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MINI-REVIEW

Current state and perspectives of truffle genetics and sustainable biotechnology

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Abstract Mycorrhizal fungi belonging to the genus *Tuber* produce, after the establishment of a productive interaction with a plant host, hypogeous fruitbodies of great economic value known as "truffles". This review summarizes the state of art on life cycle, genetic, and biotechnological investigations of *Tuber* spp. The ascocarp formation in truffles is a consequence of the activation of the sexual phase of the biological cycle. The formation of a dikaryotic secondary mycelium and the karyogamy in the ascal cell (followed by meiosis with ascospores formation) have been hypothesized by several authors but some doubts yet arise from the *Tuber* cycle by considering that a series of abnormalities have been pointed out in respect to other Ascomycetes. It is unclear if binucleated hyphal cells are derived from the fusion of mononucleated cells belonging to mycelia from different mating types or from one only. According to the karyotypes of *Tuber melanosporum*, *Tuber magnatum*, and *Tuber borchii*, the numbers of hyphal chromosomes suggest a chromosome number of eight ($2n$); these values are in the range of those of several Ascomycetes and observed for *Tuber aestivum* ($2n$=10). The importance and growth in interest during the last years in the fungi protoplasts isolation and transformation techniques can be related to current developments in *Tuber* genetics and biotechnology. *T. borchii* could be transformed through liposome-mediated delivery of genetic material as mycelial protoplasts isolation and fusion with liposomes has already been established. On the other hand, *Agrobacterium*-mediated transformation has been successfully established for *T. borchii*.

Keywords Truffles · *Tuber* spp. · Protoplasts · Karyotype · Transformation

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Introduction

The filamentous Ascomycetes *Tuber* spp. are plant-symbiotic microorganisms that colonize trees by the formation of a specialized structure, the ectomycorrhiza, where the exchange of nutrients (and other signals) between the fungus and the root system takes place. Because of their symbiotic capability, mycorrhizal fungi are thought to have played a decisive role in the evolution (and maintenance) of most present-day woody plants. An "added value" of mycorrhizal fungi belonging to the genus *Tuber* is the production, after the establishment of a productive interaction with a plant host, of hypogeous fruitbodies known as "truffles" (ectomycorrhizal fungi of economic value due to their organoleptic properties). The mycelia of mycorrhiza and sporocarp have cells containing two nuclei of uncertain origin (Pacioni and Comandini 1999). The ascocarp formation in truffles is a consequence of the activation of the sexual phase of the biological cycle (Fig. 1). The formation of a dikaryotic secondary mycelium and the karyogamy in the ascal cell (followed by meiosis with ascospores formation) have been hypothesized by several authors (Ragnelli et al. 1992; Pacioni et al. 1995; Lanfranco

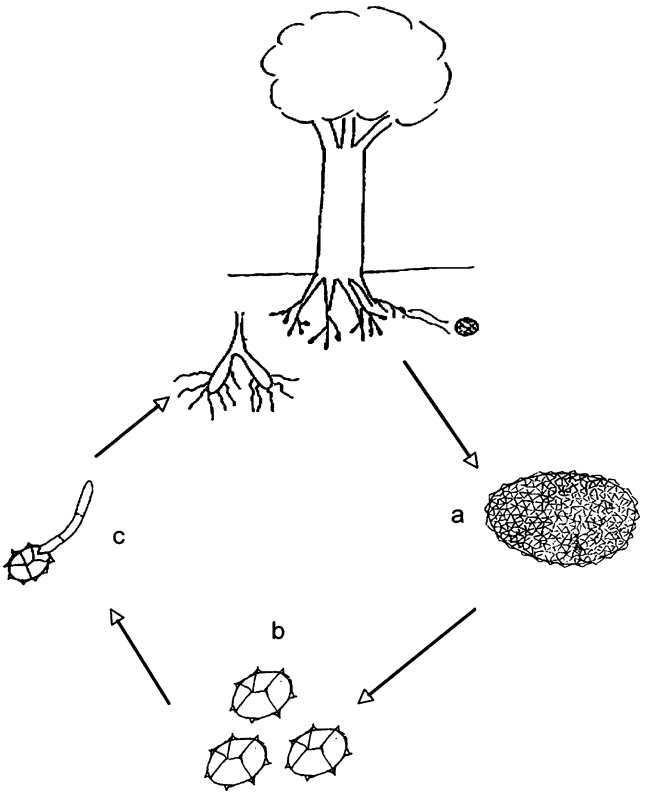

Fig. 1 *Tuber* life cycle: **a** underground ascocarp (fruiting body); **b** production of ascospores; and **c** ascospore and infection of roots (mycorrhizal symbiosis). The phase **c** and its karyological assessment are still unknown

et al. 1995). Some doubts have been raised from the *Tuber* cycle by considering that a series of abnormalities have been pointed out in respect to the Ascomycetes (Greis 1939; Marchisio 1964; Parguey-Leduc et al. 1987) such as the production of a variable number of spores in the asci with a constant total volume and the fixation of populations in homozygosis, a phenomenon explainable but not immediately compatible with an hypothetic somatogamy. Therefore, *Tuber* reproduction system might be different from that usually accepted for a dikaryotic mycelium produced via heterothallic somatogamy. The hypothesis that the two nuclei of dikaryotic hyphae of *Tuber* are the product of a self-fertilization process has been supported by the absence of heterozygosity (Pacioni et al. 1993; Urbanelli et al. 1998). There are conflicting hypotheses concerning the mating system and the ploidy level of the mycorrhizal and truffle hyphae. Recently, Paolocci et al. (2006) used polymorphic microsatellites to compare the allelic configurations of asci with those from the network of the surrounding hyphae in single *Tuber magnatum* truffles. According to these analyses, the authors provide direct evidence that *T. magnatum* outcrosses and that its life cycle is predominantly haploid.

Tuber aestivum, *Tuber melanosporum*, *T. magnatum*, and *Tuber borchii* karyotypes have been also investigated (Poma et al. 1998; Poma et al. 2002) to obtain a better information about *Tuber* cytogenetics and the reproductive cycle. Small size chromosomes image analysis of plant and fungi allows the realization of detailed karyotype also for *Tuber* species (Fukui and Iijima 1991; Fukui et al. 1998; Barry 1996). Electrophoretic karyotypes of several Ascomycetes are also known and pulsed-field gel electrophoresis allowed the analysis of genomes of a number of fungi where classical genetic analysis is not possible because of the lack of a sexual stage and cytological studies are difficult because of the small size of fungal chromosomes (Mills and McCluskey 1990; Orbach et al. 1996; Taga et al. 1998). To discern differences among species, several truffles have been characterized molecularly through the analysis of DNA, rRNA, and other polymerase chain reaction (PCR)-based techniques (Lanfranco et al. 1993; Lazzari et al. 1995; O'Donnel et al. 1997; Bertini et al. 1998).

The cited knowledges about *Tuber* mycelium have been useful to have a further understanding of the cell biology and genetics of the *T. borchii* strain, isolate ATCC 96540, used for protoplasts isolation (Poma et al. 2005). The role of fungal protoplasts in a variety of morphological and genetic investigations has always gained considerable importance; moreover, protoplast regeneration technique has been applied to the elucidation of cell wall polymer biogenesis and disposition (Hocart and Peberdy 1990; Kaul et al. 1993). Fusants between *Aspergillus*, *Penicillium*, and *Ganoderma* have been obtained by interspecies protoplast fusions (Peberdy 1995).

The fungal protoplast fusion and transformation systems have been developed as an aid for further understanding of some phenomenon such as genetic incompatibility between strains and species. Plasmids which allow the transformation of a wide range of fungal species have been constructed: they contain a gene conferring resistance to a compound toxic for most fungal species, under the control of promoter sequences which allow its expression in different backgrounds. Two different plasmid constructs have been designed using the *ble* gene fused to either a histone H4 promoter of *Phanerochaetechrysosporium* or to the glyceraldehyde-3-phosphate-dehydrogenase promoter of *Schizophyllum commune*.

Not many ectomycorrhizal fungi have been transformed; for *Laccaria laccata* and *Hebeloma cylindrosporum* a selection system using the pAN7.1 plasmid has been used (Marmaisse et al. 1995).

Generation of fungal protoplasts is essential for fusion and transformation systems so transformation of *T. borchii* mycelium protoplasts could also offer great potential for the improvement of ectomycorrhizal fungi of economic value. Grimaldi et al. (2005) have worked out basic conditions for the genetic transformation by *Agrobacterium tumefaciens*, a technique whose general suitability for gene transfer in

fungi has been demonstrated by de Groot et al. (1998) and has recently been applied even to whole mycelia (Combier et al. 2003) and fruitbodies (Chen et al. 2000).

To date, *Tuber* spp. have also started to be addressed by genomic approach. Expressed sequence tag collections have been set up for *T. borchii* (http://mycor.nancy.inra.fr/ectomycorrhizadb/; http://linuxbox.itb.cnr.it/estuber/) and are being exploited for the construction of microarrays for large-scale gene expression profiling (Lacourt et al. 2002).

Current state

According to the karyotypes of *T. aestivum*, *T. melanosporum*, *T. magnatum*, and *T. borchii* (Poma et al. 1998; Poma et al. 2002), the numbers of hyphal chromosomes suggest a chromosome number of eight (2*n*) for *T. borchii*, *T. magnatum*, and *T. melanosporum* (Table 1); these values are in the range of those of several Ascomycetes (Barry 1996) and observed for *T. aestivum* (2*n*=10), (Poma et al. 1998). The *T. borchii* mean bp (base-pairs) content calculated from the mean metaphase chromosomal size is equivalent to about 6–7 Mbp/chromosome, that is about in the range of some *Neurospora crassa* chromosomes (4–12.6 Mbp), as estimated by CHEF (Mills and McCluskey 1990). Findings by Poma et al. (2002) show that truffles share chromosomal dimensions with other fungi and considerably smaller than those of plants and animals. Measures achieved with image analysis evidenced *T. melanosporum* chromosome 1 (1.27±0.04 μm) significatively longer and larger in comparison to the other studied *Tuber* species. The shortest chromosome (0.7±0.1 μm) was founded in *T. magnatum*. In higher plants, the ratio between the longest and the shortest chromosome is an indication of the karyotype asymmetry that is related to evolution of the species group (Stebbins Ledyard 1971). Among the three studied species, the more asymmetric is *T. melanosporum*.

Karyotypes analysis shows that from the diploid presporigenous hyphae (2*n*=8), premeiotic (sporogonial cells) would arise, that after meiosis should give rise to haploid (*n*=4) spores. This suggests that hyphal dikaryotic cells might give rise to diploid synkaryotic presporal hyphae. As ectomycorrhizal mantle and ascocarpal cells contain in general two nuclei, the hypothesis of the presence of a primary mononucleate mycelium and then of a dikaryotic one could be confirmed. It is an open question whether binucleated hyphal cells are derived from the fusion of mononucleated cells belonging to mycelia characterized by different mating type or from automatic processes. Data by Poma et al. (2002) related to the results obtained by electrophoretic analysis of gene–enzyme systems (Pacioni and Pomponi 1991; Urbanelli et al. 1998), indicate *Tuber* as homozygotic and automatic.

The work by Poma et al. (2005) was undertaken to develop a procedure for the release of *T. borchii* viable protoplasts to develop applications of protoplast fusion or transformation. Functional delivery of liposome content into *T. borchii* protoplasts has been examined with a cytotoxic ribosome inactivator as saporin (Fig. 2). This molecule allows assessment of liposomes contents delivery because it requires a small number of molecules to inhibit protein synthesis of the receiving protoplast. This first report about *T. borchii* mycelial protoplasts isolation and fusion with liposomes suggests that *T. borchii* could be transformed through liposome-mediated delivery of nucleic acid. In parallel, the *Agrobacterium*-mediated transformation described by Grimaldi et al. (2005) is the first report about gene transfer and enhanced green fluorescent protein visualization in *T. borchii* mycelium in vitro. The lack in the transformed *T. borchii* of the DNA for kanamycin resistance encoded outside of the T-DNA indicates the correct *Agrobacterium* gene transfer. Many species are routinely transformed using *A. tumefaciens* and their list seems to grow daily (Pardo et al. 2002); an increasing number of agronomically and hortoculturally transgenic varieties are generated by *Agrobacterium*-mediated, as opposed to particle bombardment and liposome mediated

Table 1 Chromosome hyphal number and morphometric data of *T. borchii*, *T. magnatum*, and *T. melanosporum*

Taxon	Chromosome complement, 2*n*	Chromosome length (μm)	Surface area (μm^2)
T. borchii	8	0.95±0.11	1.9±0.4
T. magnatum	8	0.87±0.12	1.7±0.5
T. melanosporum	8	0.94±0.15	2.2±0.9

Chromosome length (μm) and surface area (μm^2) are compared as mean values±SD among the species

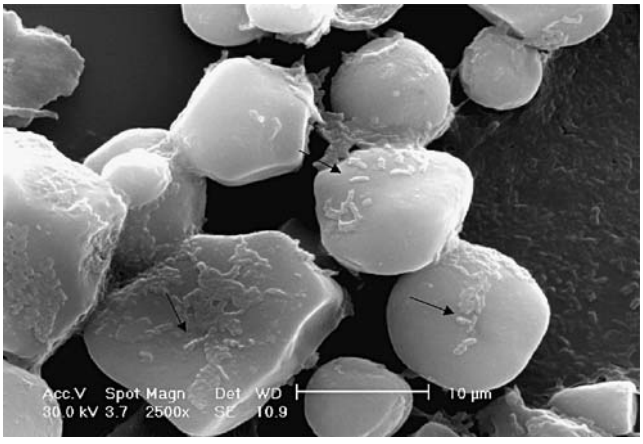

Fig. 2 *T. borchii* protoplasts [16 days in culture, incubated for 2 h with a liposomes suspension (*black arrows*), ratio protoplasts/liposomes *v/v* 1:1] observed under a scanning electron microscope

transformation. Sugui et al. (2005) reported *A. tumefaciens*-mediated transformation (ATMT) of *Aspergillus fumigatus* as a tool for insertional mutagenesis as well as for gene disruption. Transformation of fungi with DNA that does not possess homology with the fungal genome results in random integration into the fungal genome and can cause gene disruption as an insertional mutagen. A decisive advantage of insertional mutagenesis over chemical or radiation mutagenesis is that the mutated genes are tagged by the transforming DNA (T-DNA). Electroporation and biolistic methods were also used to transform *A. fumigatus*; these methods, however, resulted in a high frequency of multiple integrations and a low frequency of homologous recombination (Brakhage and Langfelder 2002). de Groot et al. (1998) used *A. tumefaciens* to deliver T-DNA containing a hygromycin resistance gene into conidia as well as spheroplasts of *Aspergillus awamori.* The transformation frequency via ATMT is higher in comparison to frequency with the traditional method, regardless of whether conidia or spheroplasts are used for transformation. Most of the transformants obtained by ATMT contained a randomly integrated single T-DNA copy. Homologous integration via ATMT is also accomplished in *A. awamori*. The frequency of homologous recombination with T-DNA containing the *pyrG* gene of *A. awamori* is higher compared to results with conventional transformation methods (Gouka et al. 1999; Casas-Flores et al. 2004). A comparison of ATMT with biolistic, electroporation, and spheroplast methods could be improved for the transformation of *T. borchii*.

Trends and prospects for the future

From our first results about liposomes delivery content into protoplasts, we are working out basic conditions for a possible *T. borchii* protoplasts transformation by liposome-mediated transfer of genetic material for the improvement of ectomycorrhizal ascomycetes. This approach will be alternative to the fact that according to Grimaldi et al. (2005), the most of the transformed hyphae have a peripheral location and a mosaic of transformed and untransformed hyphae have been produced. The problem of the enrichment and purification of transformed hyphal offshoots from the mass of untransformed hyphae remains to be solved. On the other side, protoplast fusion could be a useful system to establish if binucleated hyphal cells are derived from the fusion of mononucleated cells belonging to mycelia characterized by different mating type or from automatic processes.

But how we could achieve "sustainable" transformation of *T. borchii* according to its involvement in the symbiotic properties of the truffles ectomycorrhiza? The additional genes will be chosen because they code, i.e., for enzymes which may play a role in the symbiosis [NADP-GDH which contributes to the ammonium assimilation by the fungus (Martin and Botton 1993)] or the proB74 gene which in *L. laccata* increases its salt tolerance (Lemke 1994) and could represent a model to study the salt tolerance of mycorrhizal plants. To evaluate the expression of these additional genes, they could be tagged as knocked ones. The genetic manipulation of ectomycorrhizal fungi of the *Tuber* genus may in the future significantly contribute to the understanding of the interactions between plants and their symbiotic fungal partners.

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