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Ectomycorrhizal Fungi: State of the Art, Application and Perspectives for Research Under Consideration of Molecular Biology*

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Abstract

For any genetic work it is a prerequisite to characterize the organisms concerned. For genetic manipulation of fungi, extrachromosomal DNA is of special importance. After having detected linear plasmids in *Morchella conica* other strains capable of forming mycorrhiza were screened for comparable genetic traits. The detection of linear plasmids in mycorrhiza forming fungi allows the construction of vectors capable of replication in this group of fungi and could lead to the answer of the quetion how symbionts communicate.

Keywords: Ectomycorrhiza, linear plasmids

1. Introduction

Deforestation has, in many countries, led to soil erosion and nutrient loss which can be stopped and cured by reforestation only. The growth of forests and food crops is, however, (especially in the tropics) limited by phosphate availability. The presence of symbiotic fungi substantially enhances the growth of plants. The benefits caused by the fungi are the following:

- enhanced efficiency of phosphate uptake
- drought tolerance

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- pH tolerance (in a wide range)
- resistance to certain pathogens

The fungi concerned envelop plant roots and send out hyphae into the surrounding soil, thus increasing the total volume which can be used for water and nutrient intake.

It is a fact that mycorrhizae are more common than uninfected roots as nutrient-absorbing organs; their potential impact on forest production therefore becomes obvious.

Much of the research on mycorrhizae has been done on the following 3 types whereas the other, less frequent types are also studied, but to a lesser degree. These are classified according to their morphological peculiarities.

*Endo*mycorrhiza	
Fungi:	Mainly Phycomycetes (Endogonaceae)
Host:	Pteridophyta, Gymnospermae, Angiospermae
Hyphae:	Intracellular, vesicular, arbuscular
Spores:	Germinate on artificial media
Mycelia:	Do not grow in axenic culture
*Ecto*mycorrhiza	
Fungi:	Mainly Ascomycetes, Basidiomycetes
Host:	Gymnospermae, Angiospermae
Hyphae:	Hyphal sheath encloses roots, penetrates cortex, Hartig net in middle lamellae
Mycelia:	May grow in axenic culture
*Ectendo*mycorrhiza	
Fungi:	Ascomycetes, Basidiomycetes
Host:	Gymnospermae, Angiospermae
Hyphae:	Intracellular, Hartig net, vesicles, arbuscules?
Literature reviews	Strullu, 1985; Harley, 1984; Read, 1984

2. Significance of Ectomycorrhizae

Although ectomycorrhizae are common in only about 5% of vascular plants, these species include many of the important forest, ornamental, and nut crop trees (see Table 1). Numerous fungi can form ectomycorrhizal associations, but members of the Asco- and Basidiomycetes predominate (see

Table 2). For reasons of clarity we have given only the biotechnologically relevant groups of green plants and fungi in Table 1 and 2.

3. Practical Application

Mycorrhizal fungi are virtually ubiquitous in the soils of natural forests. The mycorrhizae found there are formed by species best suited to the prevailing conditions. There is therefore no need for the introduction of mycorrhizal fungi into these soils.

In man-made forests the situation is completely different, since trees are often planted outside their natural range, or in highly disturbed soils, or in soils lacking the appropriate mycorrhizal fungi. Drainage, fertilization, biocide treatments and burning have an additional negative effect on mycorrhizal relationships. Afforestation has, thus, made the importance of mycorrhizal research obvious. In the following section some examples of the application of ectomycorrhizal associations are presented followed by a compilation of applied ectomycorrhizae, which is by no means complete.

1. Truffles (*Tuber melanosporum*), a gourmet delight in many countries, form ectomycorrhizae predominantly with hazelnut and oak trees. From Table 3 it is evident that, with the price of the truffles being so high, they are guaranteed of high commercial interest. It is therefore not surprising that commercial truffle production has been realized in France, and that it is a quite profitable business (Grente and Delmas, 1974).
2. The pioneer work of M. Moser (1958a, 1958b, 1959, 1963, 1965) and Moser and Haselwandter (1983) has resulted in the fact that much of the practical application of ectomycorrhizal fungi has been in forestry. Artificial inoculation of the tree seedlings with ectomycorrhizal fungi is now a common practice particularly in the USA, Canada, and Europe: Table 4 gives a selection of applied ectomycorrhizal associations in afforestation procedures. As may have become evident, mycorrhizal plants are advantageously being used in the establishment of man-made forests and in the afforestation of adverse regions. Millions of pine seedlings have already been treated. According to Abelson (1985) the cost per seedling is only about 1 US cent.

4. Genetics and Molecular Biology

Until recently all we knew about the genetics of mycorrhizal fungi was by analogy to the saprophytic fungal species. The main reason for the lack of

Table 1. Biotechnologically relevant plant families with Ectomycorrhizae.

Family	Examples
Aceraceae	Acer
Betulaceae	Coryllus, Betula
Caprifoliacease	Sambucus
Fagaceae	Fagus, Quercus
Myrtaceae	Eucalyptus
Oleaceae	Fraxinus
Pinaceae	Pinus, Abies, Picea
Salicaceae	Populus, Salix
Tiliaceae	Tilia
Ulmaceae	Ulmus
Vitaceae	Vitis

Table 2. Biotechnologically relevant orders of fungi capable of forming Ectomycorrhizae.

Class	Order	Examples
Ascomycetes:	Plectascales	*Elaphomyces* spec.
	Pezizales	*Morchella* spec.
	Helotiales	*Spathularia* spec.
		Cirinans spec.
	Tuberales	*Tuber melanosporum*
		Terfezia leptoderma
Basidiomycetes:	Poriales	*Piloderma croceum*
	Agaricales	*Russula fragilis*
	Gastromycetales	*Pisolithus tinctorius*
		Lycoperdon spec.
		Scleroderma spec.

Table 3. Truffle production in France (Data derived from Grente and Delmas, 1974).

Truffle species	Biomass production	
	1892	1973
Tuber melanosporum		
Tuber aestivum	ca. 2000 t	ca. 60 t
Tuber brumale	(10 FF/kg)	(400 FF/kg)
Tuber uncinatum		

Table 4. Selected examples of applied Ectomycorrhizal inoculation.

Amanita spec.	Higher alpine regions	
Boletus edulis	Acid soil	Moser, 1958
Phlegmacium spec.	Calcereous soil	
Hebeloma cylindrosporum	Stimulation of Seedling growth	Le Tacon et al., 1980
Pisolithus tinctorius	Stimulation of Seedling growth	Marx et al., 1982; Plassard et al., 1983
Terfezia leptoderma	Calcereous soil	Dexheimer et al., 1985

knowledge is the fact that it has been impossible to obtain fruit bodies of ectomycorrhizal fungi in the laboratory. Germination of spores from different ectomycorrhizal species was obtained by N. Fries (Uppsala) but for his studies he used only spores from fruit bodies collected in nature (Fries, 1983; Fries and Birraux, 1980; Birraux and Fries, 1981).

In our opinion the work with *Hebeloma cylindrosporum* by a French group from Lyon will give deep insights into the genetics of this ectomycorrhizal fungus and, at the same time, will facilitate the concerted breeding of this species (Bruchet, Debaud, Gay, 1986). The most promising results and features are summarized below:

- Fruit body production in the laboratory in association with *Pinus pinaster*
- Tetrapolar mechanism of homogenic incompatibility
- Great variability in the offspring concerning parameters important for ectomycorrhiza formation (e.g. phosphate and nitrogen-metabolism)
- Breeding of an effective mycorrhizal fungus was facilitated.
- *H. cylindrosporum* has proved to be an efficient fungal partner in afforestation experiments with pine trees (Le Tacon eta l., 1984, 1983).

The other way to manipulate (breed) a fungus is by genetic engineering techniques. Since extrachromosomal DNA elements have been detected in a variety of fungi (for literature see Esser et al., 1986), we were enticed to investigate mycorrhizal fungi for the presence of comparable genetic traits. Nearly 40 strains comprising 12 different species were screened for the presence of extrachromosomal DNA. The results of the screening tests are summarized in Table 5 (Esser and Meinhardt, 1985).

It became obvious that, among members of the genus *Morchella*, plasmid-like DNA was routinely observed. Electron micrographs (Fig. 1) and restriction analysis led to the construction of linear physical maps of these genetic

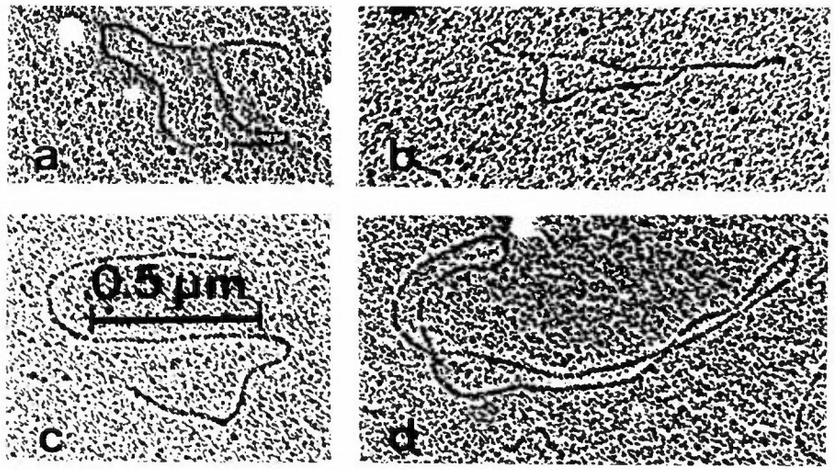

Figure 1. *Morchella conica*: Electron micrographs of the 6 kb plasmid. a and b: homoduplex structures after alkaline denaturation and self renaturation; c: linear 6 kb molecule; d: circular marker molecule (pM 2).

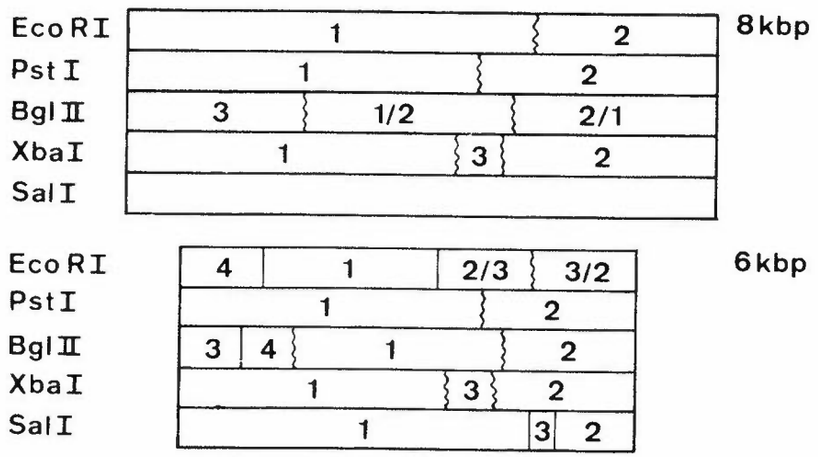

Figure 2. *Morchella conica*: Restriction maps of the linear plasmids; ξ represents sites present in both molecules; | represents restriction sites unique to either plasmid.

Table 5. Screening of various wild strains of ectomycorrhizal fungi for the presence of extrachromosomal DNA. Each DNA assay was repeated at least once.

Species	Total number of strains	Number of strains containing plasmids
Boletus elegans	2	–
Boletus luteus	1	–
Boletus variegatus	1	–
Laccaria laccata	2	–
Pisolithus tinctorius	1	(+)
Morchella conica	10	7
Morchella conica f. *elata*	3	2
Morchella costata	1	–
Morchella esculenta	7	–
Morchella hortensis	2	1
Morchella rotunda	1	–
Morchella semilibera	1	–
Gyromitra esculenta	1	–

traits (Fig. 2). As is characteristic of linear plasmids, they also have terminal-inverted repeats (Meinhardt and Esser, 1984; Esser and Meinhardt, 1986). Restriction analysis done together with hybridization experiments points to a certain degree of homology between the plasmids under investigation. Since *Morchella* is able to form ectomycorrhizae (Mayr et al., 1984; Mayr, 1982) with *Pinus silvestris*, it was tempting to use this fungus as a starting point for applying molecular biology and especially genetic engineering in the research of the fungal component of mycorrhizal associations. Two different lines of research were followed:

1. Development of a vector for the transformation of the fungus from which this DNA originated.

For this purpose as much of the DNA as possible was cloned into the *E. coli* vector pUC 9 (see Fig. 3). Because the 5' ends seem to be capped with covalently bond proteins, as is common for linear plasmids, we have not yet succeeded in cloning the terminal fragments of these molecules. We are currently under way to subclone the plasmid DNA into a vector which allows the identification of autonomously replicating sequences (ars) in yeast. These putative origins of replication will subsequently be used to construct vectors for the transformation of the fungus. For these purposes we have established a procedure to produce and regenerate protoplasts and have determined the concentration of antibiotics (chloramphenicol and G 418) useful in selection

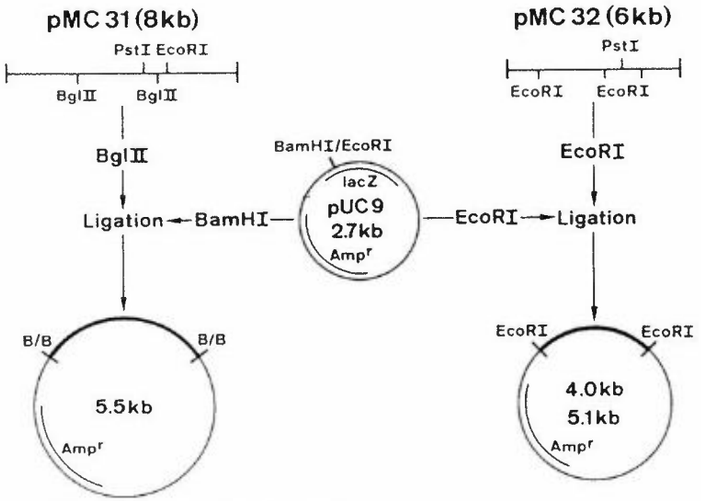

Figure 3. *Morchella conica*: Cloning of the inner parts of the linear plasmids.

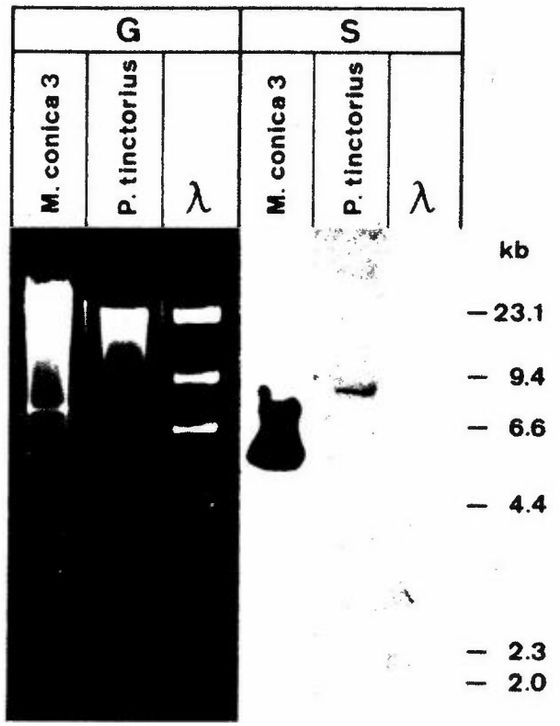

Figure 4. Cross hybridization of *Morchella* and *Pisolithus trinctorius* DNA with cloned fragments form the linear 6 kb plasmid.

Table 6. Linear plasmids in Fungi.

Species		Plasmid-name	Size (in kb)	IRS	References
Agaricus bitorquis	saprophytes	pEM	7.35	1.0	Mohan et al., 1984
		pMPJ	3.65	?	
Ascobolus immersus		pA1	6.4	1.2	Francou, 1981
Kluyveromyces lactis		pGKl2 (k2)	13.4	0.2	Gunge et al., 1981
		pGKl1 (k1)	8.8	0.18	Sor et al., 1983
Claviceps purpurea	parasites	pl1	6.6	0.35	Tudzynski et al., 1983
		pl2	5.3	?	
		p14	1.1		
Gaeumannomyces graminis var. *tritici*		–	8.7	?	Honeyman and Currier, 1983
		–	7.5		
Fusarium oxysporum					Kistler and Leong, 1986
Rhizoctonia solani	parasite of many herbs orchid myc.	pRS64	2.6	?	Hashiba et al., 1984
Morchella conica	symbionts	pMC31	8.0	?	Meinhardt and Esser, 1984
		pMC32	6.0	0.75	
Morchella elata		pME141-1	6.7	?	unpubl.
		pME141-2	6.0	?	unpubl.
Morchella hortensis		pMH1	7.8	?	unpubl.

IRS— invers repetitive sequence
? — unknown
– — no data

procedures (unpublished data).

Our goal is to establish a system for manipulating ectomycorrhizal fungi by using its own DNA for the construction of genetic vectors for gene transformation (for details see Esser et al., 1986).

2. Biological significance of the plasmids

If one looks at the linear plasmids and the organisms in which they have been found, it becomes evident that the plasmids so far described share some common features (see Table 6). Most of the liner plasmids seem to have

terminal-inverted repeats, and most of the organisms are either parasitic or symbiotic to higher plants. Quite recently it was shown that linear plasmids control the host specificity of the pathogen *Fusarium oxysporum* (Kistler and Leong, 1986).

In order to find out whether comparable genetic elements exist in different mycorrhizal species, we have hybridized isolated bulk DNA from different fungi with cloned fragments (see Fig. 4) of the plasmid DNA of *Morchella conica*. As may be seen from Fig. 4, hybridization occurs with distinct bands of low molecular weight DNA of *Pisolithus tinctorius*. The questions to be asked are: "Are similar sequences present in other mycorrhizal fungi?" and "Are there target sites in the genome of the green partners?"

5. Conclusion

Our experimental data, albeit far from comprehensive, open a pathway for the integration of genetics into the research on mycorrhizae. A concerted application of the techniques of classical genetics as shown for *Hebeloma* and of molecular genetics as shown for *Morchella* (and probably *Pisolithus*) may well, in the future, lead to an improvement of the effectiveness of the fungal component of this symbiotic association of mycorrhizae especially in afforestation but also in the cultivation of fungal crops.

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