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Differential RNA accumulation of two β-tubulin genes in arbuscular mycorrhizal fungi

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Abstract RNA was isolated from spores of different arbuscular mycorrhizal (AM) fungi and used for RT-PCR with degenerate primers for β-tubulin genes. PCR products were cloned and the sequence of several clones was analysed for each fragment. Comparison of sequences identified two loci for β-tubulin genes with different GC content and codon usage. *Btub*1 sequences were most similar to β-tubulin genes from the Oomycota, while *Btub*2 sequences showed highest similarity to sequences from the Zygomycota. RT-PCR experiments were carried out to monitor RNA accumulation patterns of *Btub*1 and *Btub*2 in asymbiotic germinating spores and in symbiotic extraradical hyphae of three different AM fungi. This indicated that *Btub*1 is constitutively expressed in *Gigaspora rosea*, but down-regulated during symbiosis in *Glomus mosseae* and *Glomus intraradices*. In contrast, *Btub*2 showed constitutive expression in the two *Glomus* species, but down-regulation in *G. rosea*. Further analysis of different fungi indicated that *Btub*2 primers could be used to specifically monitor RNA accumulation of AM fungi in environmental samples.

Keywords Gene family · *Gigaspora rosea* · *Glomus mosseae* · *Glomus intraradices* · RNA accumulation

Introduction

Arbuscular mycorrhizal (AM) fungi are obligate biotrophic root symbionts of many land plants and therefore important components of nearly all terrestrial ecosystems (Read et al. 1992). Due to their function as biofertilisers, bioregulators and bioprotectors, they influence population structures of plant communities (Van der Heijden et al. 1998) and represent potential tools for sustainable plant production (Gianinazzi et al. 1995; Lovato et al. 1996; Azcon-Aguilar and Barea 1997). Despite the fact that it is not possible to keep AM fungi in pure culture, molecular analyses have been carried out for several years in order to gain insight into their phylogenetic relationships (Simon et al. 1993), biodiversity (Franken 1998) and functioning during interaction with the host root (Franken and Requena 2000). Most sequence information has been obtained from the ribosomal RNA gene cluster, but protein-encoding genes have also been studied, because it was expected that they be regulated during mycorrhiza formation. One copy of a single gene has usually been detected, but gene families were identified in two cases, encoding H^+-ATPases (Ferrol et al. 2000) and chitin synthases (Lanfranco et al. 1999a, b; Ubalijoro et al. 2001). These genes were chosen for analysis, because H^+-ATPase activity was predominantly localised in intercellular hyphae (Gianinazzi-Pearson et al. 1991), while chitin has been shown to be unevenly distributed during AM fungal development (Bonfante-Fasolo et al. 1990). In addition to this, changes in the cytoskeleton also seem to play a role in the development of the symbiosis. Such changes were first detected on the plant side (Genre and Bonfante 1997, 1998), mirrored on the molecular level by the induction of a maize α-tubulin promoter in tobacco plants after mycorrhization (Bonfante et al. 1996). On the fungal side, a β-tubulin gene was identified and its RNA accumulation monitored (Franken et al. 1997; Bütehorn et al. 1999). However, these experiments did not distinguish between different members of a putative gene family, and therefore changes in the expression levels could probably not be detected, as one might expect from the highly dynamic microtubule development in the fungal hyphae during formation of the symbiosis (Blancaflor et al. 2001; Timonen et al. 2001).

In the present report, PCR cloning was used to identify more β-tubulin genes in AM fungi. Alignment of the sequences indicated the presence of at least two members

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of a β-tubulin gene family, and RT-PCR experiments showed differential RNA accumulation of the two gene transcripts. The primer pair for the *Btub*2 gene was tested to see if it could be used to specifically monitor RNA accumulation in glomalean fungi.

Materials and methods

Allium porrum L. pot cultures of the AM fungi *Gigaspora rosea* BEG9, *Gigaspora margarita* BEG34, *Scutellospora castanea* BEG1, *Glomus coronatum* BEG22, *Glomus geosporum* BEG11, *Glomus mosseae* BEG12, *Glomus clarum* BEG142, *Acaulospora laevis* BEG13 and *Acaulospora scrobiculata* BEG33 were obtained from the Banque Européenne des Glomales (INRA Dijon, France). After wet sieving (Gerdemann and Nicolson 1963) and purification on Percoll gradients (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, RNA of 500 spores was extracted and subsequently treated with DNase using spin columns (Qiagen, Hilden, Germany) following the protocol of Requena et al. (1999). RNA amount and quality was measured as described (Stommel et al. 2001). cDNA was synthesised from 100 ng total RNA in a volume of 25 µl with 0.5 µg oligo(dT)$_{15}$ using MMLV-RT transcriptase following the supplier's protocol (Promega, Madison, Wis.). One microlitre of the resulting single-stranded cDNA was used for amplification of β-tubulin gene fragments as described (Franken et al. 1997). Because it was not possible to derive amplification products from genomic DNA with the primer pair used, it was highly probable that the PCR fragments obtained were from cDNA. Amplification products were excised from 2% agarose gels, purified using the Geneclean system (BIO101, Vista, Calif.) and cloned into the pGEM-Teasy vector (Promega). *Escherichia coli* XL1-Blue electrocompetent cells were transformed (Sambrook et al. 1989) and clones screened by PCR (Güssow and Clackson 1989). Six clones from two independent biological samples for each isolate were sequenced twice in both directions. The nucleotide sequences were aligned to each other and with additional β-tubulin genes from the EMBL database using ClustalW (Thompson et al. 1994). Based on the alignment, phylogenetic analyses were carried out using the programs Seqboot, Dnapars and CONSENSUS from the PHYLIP package (version 3.573c; Felsenstein 1993). Based on the results, distance trees were constructed using the program TREEVIEW (version 1.5.3; Page 1996).

Glomus intraradices DAOM 181602 carrot root organ cultures were cultured in a two-compartment system (St-Arnaud et al. 1996) and used for harvesting spores (Doner and Bécard 1991). Extraradical hyphae from *G. intraradices* were obtained from the two-compartment system after cutting off the old mycelium and allowing new hyphae to grow into the second compartment for 2 weeks (Sawaki and Saito 2001). Germination of spores and harvesting of extraradical hyphae from *G. mosseae* and *G. rosea* were carried out as described (Bütehorn et al. 1999). The other fungi were cultivated in a liquid malt extract medium (Difco, Detroit, Mich.), except *Aspergillus nidulans* and *Piriformospora indica*, which were grown on a complete medium (Pontecorvo et al. 1953), and *Ustilago maydis* on potato dextrose (Difco). *Pisum sativum* was inoculated with *G. mosseae* BEG 12 (Biorize, Dijon, France) and grown under low phosphate conditions (Dumas-Gaudot et al. 1994). Total RNA was extracted as described above, and double-stranded cDNA was synthesised from 100 ng RNA using the PCR-based SMART system in 100 µl volume (BD Bioscience Clontech, Palo Alto, Calif.). Quality and quantity was analysed by agarose gel electrophoresis. cDNA samples for RNA accumulation analyses of β-tubulin genes were additionally tested by PCR in a 20 µl reaction volume with 10 µM of a primer pair for fungal *Tef* genes (Wendland and Kothe 1997) using 1 µl of the double-stranded cDNA as template and 0.5 U recombinant *Taq* polymerase (Gibco-BRL, Karlsruhe, Germany). Conditions for amplification were as described (Bütehorn et al. 2000). In parallel, cDNA from all samples was used for PCR with a primer pair for cluster 1 genes (TubI.for: TTC CGA TGA GCA TGG TGT CGA CC; TubI.rev: AGG TTG TGA TAC CGC TCA TAG C; AT: 55°C), for cluster 2 (TubII.for: GAC CTT ATC AGT ACA TCA GTT G; TubII.rev: GGC AAA TCC GAC CAT AAA GAA A; AT: 58°C) or for cluster 3 (TubIII.for: ACG GHT CCG GWG GTM TAC AAT GG; TubIII.rev1: ABG GGT TGK WSA GCT TNA RTG TNC; TubIII.rev2: WGC CTC GTT RTC RAT RCA GAA DGT C; AT: 52°C). PCR conditions were the same as for the *Tef* gene. Amplification products were separated by 2% agarose gel electrophoresis, cloned and sequenced as described above or analysed by Southern blot under standard conditions (Sambrook et al. 1989). Cluster 1 and cluster 2 β-tubulin sequences of *G. mosseae* were labelled with digoxigenin according to the supplier's protocol of (Roche, Mannheim, Germany) and served as hybridisation probes. Detection was carried out by chemiluminescense using the alkaline phosphatase-coupled digoxigenin antibody and the substrate CDP-Star (Roche).

Results

To identify β-tubulin genes expressed in AM fungi, RT-PCR cloning experiments were carried out with different isolates. Sequences of 802 bp were obtained in each case as previously described for *Gigaspora rosea* (Franken et al. 1997) and *Glomus mosseae* (Bütehorn et al. 1999) and compared with β-tubulin genes from fungi belonging to the Ascomycota, the Basidiomycota, the Zygomycta and the Oomycota. An animal sequence was taken as an outgroup. The phylogenetic analysis of the alignment showed that most AM fungal sequences could be grouped into two different clusters (Fig. 1). Two sequences from *S. castanea* and from *A. laevis* were placed in cluster 1. This cluster showed highest similarity to Oomycota β-tubulin genes. In contrast, *Glomus geosporum*, *Glomus coronatum* and *Gigaspora margarita* sequences were grouped in a different cluster (cluster 2). In the distance tree, this cluster was located close to the Zygomycota. Sequences from *Gigaspora rosea* and *Glomus mosseae* were found in both clusters. For one fungus, *Glomus clarum*, two sequences grouped neither in cluster 1 nor in cluster 2, but showed highest similarity to β-tubulin genes from the Ascomycota. The three clusters differed in their GC content. While cluster 1 and cluster 3 sequences showed around 53% or 58% GC, respectively, the value was for cluster 2 sequences between 42% and 44%. This was also mirrored by the codon usage. Most obviously, the approximately 25 leucine residues found in cluster 2 gene fragments were encoded by the codon TTA in seven to nine cases; this codon was never used in cluster 1 and cluster 3 genes.

In addition to the data obtained from this study, four cDNA fragments with similarity to β-tubulin genes from *Glomus intraradices* extraradical hyphae were found to be deposited in the database. Two of them partially overlapped the sequences presented here and showed closest similarity to β-tubulin genes from cluster 2. An additional sequence was detected in a cDNA library from *Medicago truncatula-G. intraradices* mycorrhiza. This sequence was highly similar to cluster 1 sequences. In order to

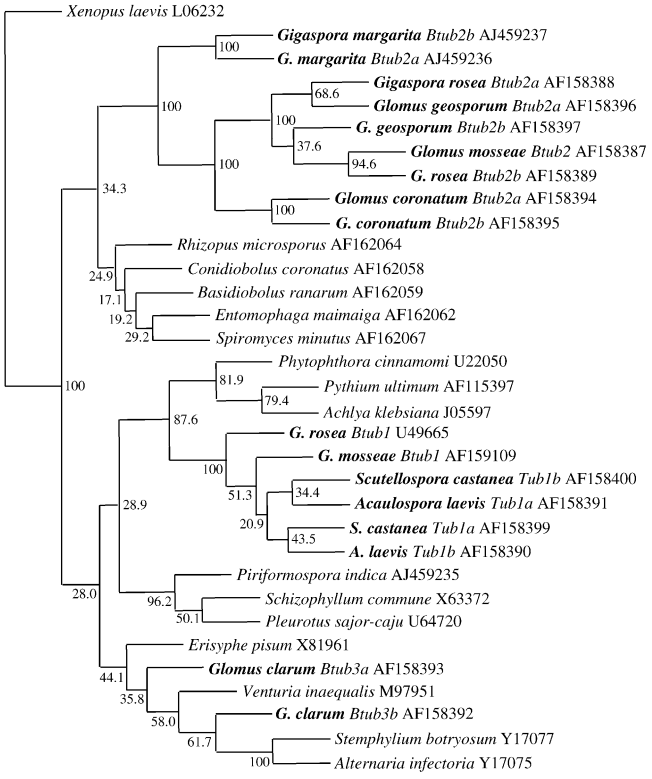

Fig. 1 Distance tree of β-tubulin gene sequences. Nucleotide sequences of β-tubulin genes derived by RT-PCR from different arbuscular mycorrhizal (AM) fungi were compared with the β-tubulin genes of other fungi belonging to the Oomycota, the Zygomycota, the Ascomycota and the Basidiomycota using the program ClustalW. The β-tubulin gene of *Xenopus laevis* was added as an outgroup. After bootstrap analysis of the data, the consensus of most parsimonious trees was calculated and is shown in the figure. Values of 500 bootstrap resamplings are indicated

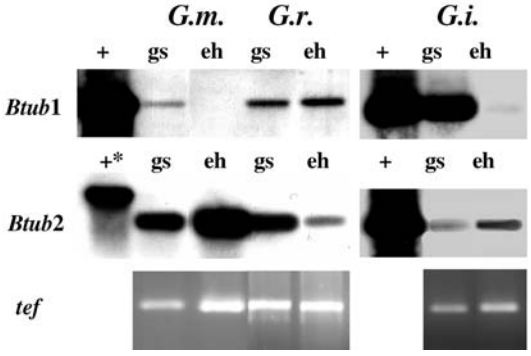

Fig. 2 RT-PCR analysis of β-tubulin genes. RNA was extracted from germinating spores (*gs*) and extraradical hyphae (*eh*) from *Glomus mosseae* (*G.m.*), *Gigaspora rosea* (*G.r.*) and *Glomus intraradices* (*G.i.*) and used for RT-PCR with primer pairs specific for the β-tubulin genes *Btub*1 and *Btub*2 or for the *Tef* gene. In the case of *Btub*1 and *Btub*2, PCR products were hybridised in Southern blot analyses to the corresponding probes from *G. mosseae*. Plasmid DNA of the *Btub*1 and *Btub*2 cDNA clones (+) or of the *Btub*2 genomic clone (+*) of *G. mosseae* were used as positive controls

identify β-tubulin cDNA belonging to the three identified clusters in *G. intraradices* in vitro-produced spores, PCR cloning using cluster-specific primer pairs was carried out. Amplification products of the correct size were obtained only with cluster 1 and cluster 2 primers, but never with the cluster 3-specific primer pair, although different tissues and conditions were tested (data not shown). Sequences of *G. intraradices* could not be included in the distance tree, because they covered a different region of the gene.

In order to analyse if genes of the two clusters are expressed not only in activated spores, but also in other stages of the AM fungal life cycle, RNA was extracted from germinated spores and from extraradical hyphae of *Glomus mosseae*, *Gigaspora rosea* and *Glomus intraradices*. cDNA was synthesised and its quality and quantity confirmed to be similar in all samples using a primer pair specific for fungal *Tef* genes encoding the translation elongation factor EF-1α (Fig. 2). This cDNA was used for PCR experiments with β-tubulin cluster-specific primer pairs, and amplification products were hybridised in Southern blot analysis to probes corresponding to either cluster 1 or cluster 2 (Fig. 2). These experiments indicated differential transcript accumulation of the two β-tubulin genes. Using *Btub*1 primers, higher amounts of RT-PCR products were obtained from germinated spores than from extraradical hyphae. This, however, was only the case in *Glomus mosseae* and *Glomus intraradices*; in *Gigaspora rosea* the amounts did not differ significantly between the two tissues. The opposite situation was observed for *Btub*2. This gene copy shows differential RNA accumulation in *G. rosea*. When RNA from asymbiotic and symbiotic tissues from the two *Glomus* species was used, *Btub*2 amplification and hybridisation resulted in similar signal intensities. Comparable results were obtained in two repetitions using independent biological samples.

In some experiments, a second band was detected in some of the samples from the three isolates used for the repetitions. Sequencing of this fragment showed that it originated from a contamination with genomic DNA because it contained a small intron (the sequence is not shown, but included in the database under the AC number AJ459248). The corresponding samples were not used for analysis of RNA accumulation.

In order to test if the primer pair for the *Btub*2 genes could also be used for analysing AM fungal transcript accumulation in environmental samples, RNA was extracted from the mycelium of a number of different fungi including five Zygomycetes. As a positive control, spores from *A. scrobiculata* were included, which were not the subject of the initial analysis, as well as *Pisum sativum-G. mosseae* mycorrhiza and the corresponding control root. RT-PCR fragments were obtained only from the AM fungus and from mycorrhizal roots (Table 1), indicating that the *Btub*2 primer pair may be specific for glomalean fungi.

Table 1 Results of RT-PCR with a primer pair specific for the *Btub*2 gene

Species	*Btub*2 PCR fragment
Acaulospora scrobiculata	Yes
Aspergillus nidulans	No
Aphanomyces euteiches	No
Piriformospora indica	No
Ustilago maydis	No
Phycomyces blakesleanus	No
Mortirella vinacea	No
Mycotypha microspora	No
Basidiobolus ranarum	No
Mucor mucedo	No
Pisum sativum control roots	No
P. sativum colonised by *G. mosseae*	Yes

Discussion

β-Tubulin gene fragments were obtained from a number of AM fungal isolates and their comparison grouped them into three different types of sequences. Using specific primers and RNA from sterile cultivated *G. intraradices* spores, the presence of type 1 and type 2 sequences was verified, but a cluster 3-type sequence could not be isolated. Because such sequences were obtained only from *G. clarum* spores of one-pot culture, these fragments might be derived from a fungal contaminant, which would – according to the similarity – belong to the Ascomycota. Such contaminants are often found in AM fungal spores from field sites (Lee and Koske 1994) and might also occasionally occur in pot cultures. Sequences from a putative Ascomycete have already been detected in DNA extracts from AM fungi (Redecker et al. 1999). Fragments with similarity to type 1 and type 2 sequences were, however, isolated from in vitro-produced *G. intraradices* spores, and RNA accumulation of the corresponding genes could be detected in growing hyphae, where contaminants are normally observable. This confirms the presence of *Btub*1 and *Btub*2 in the genomes of AM fungi. Since no experiments were carried out with single spores or single nuclei, it cannot be concluded without doubt that they represent two loci at different chromosomal positions. They might also correspond to groups of alleles at one locus existing in parallel as has been shown for rRNA sequences (Sanders et al. 1995; Hijri et al. 1999; Trouvelot et al. 1999; Kuhn et al. 2001). However, this is not likely in the case of the two β-tubulin genes because they are differentially expressed. Two sequences with slight differences for cluster 1 or cluster 2 β-tubulin genes were obtained from most isolates (Tub1a and Tub1b or Tub2a and Tub2b in Fig. 1). These might represent different alleles of the two putative loci, as in the case of a binding protein gene in different nuclei of one AM fungal spore (Kuhn et al. 2001).

Although the significance of the precise locations in the distance tree was low, the difference in the GC content and in the codon usage suggests a divergent origin of the two types of *Btub* genes. Based on the similarity, the closest relatives of the cluster 2 sequences were found among the Zygomycota. This correlates with the phylogenetic analysis based on 18S rRNA sequences as recently presented by Schüßler et al. (2001). In contrast, cluster 1 sequences were located among the β-tubulin genes of the other true fungi with closest similarity to those of the Oomycota. The origin of the cluster 1 genes remains open. One possibility could be horizontal gene transfer, which has been discussed to occur not only in bacteria, but also in fungi (Rosewich and Kistler 2000). DNA transfer during interaction has been demonstrated, for example in the case of the mycoparasite *Parasitella parasitica* and its host *Absidia glauca* (Kellner et al. 1993). Because AM fungi are colonised by a large number of different fungi (Lee and Koske 1994), mycoparasitism could have been the mechanism for the transfer of the cluster 1 sequence into the AM fungal genome. DNA transfer from a plant to a fungus has also been shown experimentally. After co-cultivation of *Aspergillus niger* with different transgenic plants, the fungus received hygromycin resistance and, in one case, this could be clearly correlated with the transfer of the corresponding *hph* gene (Hoffmann et al. 1994). Such an event is not unlikely as AM fungi and their hosts form a very close symbiosis, which includes the penetration of the plant cell wall and intracellular colonisation during arbuscule development. The cluster 1 sequences might therefore also be derived from a plant, as plants and Oomycetes are closely related and show sequence similarities among their β-tubulin genes (Mu et al. 1999).

Differential gene expression of β-tubulin genes has been detected in animals (Hoyle et al. 1995) and in plants (Artlip et al. 1995; Vassilevskaia et al. 1996), but the present study is the first time that such a phenomenon has been shown in fungi. While *Btub*1 is repressed in extraradical hyphae of *Glomus mosseae* and *G. intraradices*, when compared with germinating spores, *Btub*2 is constitutively expressed. The microtubule cytoskeleton of AM fungi is highly dynamic (Blancaflor et al. 2001; Timonen et al. 2001) and it could be speculated that the predicted two isotypes are involved in different cellular needs during asymbiotic germination or symbiotic extraradical hyphal growth. Surprisingly, the opposite expression pattern was detected in the AM fungus *Gigaspora rosea*. Instead of *Btub*1, which seemed to be constitutively transcribed, the second copy *Btub*2 was repressed in the symbiotic mycelium of this isolate. The expression of different copies of a gene family in two AM fungi was also detected in the case of the chitin synthase genes. While two class II chitin synthase genes were expressed in the extraradical mycelium of *Glomus intraradices* (Ubalijoro et al. 2001), RNA accumulation of a similar class II gene could not be detected in the same tissue of *Gigaspora margarita* (Lanfranco et al. 1999b). This shows that the different biology of various AM fungi is reflected not only in their morphology (Walker 1995; Cavagnaro et al. 2001) and physiology (Boddington and Dodd 1999; Smith et al. 2000), but also in gene expression patterns.

The distribution of AM fungi in the soil or in plant roots has been analysed by molecular methods using PCR on DNA extracts with primer pairs mainly directed towards the rRNA gene cluster (Franken 1998). In this way, the absence or presence of different isolates in mycorrhiza of field sites (Helgason et al. 1998) or microcosms (Van Tuinen et al. 1998) could be determined. However, the application of different staining techniques has shown that not all the hyphae in a mycorrhiza are active (Smith and Dickson 1991; Tisserant et al. 1993). Therefore, instead of DNA, it might be useful to analyse the occurrence of fungal RNA, which would reflect activity rather than simply the presence of organisms. Such an approach has already been carried out to assess the physiological activity of *Phanerochaete chrysosporium* in organo-polluted soil samples, where accumulation of (among others) β-tubulin RNAs could be detected (Lamar et al. 1995). Because the present study indicates that *Btub*2 PCR is specific for Glomales and the source of template can be distinguished due to the presence of a small intron, this primer pair could be used to estimate AM fungal RNA accumulation in complex root or soil samples. *Btub2* RNA accumulation in mycorrhiza has been shown at least for the two *Glomus* species (data not shown). Future experiments will reveal if cDNA fragments of *Btub*2 genes from different AM fungi can be separated, e.g. by denaturing gradient gel electrophoresis as has been used to analyse expression levels of different transgene copies (Ringel et al. 1998). This would allow RNA transcripts of a certain gene to be assigned to a specific isolate and, in this way, to relate symbiotic functions with the population structure of AM fungi.

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