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Self/nonself recognition in *Tuber melanosporum* is not mediated by a heterokaryon incompatibility system

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ABSTRACT

Vegetative incompatibility is a widespread phenomenon in filamentous ascomycetes, which limits formation of viable heterokaryons. Whether this phenomenon plays a role in maintaining the homokaryotic state of the hyphae during the vegetative growth of *Tuber* spp. Gene expression, polymorphism analysis as well as targeted *in vitro* experiments allowed us to test whether a heterokaryon incompatibility (HI) system operates in *Tuber melanosporum*. HI is controlled by different genetic systems, often involving HET domain genes and their partners whose interaction can trigger a cell death reaction. Putative homologues to HI-related genes previously characterized in *Neurospora crassa* and *Podospora anserina* were identified in the *T. melanosporum* genome. However, only two HET domain genes were found. In many other ascomycetes HET domains have been found within different genes including some members of the *NWD* (NACHT and WD-repeat associated domains) gene family of *P. anserina*. More than 50 NWD homologues were found in *T. melanosporum* but none of these contain a HET domain. All these *T. melanosporum* paralogs showed a conserved gene organization similar to the microexon genes only recently characterized in *Schistosoma mansoni*. Expression data of the annotated HI-like genes along with low allelic polymorphism suggest that they have cellular functions unrelated to HI. Moreover, morphological analyses did not provide evidence for HI reactions between pairs of genetically different *T. melanosporum* strains. Thus, the maintenance of the genetic integrity during the vegetative growth of this species likely depends on mechanisms that act before hyphal fusion.

Introduction

Truffles are ectomycorrhizal ascomycetes belonging to the pezizalean genus *Tuber*. Despite the economic and culinary importance of some of these fungi, significant insights into basic aspects of their biology have been gained only recently (Hall *et al.* 2007; Rubini *et al.* 2007). Genetic analysis revealed that *Tuber melanosporum* is a heterothallic organism and that truffle mycorrhizas are formed only by haploid strains (Rubini *et al.* 2011a, 2011b). More interestingly, in face of the fact that genetically different mycorrhizas can be initially produced on a given host plant *via* inoculation with spore suspension, no evidence of heterokaryon/dikaryon formation has ever been recorded (Paolocci *et al.* 2006; Rubini *et al.* 2011a).

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Furthermore, phenomena of strain competition can occur on artificially inoculated plants, ultimately leading to strain selection on host plant roots (Rubini *et al.* 2011a). Altogether, these results suggest that a self/nonself recognition system may operate within these species. Studies carried out with mycelia of the model species *Tuber borchii* showed that anastomoses are common between hyphae of the same strain, but rare, or absent, between hyphae of different strains (Giomaro *et al.* 2000; Iotti *et al.* 2002; Sbrana *et al.* 2007).

Self/nonself recognition among conspecifics occurs in filamentous ascomycetes during both the sexual and asexual phases. Vegetative incompatibility (VI) systems are responsible for acceptance or rejection of partners during vegetative growth and may act before, during, or after hyphal fusion (Leslie & Zeller 1996; Glass *et al.* 2000). The occurrence of VI likely constitutes a system limiting horizontal transfer of deleterious genetic elements between strains and the formation of a demarcation zone, also referred to as a barrage, may occur where two genetically distinct mycelia meet. In some cases the barrage is a consequence of heterokaryon incompatibility (HI) reactions triggered by the fusion of hyphae carrying different nuclear types (Biella *et al.* 2002; Pinan-Lucarré *et al.* 2007). HI induces a Programmed Cell Death (PCD) characterized by vacuolization and final cell lysis and leads to the formation of an unpigmented barrier line between the incompatible mycelia. However, occurrence of the barrage can be independent of HI-mediated reactions (Micali & Smith 2003; Smith *et al.* 2006). To date, the genetic bases of HI reactions have been determined in only a small number of fungi.

HI is governed by nonself recognition genes, called *het* genes (Glass & Kuldau 1992), which develop viable heterokaryons in compatible reactions or can lead to the death of the heterokaryotic cells in incompatible reactions. A *het* locus can be defined as a locus at which heteroallelism cannot be tolerated in a heterokaryon (Saupe 2000). HI genes are organized in two different genetic systems in which incompatibility is triggered by coexpression of two incompatible alleles of the same locus (allelic systems) or due to the interaction of two genes that belong to distinct loci (nonallelic systems). These genetic systems have been characterized in the model species *Neurospora crassa* and *Podospora anserina*. In *N. crassa*, examples of HI that is controlled by nonallelic systems include the interaction of two different couples of linked genes (*pin-c*/*het-c* and *het-6*/*un-24*) (Kaneko *et al.* 2006; Micali & Smith 2006). Nonallelic incompatibility in this species can also be triggered by expression of the *tol* gene in heterokaryotic cells containing both MAT-A1 and MAT-a1 polypeptides highlighting the existence of a mating-type-associated incompatibility during vegetative growth (Shiu & Glass 1999). The unique allelic system characterized to date involves the *het-s* locus from *P. anserina*, which has two alternative allelic specificities (*het-s* and *het-S*). Cell death reaction occurs only when the prion form of *het-s* interacts with the product of the antagonistic allele *het-S* (Coustou *et al.* 1997). In *P. anserina* at least three nonallelic systems are also active, involving interactions between multiallelic loci *het-e* and *het-c2*, *het-d* and *het-c2*, *het-r* and an as yet uncharacterized *het-v* (Espagne *et al.* 2002; Chevanne *et al.* 2009). The majority of these HI systems share a protein with a fungus-specific domain termed HET, encoded either by the *het* gene itself or by another gene involved in the system. The HET domain has a wide distribution in filamentous ascomycetes (Fedorova *et al.* 2005; Pál *et al.* 2007) and plays a fundamental role as mediator of HI-associated PCD. In fact, point mutations in the HET domain of *N. crassa pin-c* and *P. anserina het-e* genes suppress HI reactions (Kaneko *et al.* 2006; Paoletti & Clavé 2007). Molecular characterization of *het*-like genes has also been performed in other ascomycetes, such as *Botrytis cinerea* (Fournier *et al.* 2003), *Fusarium proliferatum* (Kerényi *et al.* 2006), and *Aspergillus niger* (Van Diepeningen *et al.* 2008), but the homologues examined did not exhibit endogenous HI characteristics in these species.

To date no *het* genes have been identified in *Tuber* spp. (Kagan-Zur & Roth-Bejerano 2008). Here we took advantage of the recently released *T. melanosporum* genome sequence (Martin *et al.* 2010) to search for putative inducers and mediators of the HI reaction, and of genetically different isolates for targeted *in vitro* experiments to test whether VI systems operate in this plant-symbiotic ascomycete.

Materials and methods

In silico *analyses*

The genes involved in HI listed in Table 1 were used to identify homologous gene models in the draft genome of *Tuber melanosporum* Mel28. Searches were performed against genome databases of *T. melanosporum* using BLASTP and TBLASTN algorithms (Altschul *et al.* 1997) as incorporated in the INRA TuberDB (http://mycor.nancy.inra.fr/IMGC/TuberGenome/). Gene models with an E-value of $<e^{-5}$ were selected by the annotation pipeline (Martin *et al.* 2010), manually reviewed and curated based on expressed sequences tag (EST) support available at the Genoscope Genome Portal for *T. melanosporum* (https://www.genoscope.cns.fr/secure-nda/Tuber/html/entry_ggb.html) and at the INRA TuberDB. Manual annotation was carried out using the ARTEMIS software (http://www.sanger.ac.uk/Software/Artemis/) and gene models were corrected when necessary. The sequences were aligned by Multalin (Corpet 1988) followed by manual alignment to correct obvious sequence mismatches. Annotations are available at INRA TuberDB (http://mycor.nancy.inra.fr/cgi-bin/gbrowse/tuberDB/).

The putative *T. melanosporum* proteins were characterized based on conserved domains revealed by the HMMer program with the Pfam database (version 24) (Finn *et al.* 2010) and identity and similarity values obtained using the European Molecular Biology Open Software Suite (EMBOSS) Pairwise Alignment Algorithm at the EMBL-EBI website (http://www.ebi.ac.uk/emboss/align/) with the Needle program and default settings selected. Signal properties were analyzed using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/) prediction algorithms. Bi-directional best hit analyses were also performed using sequences of the main domains involved in HI as a query for a Blastp search against the *T. melanosporum* gene model dataset. Sequences of the HET (PF06985), HET-C (PF07217), HET-S 218–298 (PF11558), and NACHT (PF05729) domains were downloaded from the Pfam database