboring trees, is needed for fertilization to occur (6). The fertilization process gives rise to the ascocarp (fruit body, truffle) (7) which is made of the dikaryotic hyphae and the gleba, a sterile haploid mycelium of uniparental origin. Inside the mature ascocarp (8), the dikaryotic hyphae generate the asci where karyogamy takes place to form the zygotes; these diploid nuclei undergo meiosis to produce the haploid ascospores

In ascomycetes, there are two primary sexual reproductive modes: heterothallism and homothallism. In heterothallic ascomycetes, the two *MAT* genes occur in different strains; thus, heterothallic ascomycetes are self-sterile, and crossing between strains of opposite mating types is required. Incidentally, the two alternative forms of the *MAT* locus in these fungi are not two allelic versions *sensu stricto* and are instead referred to as idiomorphs (Metzenberg and Glass 1990).

In homothallic ascomycetes, a single strain harbors both *MAT* genes. Typically, the two genes are closely linked or fused within the same locus, but in a few species, such as *Aspergillus nidulans* (Eidam) G. Winter, they may reside at different loci in a single strain (Galagan et al. 2005). Therefore, homothallic ascomycetes do not have distinct sexes and are capable of selfing or crossing with any other individuals of the same species.

An *in silico* analysis of the *T. melanosporum* genome for orthologs of the *MAT1-2-1* and *MAT1-1-1* genes present in other ascomycetes revealed that the sequenced strain (Mel28) contains only the *MAT1-2-1* gene, indicating a heterothallic organization. In line with this hypothesis, only a subset of truffles produced the expected amplicon when their gleba was amplified with primers specific for *MAT1-2-1* gene. Using other PCR-based strategies, the *MAT* locus from a gleba (me206) that lacked the *MAT1-2-1* gene was cloned, and the second *T. melanosporum* mating type gene (*MAT1-1-1*) was identified therein (Rubini et al. 2011b).

The sequence comparison of the complete *MAT* regions from the me206 and Mel28 strains showed that the length of the *MAT1-2* and *MAT1-1* idiomorphic regions were approximately 7,430 bp and 5,550 bp, respectively. Although these regions are considerable in length, each *T. melanosporum* idiomorph contains only a single *MAT* gene, a situation that typifies most but not all ascomycetes (Debuchy et al. 2010). The genomic regions flanking the *MAT* locus are conserved among ascomycetes (Butler et al. 2004), and the presence of genes, such as *APN2* and *SLA2*, linked to the *MAT* locus in Pezizomycotina and some Saccharomycotina species (Butler 2007; Martin et al. 2010c) is regarded as a mark of a conserved evolutionary origin of the *MAT* locus. Interestingly, these two genes are not linked to the *MAT* locus in *T. melanosporum*. Among the genes that are linked to the *MAT* locus in *T. melanosporum*, only *COX13*, encoding for cytochrome c oxidase, resides near the *MAT* locus in other ascomycetes such as *Neurospora crassa* Shear & B. O. Dodge and *Gibberella zeae* (Schwein.) Petch (Butler 2007). This low level of synteny around the *MAT* locus likely reflects the extensive TE-driven rearrangements that the *T. melanosporum* genome has undergone (Martin et al. 2010a).

In conclusion, the analysis of the organization of the *MAT* locus in the *T. melanosporum* genome has provided mycologists some definitive evidence concerning the reproductive biology of this fungal species. The information garnered from *T. melanosporum* has also provided some clues for the characterization of the reproductive strategies employed by other *Tuber* spp. (Martin et al. 2010b).

4.6.3 *The Pheromone Receptor System in* T. melanosporum

The mating type genes encode regulatory proteins responsible for the determination of cell specificity. The mechanism of mating type determination of sexual specificity is particularly well studied in *S. cerevisiae*. In budding yeast, the recognition between cells of opposite mating types is mediated by diffusible a-factor and α-factor peptide pheromones that are produced in a mating type-specific manner. The pheromones in this yeast species are sensed by the specific G protein-coupled

receptors STE2 and STE3 that, through a mitogen-activated protein kinase (MAPK) cascade, trigger the expression of the homeodomain transcription factor *STE12*, which, in turn, activates the mating response (Leberer et al. 1997; Elion 2000).

A pheromone receptor system similar to that of *S. cerevisiae* has been described in many filamentous ascomycetes (for a recent review, see Pöggeler 2011). In heterothallic species, such as *N. crassa*, fertilization is preceded by the development of specialized male and female structures called antheridia and ascogonia, respectively. In ascogonia, a specialized hypha, the trichogyne, grows toward the hyphae of the opposite mating type attracted to the pheromone that the sexual partner emits (Bistis 1981; Kim and Borkovich 2006). Pheromones and receptors have also been identified in homothallic fungi, but in some of these fungi, the pheromone receptor system is not required for fertilization and is instead involved in later stages of sexual development (Mayrhofer et al. 2006).

Although genetic analyses have shown that *T. melanosporum* is heterothallic, the sexual fertilization step in this and other *Tuber* spp. remains elusive (Rubini et al. 2007). Indeed, fertilization structures such as antheridia have never been observed in any truffle species, and the presence of ascogonia has been reported only once (Callot 1999). Nevertheless, the majority of the genes controlling sexual reproduction in yeasts and other filamentous ascomycetes are conserved in *T. melanosporum*. In particular, its genome contains genes with sequences and structural features similar to the α-factor pheromone and the *STE2* and *STE3* receptors of *S. cerevisiae* (Martin et al. 2010a; Rubini et al. 2011b). Sequence similarity-based searches failed to identify any putative a-factor pheromone precursor genes, but this is consistent with the short length of this gene and the low level of conservation among a-factor pheromone precursor genes of different ascomycetes (Pöggeler 2011). The two-pheromone peptides are produced from larger protein precursors following different maturation pathways, and genes involved in both pathways are present in *T. melanosporum*. More specifically, the following genes have been identified: homologs of *KEX1* and *KEX2*, which encode putative carboxypeptidases; a homolog of *STE13*, which encodes a diaminopeptidase responsible for α-pheromone processing; and components of the a-factor pheromone-processing pathway (Martin et al. 2010a). Similarly, homologs of the following are present: G protein α, β, and γ subunits; components of the MAPK cascade, *STE20*, *STE11*, *STE7*, and *FUS3*; and the genes encoding the transcription factors STE11-HMG and STE12, which are associated with the pheromone pathways of *Schizosaccharomyces pombe* and *S. cerevisiae*, respectively.

The *in silico* identification of all of these genes suggests that a pheromone pathway operates in *T. melanosporum*. What remains to be identified are the specific biotic and abiotic factors that trigger the sexual pathway in this species. The availability of powerful tools, such as whole-genome custom oligoarrays (Martin et al. 2010a) and RNA-seq-based methodology (Tisserant et al. 2011), will allow for the investigation of the effects of diverse factors on the induction of sexual reproduction. Using a whole-genome oligoarray, Zampieri et al. (2010) showed that the cold stress response in *T. melanosporum* mycelia involves extensive transcriptomic changes and suggested that a cold period is necessary to initiate the development of truffle fruiting bodies.

4.6.4 *From the Genome to the Field: The Tracing of* T. melanosporum *Strains of Opposite Mating Type Reveals an Unexpected Biased Distribution*

Truffle production in both natural and cultivated truffle grounds is highly unpredictable. Until very recently, environmental determinants were the only factors considered to affect truffle fruiting. The finding that *T. melanosporum* is a heterothallic fungus has raised the question of whether the spatiotemporal distribution of strains of opposite mating types might, in addition to pedoclimatic conditions, represent a limiting factor for the occurrence of fruiting.

The availability of commercial kits for the isolation of soil DNA, the development of quick procedures for typing single truffle ectomycorrhizas (Paolocci et al. 1999), and the use of mating type-specific primers (Rubini et al. 2011b; Martin et al. 2010b) have made it possible to monitor the sexual identity of *T. melanosporum* strains at sites where truffles are naturally produced (Rubini et al. 2011a). In this study, single mycorrhizas and fruiting bodies collected beneath twelve productive sites within the same truffle ground were genotyped using *MAT*-specific primers and SSR markers (Rubini et al. 2011a). It was observed that strains with opposite mating type were never present on the same root apparatus and that a given fungal strain might spread to nearby plants by vegetative propagation to give rise to a genet. A balanced presence of mycelia of the two opposite mating types was the expected distribution for a heterothallic fungus, and this biased distribution of strains on productive host plants was a surprising result.

The same study revealed that the gleba of the harvested fruiting bodies had the same genotype as the ectomycorrhizas collected from the same site. This finding suggested that the root resident strain, regardless of its mating type, always acts as the female partner in the cross. However, the pool of spores for most of the truffles analyzed had alleles that were different from those exhibited by the mycorrhizas collected at all of the productive sites, which suggested that the male partner might not necessarily be represented by a root resident strain. Indeed, both mating types of *T. melanosporum* were detected in soil samples around a productive tree. In accordance with these findings, recent studies investigating *T. melanosporum* strain biodiversity using the polymorphism within the ITS region revealed the presence of different haplotypes in the soils of productive truffle grounds and a higher level of strain biodiversity in the soil than those displayed by the fruit bodies (Napoli et al. 2010; Mello et al. 2011). Because the saprotrophic activity of *T. melanosporum* is limited (Martin et al. 2010a; Iotti et al. 2002), the persistence and spread of its free-living mycelia in the soil is expected to be more limited, spatially and temporally, compared to strains that reside in roots, and this may negatively interfere with truffle production.

As the "ideal situation" of the co-occurrence of both *MAT1-2-1* and *MAT1-1-1* strains is not met on naturally colonized host plants, could it be achieved by artificially inoculating host plants? To address this question, host plants were inoculated under controlled conditions with single *T. melanosporum* fruiting bodies as spore donors and 6 and 19 months postinoculation (pi), the screening for

the presence of both mating types performed (Rubini et al. 2011a). Mycorrhizas of both mating types were formed and, at 6 months pi, the ratio of the two types was approximately similar in all inoculated plants. The screening performed at 19 months pi, however, indicated a drastically different situation: the majority of the plants had mycorrhizas that were all of the same mating type; of the few plants that displayed both mating types, there was a marked prevalence of one of the two types. Thus, under forced conditions, *T. melanosporum* strains seem to compete with each other for the colonization of host plant roots, and as a result, only a single strain persists on a given plant. Remarkably, the distribution pattern observed on artificially inoculated host plants strongly resembled the distribution patterns found on plants from a site where truffles are naturally produced. Taken together, these data indicate that a competition occurs between *T. melanosporum* strains for the colonization of their host plants and corroborate the view that an unbalanced representation of both mating types may be the main factor limiting *T. melanosporum* production in both artificial and natural sites (Rubini et al. 2011a).

The genetic basis underlying competition phenomena between truffle mycelia of different mating types has yet to be elucidated. It might be somehow related to self/nonself recognition phenomena. At this regard, Iotti and coworkers (2012) showed that orthologs of the genes known to control heterokaryon incompatibility (HI) in other fungal species are present in the *T. melanosporum* genome, although they lack the key functional domains involved in the HI process. Moreover, as in vitro dual culture experiments between pairs of genetically different *T. melanosporum* strains revealed neither the existence of any HI reaction nor the formation of anastomoses, it has been argued that in this truffle species any vegetative incompatibility-related phenomena might depend on mechanisms that act before hyphal contact (Iotti et al. 2012).

If the results concerning the biased distribution of the two fungal sexes on host plants are confirmed by additional experiments, then many practices in the cultivation of this fungus will need to be carefully reconsidered. For example, *T. melanosporum* orchards should be set taking into consideration the mating type of the fungal strain(s) on the host roots to maximize the chances of the appropriate strains mating with each other. Additionally, the screening of artificially inoculated host plants would be recommended to certify the *Tuber* spp. on their roots along with the mating types present.

Finally, because of the possibility that other *Tuber* spp. of economic interest are also heterothallic (Martin et al. 2010b), parallel studies should be undertaken to determine whether an unbalanced distribution of strains with opposite mating type is a common feature among other *Tuber* spp.

4.7 Conclusions

The last few years have seen a tremendous increase in our knowledge on *T. melanosporum* biology, much of which can be attributed to the sequencing of its genome. *Tuber* spp. are obligate symbiotic fungi, whose life cycles cannot be entirely

reproduced under controlled conditions. In light of this fact, our improved understanding of *T. melanosporum* population genetics, the progress made in characterizing the genes and pathways committed to symbiosis and reproduction and the wealth of recently developed genetic tools and screening methodologies will allow mycologists to address relevant issues concerning this and other *Tuber* spp. along with other symbiotic ascomycetes. Indeed, *T. melanosporum* is now a reference species among ascomycetes that can be used to understand how the ectosymbiotic lifestyle has been acquired by these fungi and for understanding the propagation strategies used by heterothallic species. In summary, *T. melanosporum* genomics is crucial for addressing both fundamental and applied questions concerning ascomycete fungi that are of outstanding ecological and economical relevance.

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