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EST-library construction using spore RNA of the arbuscular mycorrhizal fungus *Gigaspora rosea*

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Abstract RNA was extracted from activated spores of the arbuscular mycorrhizal fungus *Gigaspora rosea*. Double-stranded cDNA was synthesised, digested and cloned into the vector lambda-ZAP express. Of the 1,500 clones obtained, 1.5% carried inserts of the rRNA gene cluster. After excision, inserts from 50 randomly selected clones were sequenced. Database searches revealed that 62% of the clones had similarities to already known sequences. These mainly code for proteins involved in translation and protein processing, replication and the cell cycle and cell signal transduction. One fragment probably belonged to a metallothionein-encoding gene which may be involved in heavy-metal binding. The method presented is an easy and rapid way to obtain short fragments of coding regions for expressed sequence tag libraries.

Keywords Arbuscular mycorrhizal fungi · cDNA library · Activated spores · Expressed sequence tags · *Gigaspora rosea*

Introduction

Both understanding the role of arbuscular mycorrhizal (AM) fungi (Glomales, Zygomycota) in the environment and their application in plant production have been hampered by the lack of large amounts of pure material. However, application of modern molecular techniques has resulted recently in the cloning of the first genes and gene fragments. Genomic DNA isolated from chlamydospores, which can be obtained from soil and which contain many nuclei, has been used successfully for the construction of libraries (Zézé et al. 1994; Franken and Gianinazzi-Pearson 1996; van Buuren et al. 1999) and for PCR amplification (Kaldorf et al. 1994; Lanfranco et al. 1999; Ferrol et al. 2000). RNA from AM fungal spores has been used also in RT-PCR experiments to obtain fragments of several genes (Franken et al. 1997, 1998). There is now a need for more data to extend the genetic analysis of AM fungal development. Large databases are currently being established for various model organisms by extensive sequence analysis of clones from cDNA libraries. The clones are spotted onto supports for hybridisation with labelled cDNA synthesised from RNA obtained from tissues subjected to various environmental signals or during certain developmental stages. Mass sequencing and analysis of cDNA arrays results in a broad overview of the different functions expressed in an organism in a given situation. We decided to construct expressed sequence tag (EST) libraries from AM fungi within the framework of an EU project established to support the European Bank of Glomales with all kinds of information. Here we present a convenient method for constructing such EST libraries using the AM fungus *Gigaspora rosea* as model.

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Materials and methods

Soil with spores of the AM fungus *Gigaspora rosea* BEG 9 from a 6-month-old pot culture with *Allium porrum* L as host were obtained from Biorize (Dijon, France). After wet-sieving (Gerdemann and Nicolson 1963) and passage through a percoll gradient (Hosny et al. 1996), spores were collected in sterile water and surface sterilised in 2% chloramine T/0.03% streptomycin for 20 min. After incubation for 1 week at 4°C in sterile water, four samples, each containing 500 spores, were subjected to RNA extraction and subsequent DNase treatment using spin columns (Qiagen, Hilden, Germany) following the protocol of Requena et al. (1999). RNA amount and quality were measured by absorbance (Franken et al. 1997) and Northern blot analysis under standard conditions (Sambrook et al. 1989) using the rRNA gene of *Glomus mosseae* BEG 12 as probe (Franken and Gianinazzi-Pearson 1996), labelled with digoxigenin according to the manufacturer's protocol (Roche, Mannheim, Germany). Detection was by chemiluminescence using the alkaline phosphatase-coupled digoxigenin antibody and the substrate CDP-Star.

cDNA was synthesised from 5 µg RNA in 25 µl with 50 µg oligo(dT)$_{15}$ using MMLV-RT transcriptase and DNA polymerase I following the protocol of the supplier (Promega, Madison, Wis.). Double-stranded cDNA was totally digested with the restriction enzyme *Sau*3A I, purified by Gene Clean III (BIO101, La Jolla, Calif) and ligated into the lambda ZAP express vector predigested with *Bam*H I (Stratagene, La Jolla, Calif). After packaging and plating of recombinant phage DNA (GigaPlusII packaging extract, Stratagene), the library was amplified. Approximately 1,500 clones were plated, transferred to nitrocellulose membranes and hybridised to the rRNA probe described above following the protocol of Boehringer. Detection was performed with the colorimetric detection reagents NBT and BCIP following the protocol of Roche (Mannheim, Germany). To characterise the library, phagemids were excised from the phage vectors and used for transfection of *E. coli* cells (Stratagene protocol). Plasmid DNA was extracted from randomly picked clones using spin columns and inserts were sequenced by MediGenomix (Martinsried, Germany) and MWG (Ebersberg, Germany). Searches for similarities were carried out by BLASTX (Gish and States 1993). Clones are deposited in the BEG database (http://wwwbio.ukc.ac.uk/beg/) with links to the EMBL for their sequences.

Results and discussion

Gigaspora rosea spores were activated by incubation for 1 week at 4°C (Franken et al. 1997) and used subsequently for RNA extraction. A mean value of 2 µg/500 spores was obtained, which is in the range previously described for *Gigaspora rosea* (Franken et al. 1997). The quality of the RNA was confirmed by hybridising 200 ng of RNA in a Northern blot with the rRNA gene cluster of *Glomus mosseae* as probe (Fig. 1) and an EST library was constructed following the strategy outlined in Fig. 2. Double-stranded cDNA synthesised from 5 µg of RNA was totally digested and cloned into a phage vector, which resulted in a library of 1,500 clones. Initial characterisation by plaque hybridisation revealed that 1.5% of the clones carried inserts of the rRNA gene cluster of *Gigaspora rosea*. This is probably due to poly(A) stretches in the rRNA, which can hybridise to the oligo(dT) during cDNA synthesis. This has been reported previously (e. g. Martin-Laurent et al. 1997).

Further analysis of the library was carried out with 100 randomly selected clones. Inserts were in the range

Table 1 Homology to known proteins of the gene products deduced from 26 of 50 sequenced ESTs together with length of the cDNA fragments, the number of clones with identical sequences, the percentage of identical amino acids and the length (amino acids) of the alignment, as well as the accession number of the insert sequences

Protein class	Size (bp)	Gene function	No. of clones	Identity/ length	Accession number
From transcripts to proteins	288	Nucleoporin (nuclear pore protein)	3	47%/90	AF090457
	232	RNA Pol. I-specific transcription initiation factor	1	33%/59	AF090458
	134	60S Ribosomal protein L20	1	72%/44	BE057059
	199	Eukaryotic translation initiation factor 3 subunit 7 (EIF-3 ζ)	2	60%/61	BE057047
	181	Protein translation factor eIF-2A	1	53%/65	AF090462
	268	T-complex protein-1 ζ-1 subunit, chaperon family	1	82%/70	BE057054
	429	Dolichyl-diphospho-oligosaccharide-protein glycosyltransferase subunit	1	34%/146	BE057044
DNA synthesis and cell cycle	352	BRAHMA ortholog BE057043 (DNA helicase superfamily II)	1	33%/74	BE057058
	517	ATP-dependent RNA helicase CDC28	1	74%/62	BE057061
	359	Purine nucleoside phosphorylase	1	66%/42	BE057033
	164	Ubiquitin precursor I	1	100%/53	AF090460
	291	Polyubiquitin	1	98%/52	BE057048
	106	Ubiquitin-conjugating enzyme	1	66%/21	BE057026
	208	Microtubule-associated protein (chromatin assembly factor)	3	51%/39	AF090461
Signalling	139	Mitogen-activated-protein (MAP) kinase	1	93%/46	BE057050
	276	P13/14 kinase like protein	1	43%/90	BE057053
	115	Protein tyrosine phosphatase	1	61%/34	BE057043
	192	Histidine protein kinase	1	52%/35	BE057042
	182	Calcium P-type ATPase	1	64%/53	BE057024
Transport	111	Vesicle transport v-snare protein	1	66%/36	BE057039
	385	Efflux protein	1	28%/129	BE057060
Respiration	107	NADH oxidoreductase complex I subunit	1	45%/35	BE057045
	117	Methyltransferase (ubichinon biosynthesis)	1	45%/39	BE057063
Others	343	Metallothionein	1	39%/48	BE057027
	149	Homogentisate-1,2-dioxygenase (phenylalanine catabolism)	1	68%/29	BE057028
	133	Malonyl CoA-acyl carrier protein transacylase (fatty acid biosynthesis)	1	56%/23	AF090463

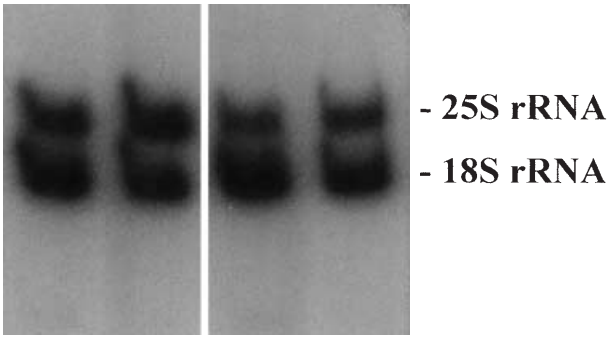

Fig. 1 Northern blot analysis. Aliquots (200 ng) of four RNA extracts from activated spores of *Gigaspora rosea* were hybridised in a Northern blot analysis with a digoxigenin-labelled probe derived from the rRNA gene cluster of *Glomus mosseae*

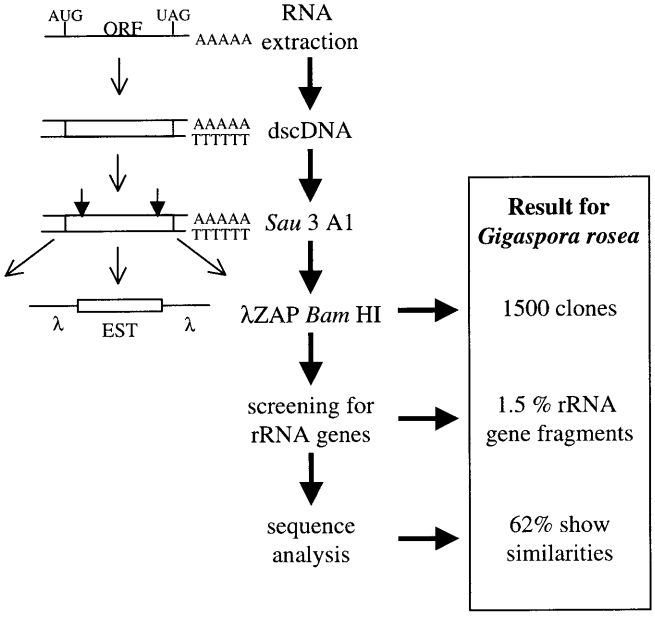

Fig. 2 Expressed sequence tags (EST) library construction: the strategy for obtaining and analysing libraries of ESTs and the results obtained for *Gigaspora rosea* activated spore RNA. Double-stranded cDNA is synthesised from total RNA and digested with *Sau*3A I. Only fragments with the restriction enzyme site at both ends are cloned into the phage vector. This leads in most cases to removal of the 5′ and 3′ untranscribed regions and the outermost ends of the coding part of the genes. Clones are screened for rRNA genes and inserts are sequenced

of 107–517 nt and sequencing of 50 clones revealed a mean size of 229 nt (Table 1). The GC content of the fragments was 32–46% with a mean value of 38%. Similar values have been obtained e.g. for *GvPT* of *Glomus versiforme* (Harrison and van Buuren 1995) and for *GmFox1* of *Glomus mosseae* (Requena et al. 1999), both with about 40% GC content in the coding regions. A GC content of only 23% was reported for the whole genome of *Gigaspora rosea* (Hosny et al. 1997), but this was probably due to the presence of repetitive sequences with a very high AT content, which would also contribute to the large genome size. However, the present study shows that the composition of the coding region also contributes to the low GC content relative to other fungal organisms. One exception among the EST sequences of *Gigaspora rosea*, with a GC content of 51%, was a fragment of a gene encoding a translation elongation factor. These genes are known to deviate from the normal codon usage (e.g. Shearer 1995), which could give a high GC content.

Screening the databases for homologies revealed no similarity with an already known sequence for 16 inserts and similarity to hypothetical genes derived from sequencing projects for three inserts. Among the remainder (Table 1), 10 inserts were obtained from genes probably involved in the processing of mRNA stored in the nucleus and its translation to functional proteins. One fragment with homology to a nucleoporin-encoding gene was obtained three times and a second fragment similar to a translation initiation factor gene was present in two copies. Other fragments may belong to genes encoding e.g. a transcription factor for the ribosomal RNA-transcribing polymerase I, a protein belonging to the chaperon family and enzymes involved in posttranslational modifications such as phosphorylation or transfer of oligosaccharides. Protein synthesis is probably the most important function during the first steps of germination. A differential display analysis of *Gigaspora rosea* has shown that RNA accumulation patterns do not change during initial hyphal development, which suggests that all transcripts are already present in the activated spore and the corresponding information is simply translated into proteins (Franken et al. 2000). This agrees with the finding that inhibitors of translation prevent the germination of AM fungal spores (Hepper 1979; Beilby 1983). Translation of mRNA stored in spores has also been shown in other fungi (Van Etten and Freer 1978; Smith and Burke 1979; Linz and Orlowski 1982).

A second group of nine fragments were similar to genes involved in DNA synthesis and the cell cycle. Of these, three fragments were probably derived from a gene encoding a chromatin assembly factor and two from ubiquitin-encoding genes. The latter differed in their DNA sequence by 12.3%, which indicates the presence of at least two loci or two alleles of one locus. This has been shown already for the rRNA genes, where various alleles are present in the different nuclei of one spore (Trouvelot et al. 1999).

The remaining ESTs showed homologies to genes involved in signal transduction, transport processes, respiration and general metabolic activities. One deduced amino acid sequence was similar to metallothioneins and contained the typical cysteine-rich motif (Fig. 3). Metallothioneins have been shown to contribute to cadmium partitioning in transgenic tobacco plants (Deborne et al. 1998). They may thus be the basis of the reported increase in the tolerance of plants for heavy metals in contaminated soil brought about by AM fungi (Galli et al. 1994).

In summary, the extent of similarities detected for the cDNA fragments in this study is high relative to the analyses of other EST libraries. This may be due to the re-

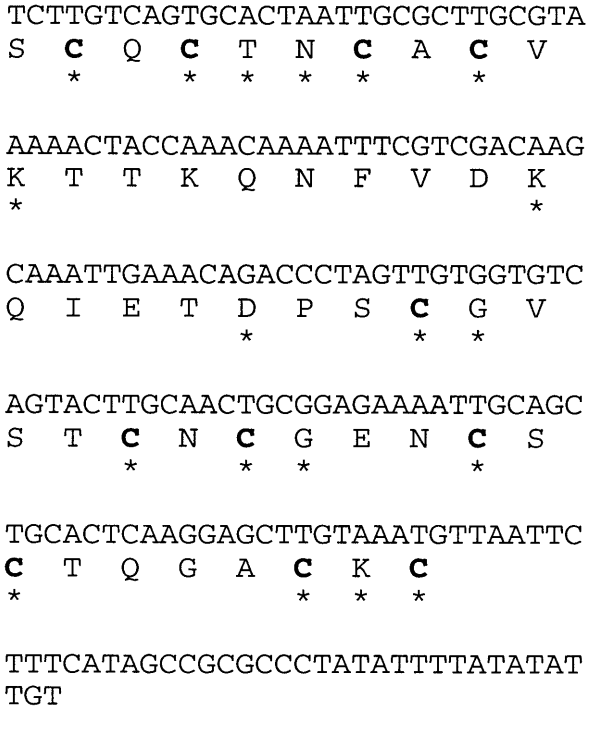

Fig. 3 EST Giroasp14 (EST *Gigaspora rosea* activated spore no. 14). The sequence of a cDNA fragment is shown together with the deduced amino acid sequence. Cysteine residues, which are supposed to be involved in metal binding, are shown in *bold.* Residues conserved in metallothioneins of different organisms are indicated by *stars*

moval of 3′- and 5′-sequences of the double-stranded cDNA by restriction enzyme digestion (Fig. 2), which results in sequence information located more in the centre of open reading frames. In contrast, in most EST-sequencing projects untranslated regions and the ends of coding regions are sequenced. These regions are often less conserved between different organisms than the central parts. Hence, we recommend the strategy outlined in Fig. 2 as useful for establishing EST libraries containing short inserts from known genes. The EST fragments obtained can be used further for screening libraries or for the design of specific primer pairs in order to analyse the expression of the corresponding genes by quantitative RT-PCR. This strategy is probably appropriate for most AM fungi, since all isolates tested so far contained RNA in their dormant or activated spores (Franken et al. 2000). Where spore RNA content is relatively low, a PCR amplification step should be included in the cDNA synthesis, as carried out by Lanfranco et al. (2000) for establishing a cDNA library from RNA of *Gigaspora margarita* presymbiotic mycelium. Libraries are currently under construction for other AM fungi, such as *Scutellospora castanea* BEG1 and *Glomus mosseae* BEG 12. The latest results can be found at www.bio.ukc.ac.uk/beg and www.uni-marburg.de/mpi/franken/est.

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