Mycorrhiza (2002) 12:67–74
DOI 10.1007/s00572-001-0149-2

ORIGINAL PAPER

**Ignacio E. Maldonado-Mendoza · Gary R. Dewbre
Marianne L. van Buuren · Wayne K. Versaw
Maria J. Harrison**

Methods to estimate the proportion of plant and fungal RNA in an arbuscular mycorrhiza

Accepted: 16 November 2001 / Published online: 6 February 2002

Abstract Arbuscular mycorrhizas are endosymbiotic associations formed between obligately biotrophic arbuscular mycorrhizal (AM) fungi and plant roots. The fungus and plant coexist in intimate contact as the fungus grows within the cortex of the root. RNA isolated from arbuscular mycorrhizas contains transcripts from both eukaryotic genomes. It is essential to be able to estimate the relative levels of fungal and plant RNA so that changes in plant and fungal gene expression can be evaluated during development of the AM symbiosis. Here we describe the design and use of specific plant and fungal internal transcribed spacer sequences and 18S rRNA probes to distinguish and quantify the relative levels of RNA of plant and fungal origin in samples from arbuscular mycorrhizas. We present two different methods. The first employs the most traditional method of transcript level analysis, namely northern blot analysis. The second one uses ribonuclease protection assays, which permit the analysis of transcript levels in a very small amount of tissue and are proving to be suitable for the analysis of gene expression in AM fungi. Analysis of tissues from a developing mycorrhiza showed that the relative levels of fungal RNA increased gradually as colonization of the root system progressed, reaching 5–12% in the most highly colonized samples.

Keywords *Medicago truncatula* · *Glomus versiforme* · 18S rRNA · Internal transcribed spacer · Symbiosis · Plant–microbe interaction

I.E. Maldonado-Mendoza · G.R. Dewbre · M.L. van Buuren
W.K. Versaw · M.J. Harrison (✉)
The Samuel Roberts Noble Foundation, Plant Biology Division,
2510 Sam Noble Parkway, Ardmore, OK 73402, USA
e-mail: mjharrison@noble.org
Tel.: +1-580-2235810, Fax: +1-580-2217380

Present addresses:
I.E. Maldonado-Mendoza, CIIDIR-Sinaloa,
Km 1.0 Carretera a Las Glorias AP 280, Guasave, Sinaloa,
Mexico 81101

M.L. van Buuren, Department of Agroenvironmental Science and Technology (DISTA), Via Filippo Re, 6–8, 40126 Bologna, Italy

Introduction

Plants have evolved a range of mechanisms to access phosphate from low-nutrient environments, one of the most widespread being the formation of symbiotic associations with arbuscular mycorrhizal (AM) fungi (Smith and Read 1997). Development of an AM symbiosis is a complex process and requires the coordinate differentiation of both symbionts. The underlying alterations in gene expression have been the focus of several investigations (Gianinazzi-Pearson et al. 2000; Krajinski et al. 2000; Lanfranco et al. 1999; Requena et al. 1999; Roussel et al. 2001; van Buuren et al. 1999). Analysis of individual transcript levels in the arbuscular mycorrhiza are complicated as isolated RNA comprises both plant and fungal RNAs and the ratio changes depending on the extent of fungal colonization of the root. Steady-state transcript levels are usually calculated relative to a constitutively expressed RNA, frequently rRNA. However, for calculations of transcript levels in mycorrhizal RNA, it is more appropriate to normalize the transcript levels to a constitutive transcript from the appropriate symbiont rather than from the total mycorrhizal RNA. Otherwise, apparent changes in a plant transcript in mycorrhizal RNA samples could be the result of variation in the amount of plant RNA present in the samples rather than a true change of expression. Likewise, the induction of a fungal gene in the mycorrhiza cannot be assessed unless the amount of fungal RNA in the mycorrhizal RNA sample can be standardized. Therefore, probes that recognize constitutively expressed transcripts from each symbiont are required. This has been achieved for ectomycorrhizas, where the use of 5.8S-internal transcribed spacer (ITS) probes specific for *Pisolithus* and *Eucalyptus* permitted the estimation of the relative amounts of fungal and plant RNAs in the ectomycorrhiza (Diaz et al. 1997). However, probes specific for constitutive transcripts from the symbionts of an arbuscular mycorrhiza have not been reported. Although there are a number of constitutively expressed plant genes that are widely used for normalizing plant transcripts, not all of these are suitable for

use in mycorrhizal roots. For example, plant tubulin, which is frequently used as a standard, is actually induced during the symbiosis and, therefore, is not appropriate (Bonfante et al. 1996). The choice of constitutive fungal transcripts is severely hampered by the lack of information about the AM fungal genome. However, this is improving rapidly with the advent of EST sequencing (Sawaki and Saito 2001).

The genomes of eukaryotes, especially those of higher plants, contain numerous copies of ribosomal RNA genes (Long and David 1980; Mandal 1984). These are arranged in one or more tandemly repeated gene clusters. One transcribed unit consists of the highly conserved 18S, 5.8S, and 25S rRNA coding regions and the ITS, which are highly divergent regions (Franken and Gianinazzi-Pearson 1996; Lloyd-MacGilp et al. 1996; Simon 1996; Simon et al. 1993). Each rDNA transcription unit is separated from the adjacent repeat by an intergenic spacer (IGS) located between the 3′ end of the 25S gene of one repeat and the 5′ terminus of the 18S gene of the next repeat. It is calculated that the haploid genome of plants can contain 200–22,000 repeating units (Rogers and Bendich 1987).

Here we demonstrate the design and use of specific ITS and 18S rRNA probes in northern blot analysis and ribonuclease protection assays (RPA) to estimate the relative amount of *Glomus versiforme* and *Medicago truncatula* RNA present in samples of RNA from mycorrhizal roots of *M. truncatula*/*G. versiforme*. These two approaches gave comparable results and the inclusion of either method in gene expression studies will assist in the evaluation of plant and fungal gene expression in mycorrhizas. There are, however, important differences in the sensitivity of these methods.

Materials and methods

Plant growth and colonization by mycorrhizal fungi

Stock cultures of *G. versiforme* were prepared and maintained according to Harrison and Dixon (1993). *M. truncatula* Gaertn. "Jemalong" growth, colonization procedures, and time-course experiments were set up as described previously (van Buuren et al. 1999). Two-week-old seedlings (7–10 plants per 11-cm pot) were inoculated with 5,000 spores from *G. versiforme* sporocarps. Control plants were mock-inoculated with the final distilled water wash from the spore sterilization procedure. The plants were fertilized weekly with half-strength Hoagland's (Arnon and Hoagland 1940) solution containing 20 µM phosphate. Control plants did not show any colonization. Roots were harvested at 8, 15, 22, 29, and 44 days and colonization levels were 12%, 20%, 57%, 71% and 72%, respectively (van Buuren et al. 1999). In a second experiment, samples were taken at 29 and 44 days and showed 30% and 61% colonization, respectively, as assessed by a modified gridline intersect method (McGonigle et al. 1990).

Isolation of RNA

RNA was extracted from non-mycorrhizal roots, mycorrhizal roots and fungal spores as described previously (Harrison and Dixon 1993; Harrison and van Buuren 1995).

Cloning of *M. truncatula* and *G. versiforme* 18S rRNAs

Reverse transcription reactions containing either RNA extracted from approximately 100 *G. versiforme* spores (Harrison and van Buuren 1995) or 1 µg of total RNA from *M. truncatula* roots were carried out as described by Harrison (1995) and Kawasaki (1990). 18S rRNA sequences were amplified from *M. truncatula* (NS1, 5′GTAGCTATATGCTTGTCTC3′; and NS8, 5′TCCGCAGGTTCACCTACGGA3′) (White et al. 1990) and *G. versiforme* (NS1 and NS21 (Simon et al. 1992), 5′AATATACGCTATTGGAGCTGG3′) by PCR. The PCR reactions contained 1/10 of the reverse transcription reaction, 0.2 mM dNTPs, 40 pmol primers, 1× PCR reaction buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) and 6 units of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis) in a total volume of 50 µl. The PCR cycle was performed as follows: 95°C/2 min, 35 cycles (95°C/1 min, annealing 50°C/1 min, elongation 72°C/2 min), and termination 72°C/10 min. The RT-PCR fragments obtained from *G. versiforme* spore RNA (590 bp) and *M. truncatula* root RNA (1.781 kb) (Fig. 1A) were cloned into the T-modified *Eco*RV site of the pT7 PCR T-vector (Novagen, Madison, Wis., USA). The *M. truncatula* 1.781-kb fragment was digested with restriction enzymes *Sal*I and *Bam*HI to generate a 329-bp fragment, which was subcloned in pBlueScript-SKII$^+$ (Stratagene, La Jolla, Calif., USA). Clones were sequenced on both strands by dideoxy sequencing on an ABI 373A automated sequencer (Applied Biosystems, Foster City, Calif., USA) (Fig. 1B).

Cloning of the *M. truncatula* and *G. versiforme* ITS regions

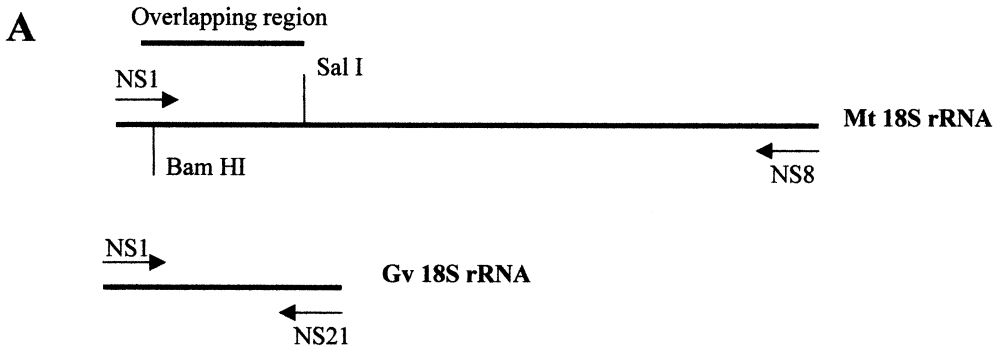
ITS regions were amplified from genomic DNA of *M. truncatula* and *G. versiforme* using ITS1/ITS4 primers (White et al. 1990). The fragments were 705 bp and 544 bp in length, respectively, and were cloned and sequenced to confirm their identity. Short, gene-specific ITS probes were generated from the large ITS clones by PCR. Primers Gv-ITS-A (5′CGCGGATTTGAGTTTTCCAGTA3′) and Gv-ITS-B (5′AAATATATACTCGCACA3′) were used to amplify a 175-bp fragment from the *G. versiforme* ITS clone and primers Mt-ITS-A (5′TGGCCTCCCGTGAGCTCTG3′) and Mt-ITS-B (5′TAAAAGAGTCCACATTC3′) were used to amplify a 132-bp fragment from the *M. truncatula* ITS clone (Fig. 1C). These short ITS fragments were used as probes on northern blots of *G. versiforme* spore RNA and *M. truncatula* root RNA.

Northern blot analysis

Aliquots (10 µg) of total RNA from non-mycorrhizal and mycorrhizal roots were fractionated on agarose-formaldehyde gels (Sambrook et al. 1989) and transferred to Gene Screen Plus membranes (Gene Screen, Boston, Mass., USA). 18S rRNA fragments from *M. truncatula* (329 bp) and *G. versiforme* (590 bp) were labeled with ^{32}P-dATP by random priming and used as probes. Blots were hybridized overnight at 42°C in a solution containing 50% formamide, 6× SSC, 5× Denhardt's reagent, 0.5% SDS and 100 µg/ml denatured, fragmented salmon sperm DNA. The blots probed with the 18S rRNA probes were washed twice in 2× SSC, 0.1% SDS for 20 min at 50°C, twice in 1× SSC, 0.1% SDS for 20 min at 60°C, and twice in 0.1× SSC for 20 min at 68°C. The blots probed with the ITS probes were washed once in 2× SSC, 0.1% SDS for 20 min at 42°C, and once in 0.1× SSC, 0.1% SDS for 20 min at 42°C.

Ribonuclease protection assay

Antisense transcripts were prepared in vitro from *M. truncatula* (329 bp) and *G. versiforme* (590 bp) 18S rRNA clones in the presence of 5 µM of ^{32}P-UTP using a MAXIscript kit (Ambion, Austin, Tex., USA). Using the Direct Protect Kit (Ambion), RPA was performed on 1 µg of total RNA or by direct protection of



B

```
1   GTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAACCAATTTATA Gv 18S probe
                                  : : :::
201 T----------------------------CGACGCA------------------------ Mt 18S probe

    CAGGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGATAGTCCAATTACT
                      :::::
    -----------------GGCTC---------------------------------------

    ACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCTAAAAGTCCCGACTTCTGGAAG
                                          :::                :::
    ----------------------------------TGC---------------CTG----

    GGATGTATTTATTAGATAAAAAGCCAATAGCCCGCAAGGGTTTCTCCTTGGTGAATCATG
                                                 :: ::  ::: ::: :::::
    ----------------------------------------TTGCT--TTGATGATTCATG

    ATAACTTTTCGAATCGTATGGTC-TTGAACCGGCGATGAATCATTCAAATTTCTGCCCTA
    ::::::  ::: :::: : :: : :::  : ::::: : ::::::::::::::::::::
    ATAACTCGTCGGATCGCACGGCCCTTGTGCTGGCGACGCATCATTCAAATTTCTGCCCTA

    TCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTTTTAACGGGTAACGGGGAATTAGG
    :::::::::::::::::::::: :::::::::::::  : :::::: :::: ::::::::
    TCAACTTTCGATGGTAGGATAGTGGCCTACCATGGTGGTGACGGGTGACGGAGAATTAGG

    GTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCG
    :::::::::::::::::::::::::::::::: :::::::::::::::::::::::::::
    GTTCGATTCCGGAGAGGGAGCCTGAGAAACGGGTACCACATCCAAGGAAGGCAGCAGGCG

    CGCAAATTACCCAATCCCGACACGGGG-AGGTAGTGACAATAAATAACAATACGGGGCTC
    :::::::::::::::::  :::::::: :::::::::::::::::::::::::: ::::::
    CGCAAATTACCCAATCCTAACACGGGGGAGGTAGTGACAATAAATAACAATACCGGGCTC

    ATTTGGGTCTCGTAATTGGAATGAGTACAATTTAAATCTCTTAACGAGGAACAATTGGAG
    ::: : :::: :::::::::::::::::::: :::::: :::::::::
    ATT-GAGTCTGGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAG      525

    GGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAACTCCAATAGCGTATATT 590
```

C

```
451 TTCCTATTGAATAGGTATTGCGTGCAGGGTGAATATGTTGGCCTCCCGTG  Gv ITS
    :::         :: : :   ::         :  ::: :::  :  : :
310 TTCGCGGATTTGAGTTTTCCAGTATTTATAAATAATGCTGG..TAACTTT  Mt ITS

    AGCTCTGTCTCGTGGTTGGTTGAAAATTGAGACCTTGGTAGGGTGTGCCA
    :    : : :     :::::  ::  :  : :                 :
    AAAATTATTTATAACTTGGTACAAGTTGAAAA.................CG

    TGATAGATGGTGGATGTGCGACCCACGAGACCAAATCATGTGCAGCTCTA
    :: :: :::   : :  ::   : ::  : ::  ::: :       : ::
    TGCTATATGTGTGGTTCGCTGACAACTTGTCC..ATCTTTATATATTATA

    TTGAATGTGGACTCTTTTACCCACATGTGTTTTGTAACGCTCGTGATGAG  650
          : :  : :::::      ::::       :   :  :  :  :
    CGCGTGCTTGGTTTTTTTAAAGCTGTGTGCGAGTATATATTTTTTTTATG  489
```

Fig. 1 **A** Physical map of PCR fragments generated by RT-PCR from *Glomus versiforme* and *Medicago truncatula*, showing the overlapping region used to construct antisense RNA probes. **B** Comparison of the *M. truncatula* (324 bp), and *G. versiforme* (590 bp) 18S rRNA regions used as probes in northern analysis and ribonuclease protection assays. *Double dots* indicate identities. **C** Comparison of the *M. truncatula* and *G. versiforme* ITS regions used as probes on northern blots. *Double dots* indicate identities. The underlined sequences indicate the primers used to amplify the Gv ITS probe. *Bold sequences* indicate the primers used to amplify the Mt ITS probe

fungal spores. Direct protection was achieved by preparing an extract of fungal material and using this to hybridize directly to the probe without isolating RNA. The RPA was performed according to the manufacturer's instructions, except that RNase digestion was for 1 h at 37°C. The samples were resuspended by vigorous vortexing in 10 µl of non-denaturing RNA loading buffer, loaded onto non-denaturing acrylamide gels and separated via electrophoresis. The sizes of the protected bands were confirmed using denaturing (8 M urea) acrylamide gels (data not shown). The full-length RNase protected bands (*M. truncatula*, 329 bp and *G. versiforme*, 590 bp) were quantified by PhosphorImager analysis (Image Quant Software, PhosphorImager SF, Molecular Dynamics, Sunnyvale, Calif., USA). Protected products smaller than the probes result from cross-hybridization of the probe with transcripts of similar sequence. These products were not quantified. To compare relative levels of fungal and plant 18S rRNA, only ^{32}P-UTP was used in the in vitro transcription reactions. An accurate comparison can be made between the different probes by comparison of their specific activities. Each reaction contained 2×10^6 cpm of probe. Differences in hybridization efficiencies between different size probes were minimized by incubating them overnight (16 h) at 42°C with the Direct Protect extracts. In reactions containing both probes, an excess of probe (2 ng each) was used to avoid competition between the probes.

Normalization of sample values for RPA

Calculations were based on the results obtained by PhosphorImager analysis. To be able to compare plant and fungal rRNA, both 18S rRNA probes were prepared by labeling them homogeneously with ^{32}P-UTP. Each probe had a different specific activity because the number of ^{32}P-UTP molecules incorporated into each probe was different. The *M. truncatula* 18S rRNA probe (329 bp) contained 87 ^{32}P-UTP molecules, while the *G. versiforme* 18S rRNA probe (590 bp) contained 180 ^{32}P-UTPs. This means that the *G. versiforme* probe/18S rRNA hybrids had a DU (densitometric units obtained by PhosphorImager analysis) value 2.069-fold higher than the *M. truncatula* probe/18S rRNA hybrids. This value was thus used as a correction factor. For example, a *G. versiforme* probe/18S rRNA hybrid band that gave a value of 100 DU in fact represented only 48.3 DU (100 DU/2.069) and this value was compared with the value from the *M. truncatula* probe/18S rRNA hybrid band. The formula employed to calculate the relative value of *M. truncatula* 18S rRNA versus *G. versiforme* 18S rRNA is as follows:

$$\%\ \text{of Gv 18S rRNA in a sample} = \frac{100\ (\text{Gv 18S rRNA D.U./2.069})}{\text{Mt 18S rRNA D.U.} + \text{Gv 18S rRNA D.U./2.069}} \quad (1)$$

In our experiments, addition of both probes to the same sample or to independent probes gave the same result. Assessment of specific fungal or plant gene expression could then be made relative to the 18S rRNA from that species.

Results and discussion

Cloning of *M. truncatula* and *G. versiforme* 18S rRNAs

Utilizing the NS1/NS8 and NS1/NS21 primer pairs, 18S rRNA sequences were amplified from *M. truncatula* and *G. versiforme* cDNA prepared from root or spore RNA, respectively. The amplified fragments were sequenced and further cloned into pT7 vector (Fig. 1A, B). The *G. versiforme* 18S rRNA sequence showed six mismatches with respect to the sequence of the gene previously described by Gehrig et al. (1996). Because the genome of a single AM fungus has been demonstrated to contain multiple 18S rRNA sequences, it is likely that mismatches are due to real differences between 18S rRNA genes (Franken and Gianinazzi-Pearson 1996; Lloyd-MacGilp et al. 1996; Sanders et al. 1995), although they could also be changes introduced by PCR (Ueda and Mikata 1997). As our sequence was obtained by PCR and was the only sequence obtained, it is most probable that this particular rRNA species represents the most common rRNA species within our isolate of *G. versiforme*.

Amplification of *M. truncatula* 18S rRNA produced a 1.781-kb product (GenBank accession number AF093507) which shares 97% identity with that of *Pisum sativum* (Genbank accession number U43011), confirming its identity as an 18S rRNA gene. A comparison of the overlapping regions of the *M. truncatula* and *G. versiforme* 18S rRNA sequences revealed that they are 90% identical (Fig. 1B).

The *G. versiforme* 18S rRNA probe cross-hybridizes with *M. truncatula* 18S sequences on northern blots

Northern blots containing RNA from non-mycorrhizal roots (*M. truncatula*) and mycorrhizal roots (*M. truncatula*/*G. versiforme*) from a time-course experiment were hybridized under high stringency conditions with the 18S rRNA probes from *M. truncatula* and *G. versiforme*, respectively (Fig. 2A). Both probes hybridized to all samples of RNA. Thus, it is clear that the *G. versiforme* probe cross hybridizes to the *M. truncatula* 18S rRNA sequence, even under high stringency conditions. In contrast to a previous study, where 18S probes of similar length to the ones described in this work were able to differentiate tomato and *Glomus intraradices* transcripts (David et al. 1998), these *M. truncatula* and *G. versiforme* 18S probes cannot be used to distinguish between *M. truncatula* and *G. versiforme* 18S rRNA.

ITS probes can be used on northern blots to distinguish between ITS sequences of *M. truncatula* and *G. versiforme*

ITS probes have been used on northern blots to estimate levels of fungal RNA in samples of ectomycorrhizal RNA (Diaz et al. 1997). However, the fungal biomass is considerably higher in ectomycorrhizas than in arbuscular mycorrhizas and it was not clear whether this approach would be useful for estimates of the proportions of plant and fungal transcripts in arbuscular mycorrhizas. To test this approach, ITS regions were first amplified from genomic DNA from *M. truncatula* and *G. versiforme* using ITS1/ITS4 primers (White et al. 1990). This resulted in fragments of 705 bp and 544 bp, respectively. The *G. versiforme* ITS sequence (AF246141) shares 93% identity with an ITS sequence from *G. intraradices* (AF185695) and the *M. truncatula*

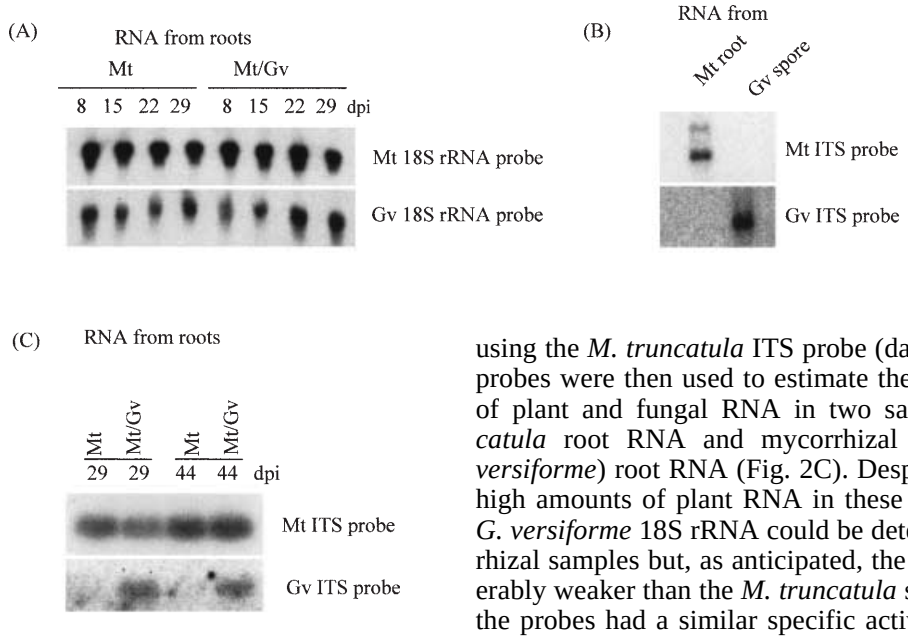


Fig. 2 **A** Northern blot analysis of RNA from roots of *M. truncatula* (*Mt*) and from *M. truncatula* colonized with *G. versiforme* (*Mt*/*Gv*). Roots were mock inoculated (*Mt*) or inoculated with *G. versiforme* (*Mt*/*Gv*) at day 0 and harvested at 8, 15, 22 and 29 days post-inoculation (*dpi*). The blots were hybridized with the *M. truncatula* 18S rRNA (*upper panel*) and *G. versiforme* 18S rRNA (*lower panel*) probes. Two blots were used and the presence of RNA was verified by staining the gel with ethidium bromide. **B** Northern blot analysis of RNA from roots of *M. truncatula* (*Mt*) and *G. versiforme* spores (*Gv*). The blots were hybridized with the *M. truncatula* ITS (*upper panel*) and *G. versiforme* ITS (*lower panel*) probes. **C** Northern blot analysis of RNA from roots of *M. truncatula* (*Mt*) and from *M. truncatula* colonized with *G. versiforme* (*Mt*/*Gv*). Roots were mock inoculated (*Mt*) or inoculated with *G. versiforme* (*Mt*/*Gv*) at day 0 and harvested at 29 and 44 days post-inoculation (*dpi*). The blots were hybridized with the *M. truncatula* ITS (*upper panel*) and *G. versiforme* ITS (*lower panel*) probes

Table 1 Colonization (% root length colonized) and relative amounts (%) of fungal and plant RNA in samples of RNA from mycorrhizal root samples as estimated by northern blot analysis

Days post-inoculation	Colonization	Fungal RNA	Plant RNA
29	30	2.9	97.1
44	61	5.4	94.6

ITS sequence (AF233339) shares 98% nucleotide sequence identity with a *M. sativa* ITS sequence (AF074399). The *M. truncatula* and *G. versiforme* ITS sequences share 52% identity. Shorter gene-specific ITS probes, generated from the larger ITS clones by PCR, were used as probes on northern blots of *G. versiforme* spore RNA and *M. truncatula* root RNA. The probes were specific; the *M. truncatula* ITS probe hybridized to RNA from *M. truncatula* root RNA but not to *G. versiforme* RNA from spores and vice versa (Fig. 2B). Southern blot analysis confirmed these results when using the *M. truncatula* ITS probe (data not shown). The probes were then used to estimate the relative quantities of plant and fungal RNA in two samples of *M. truncatula* root RNA and mycorrhizal (*M. truncatula*/*G. versiforme*) root RNA (Fig. 2C). Despite the presence of high amounts of plant RNA in these RNA samples, the *G. versiforme* 18S rRNA could be detected in the mycorrhizal samples but, as anticipated, the signal was considerably weaker than the *M. truncatula* signal, even though the probes had a similar specific activity. Densitometric analysis of this northern blot revealed that the RNA from mycorrhizal roots sampled at 29 and 44 days post-inoculation contained 2.9% and 5.4% fungal RNA, respectively (Table 1). The extent of colonization of these root samples was estimated to be 30% and 61% root length, respectively (Table 1). These results show that it is possible to estimate the proportion of fungal RNA in a mycorrhiza when the roots are relatively well colonized, but not at the early stages of development of the mycorrhiza, when the amount of fungal biomass is low, or in samples from mycorrhizal mutant interactions where the fungal biomass may also be low (data not shown). In order to develop more sensitive methods of estimating the proportions of fungal and plant RNA in arbuscular mycorrhizas, we assessed the use of 18S rRNA probes in RPAs.

18S rRNA probes can be used in RPAs to distinguish between 18S rRNA of plant and fungal origin in mycorrhizal roots

RPAs enable the discrimination of closely related transcripts, such as those arising from different members of multi-gene families (Alonso-Prados et al. 1998), or sequences that differ in their transcription initiation or termination sites (Samartzidou and Widger 1998). In this method, a radio-labeled RNA probe is hybridized with an RNA sample and then subjected to digestion with a cocktail of RNAases that cleave the mismatched hybrids. Theoretically, the RNAases are able to identify single mismatches between the RNA-RNA hybrids and, therefore, only hybrids which match exactly will remain intact. The products are separated by gel electrophoresis and the full-length protected fragment, representing the transcript with an identical sequence to the probe, is quantified via PhosphorImager analysis. Other labeled bands, resulting from RNAse digestion of mismatched

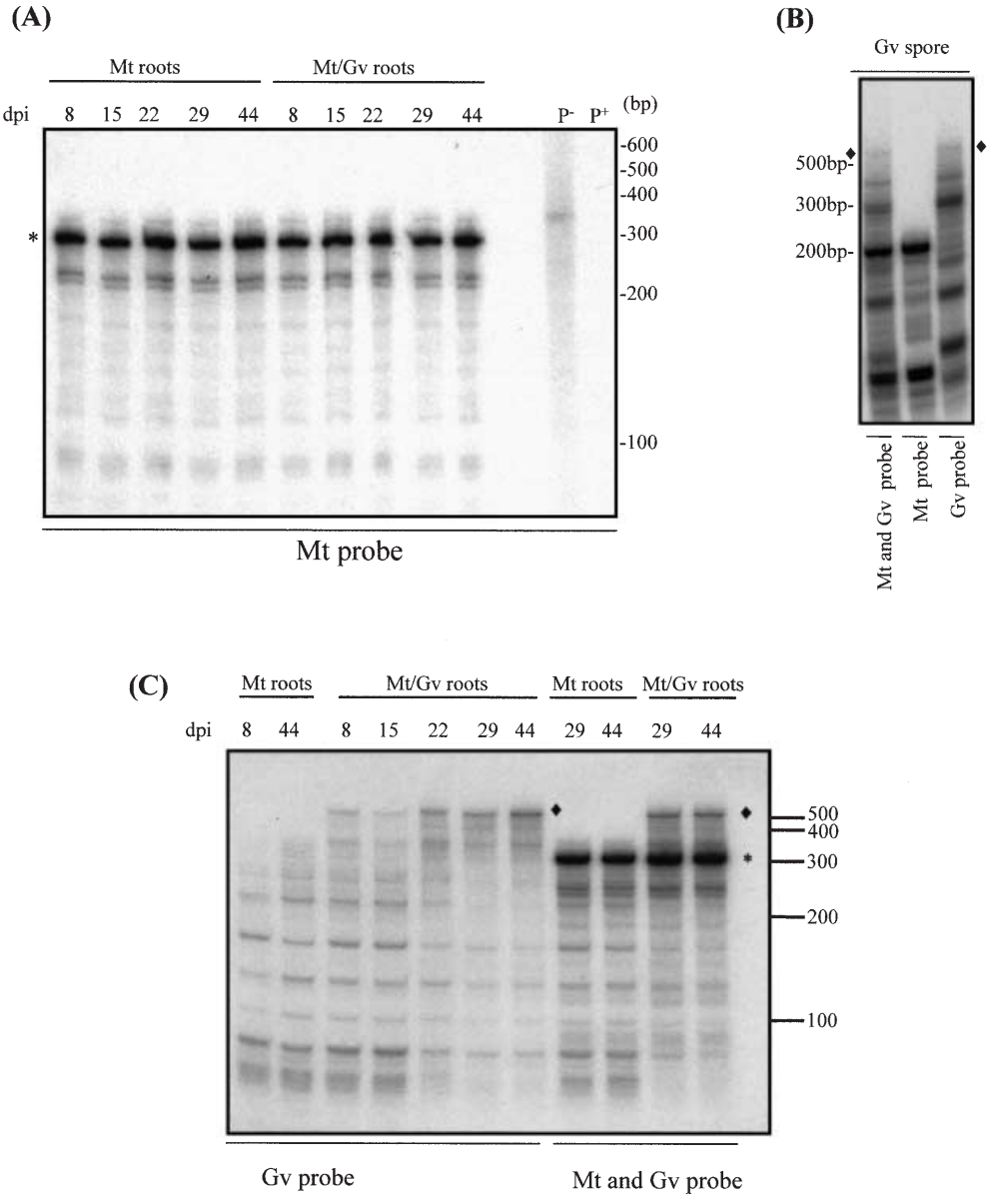


Fig. 3 **A** Ribonuclease protection assays (RPA) with RNA from roots of *M. truncatula* (*Mt*) and from *M. truncatula* colonized with *G. versiforme* (*Mt*/*Gv*) using the *M. truncatula* 18S rRNA probe. The roots were mock inoculated (*Mt*) or inoculated with *G. versiforme* (*Mt*/*Gv*) at day 0 and harvested at 29 and 44 days post-inoculation (*dpi*) (P^- probe without RNase, P^+ probe with RNase). **B** RPA with RNA from *G. versiforme* spores using the *G. versiforme* 18S rRNA probe alone (*right panel*) or the *M. truncatula* 18S rRNA probe alone (*middle panel*) or both probes (*left panel*). **C** RPA with RNA from roots of *M. truncatula* (*Mt*) and from *M. truncatula* colonized with *G. versiforme* (*Mt*/*Gv*) using either the *G. versiforme* 18S rRNA probe alone or the *M. truncatula* 18S rRNA and *G. versiforme* 18S rRNA probes simultaneously. The *G. versiforme* 18S rRNA protected product is indicated with a *diamond*. The *M. truncatula* 18S rRNA protected product is indicated with an *asterisk*

hybrids, are usually visible on the gel. These bands are smaller than the specific band and are not quantified and, furthermore, do not interfere with quantification.

To utilize this strategy to distinguish *M. truncatula* and *G. versiforme* 18S RNAs, we designed short *M. truncatula* and *G. versiforme* probes suitable for use in an RPA. The probes corresponded to partially overlapping regions so that the effects of possible differences in turnover rates could be minimized. We then confirmed the specificity of each probe in the RPA. The *M. truncatula* probe protected a fragment of the expected size (329 bp) corresponding to the plant 18S rRNA in samples of RNA from non-mycorrhizal roots (*M. truncatula*) and mycorrhizal roots (*M. truncatula*/*G. versiforme*) (Fig. 3A). This plant-specific band was not seen in samples of RNA from *G. versiforme* spores (Fig. 3B). The *G. versiforme* probe was slightly larger and protected a fragment of the expected size (590 bp) from samples of RNA from *G. versiforme* spores (Fig. 3B) and from mycorrhizal (*M. truncatula*/*G. versiforme*) roots (Fig. 3C), but not from non-mycorrhizal *M. truncatula* roots (Fig. 3C). When both plant and fungal probes were used simultaneously in an RPA reaction, RNA from non-mycorrhizal *M. truncatula* tissues produced only the plant-specific rRNA band (329 bp) while, as expected, the mycorrhizal tissues showed the presence of both the plant- and fungal-specific rRNA bands (Fig. 3C). Thus, both probes were specific and did not fully cross-protect rRNA from the other species. In addition, the protected products obtained with each probe were different in size and easily distinguished.

In most of the assays, mismatched hybrids were also visible, even when an *M. truncatula* probe was used on *M. truncatula* RNA alone or when the *G. versiforme* probe was used on spore RNA. These products were probably due to the presence of other rRNA genes that

Table 2 Colonization (% root length colonized) and relative amounts (%) of fungal and plant RNA in samples of RNA from mycorrhizal root samples as estimated by ribonuclease protection assay (*ND* not determined)

Days post-inoculation	Colonization	Plant and fungal probes added simultaneously		Plant and fungal probes added separately	
		Fungal RNA	Plant RNA	Fungal RNA	Plant RNA
8	12	ND	ND	4	96
15	20	ND	ND	2.6	97.4
22	57	6.7	93.3	7.2	92.8
29	71	11	89	10.9	89.1
44	72	11	89	11.9	88.1

correspond to different ribosomal DNA subunits in the plant or fungal genome and which are not identical in sequence to the probe. Such products are expected for genes that are members of multi-gene families (Alonso-Prados et al. 1998).

Quantification of the relative levels of 18S rRNA of plant and fungal origin in mycorrhizal roots

RPAs were performed either with both probes in a single reaction or with separate reactions for each probe (shown in part in Figure 3B, C). RNA from mycorrhizal roots sampled at 8, 15, 22, 29 and 44 days post-inoculation contained 4, 2.6, 7.2, 10.9 and 11.9% fungal RNA, respectively (Table 2). The extent of colonization of these root samples was estimated to be approximately 12, 20, 57, 71 and 72% root length, respectively (van Buuren et al. 1999). The results obtained by analysis of the RNA in two separate reactions, one for each probe, agreed with those obtained from a single reaction containing both probes (Table 2). The inclusion of both probes in a single reaction eliminates the possibility of manipulation errors between different samples and is, therefore, the method of choice.

In both northern blot analysis and RPA experiments, an overall increase in the relative amount of fungal RNA was observed as the fungus colonized the roots (Tables 1, 2). However, even in RNA samples from fairly highly colonized roots, the proportion of RNA from the fungal symbiont was significantly lower than that of the plant. This is likely the result of large differences in plant and fungal biomass within the mycorrhiza. Previously, Toth et al. (1991) estimated that the fungal symbiont comprised 4.3% of the total biomass of onion mycorrhizal roots. These roots showed 76% root length colonized. Our estimates of the proportion of fungal and plant RNA in our mycorrhizas are consistent with these data.

It is also clear from these data (Tables 1, 2) that estimates of the relative proportions of fungal and plant RNA in different mycorrhizas cannot be predicted from estimates of root length colonized. The reason for this is that the ratio of arbuscules, intra-radical hyphae, vesicles and extra-radical hyphae varies between samples and this is not reflected in the overall estimates of root length colonized. Consequently, we recommend that the proportion of fungal and plant RNA be determined for each new mycorrhizal RNA sample. It may be possible to predict the proportions of fungal and plant RNA from more detailed estimates of root length colonized, particularly if the intensity of arbuscules, vesicles, intra-radical and extra-radical hyphae are estimated individually.

The proportions of plant and fungal RNAs estimated via northern blot analyses are comparable to the data from the RPA analysis, further supporting the validity of the two methods. However, the RPA analysis shows greater sensitivity. The RPA technique has allowed quantification of *G. intraradices* phosphate transporter transcripts in the external hyphae of *M. truncatula*/*G. intraradices* mycorrhizas. The amount of fungal material employed in these experiments was extremely low, usually in the order of 1–5 mg of tissue (Maldonado-Mendoza and Harrison 1997).

In conclusion, these techniques enable direct estimations of the amount of fungal relative to plant RNA in RNA samples from arbuscular mycorrhizas. The inclusion of these estimates in gene expression studies allows evaluation of alterations in individual plant and fungal transcript levels during development of the AM symbiosis. The 18S rRNA genes and ITS sequences are highly conserved and it should be possible to design specific probes for other combinations of plants and AM fungi using the regions selected here as a guide.

Acknowledgements The authors thank Laura Blaylock for assistance with the mycorrhizal experiments and J. De Miranda and F. McAlister for reviewing the manuscript. The work was supported by the Samuel Roberts Noble Foundation.

References

Alonso-Prados JL, Aranda MA, Malpica JM, Garcia-Arenal F, Fraile F (1998) Satellite RNA of cucumber mosaic cucumovirus spreads epidemically in natural populations of its helper virus. Phytopathology 86:520–524

Arnon DI, Hoagland DR (1940) Crop production in artificial culture solutions and in soils with special reference to factors influencing yields and absorption of inorganic nutrients. Soil Sci 50:463–483

Bonfante P, Bergero R, Uribe X, Romera C, Rigau J, Puigdomenech P (1996) Transcriptional activation of a maize α-tubulin gene in mycorrhizal maize and transgenic tobacco plants. Plant J 9:737–743

David R, Itzhaki H, Ginzberg I, Gafni Y, Galili G, Kapulnik Y (1998) Suppression of tobacco basic chitinase gene expression in response to colonization by the arbuscular mycorrhizal fungus *Glomus intraradices*. Mol Plant-Microbe Interact 11:489–497

Diaz EC, Tagu D, Martin F (1997) Ribosomal DNA internal transcribed spacers to estimate the proportion of *Pisolithus tinctorius* and *Eucalyptus globulus* RNAs in ectomycorrhiza. Appl Environ Microbiol 63:840–843

Franken P, Gianinazzi-Pearson V (1996) Construction of genomic phage libraries of the arbuscular mycorrhizal fungi *Glomus mosseae* and *Scutellospora castanea* and isolation of ribosomal RNA genes. Mycorrhiza 6:167–173

Gehrig H, Schussler A, Kluge M (1996) *Geosiphon pyriforme*, a fungus forming endocytobiosis with *Nostoc* (cyanobacteria), is an ancestral member of the Glomales: evidence by SSU rRNA analysis. J Mol Evol 43:71–81

Gianinazzi-Pearson V, Arnould C, Oufattole M, Arango M, Gianinazzi S (2000) Differential activation of H+-ATPase genes by an arbuscular mycorrhizal fungus in root cells of transgenic tobacco. Planta 211:609–613

Harrison MJ (1996) A sugar transporter from *Medicago truncatula*: altered expression pattern in roots during vesicular-arbuscular (VA) mycorrhizal associations. Plant J 9: 491–505

Harrison MJ, Dixon RA (1993) Isoflavonoid accumulation and expression of defense gene transcripts during the establishment of vesicular-arbuscular mycorrhizal associations in roots of *Medicago truncatula*. Mol Plant-Microbe Interact 6:643–654

Harrison MJ, van Buuren ML (1995) A phosphate transporter from the mycorrhizal fungus *Glomus versiforme*. Nature378:626–629

Kawasaki ES (1990) Amplification of RNA. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols. A guide to methods and applications. Academic, San Diego, pp 21–27

Krajinski F, Biela A, Schubert D, Gianinazzi-Pearson V, Kaldenhof R, Franken P (2000) Arbuscular mycorrhiza development regulates the mRNA abundance of *Mtaqp1* encoding a mercury-insensitive aquaporin of *Medicago truncatula*. Planta 211:85–90

Lanfranco L, Carnero L, Bonfante P (1999) Chitin synthase genes in the arbuscular mycorrhizal fungus *Glomus versiforme*: full sequence of a gene encoding a class IV chitin synthase. FEMS Microbiol Lett 170:59–69

Lloyd-MacGilp SA, Chambers SM, Dodd JC, Fitter AH, Walker C, Young JPW (1996) Diversity of the ribosomal internal transcribed spacers within and among isolates of *Glomus mosseae* and related mycorrhizal fungi. New Phytol 133:103–111

Long EO, David IB (1980) Repeated genes in eukaryotes. Annu Rev Biochem 49:747–764

Maldonado-Mendoza IE, Harrison MJ (1997) Establishment of monoxenic root organ cultures for the study of gene expression of fungal phosphate transporters in the arbuscular mycorrhizal association. In: Flores HE, Lynch JP (eds) Radical biology: advances and perspectives on the function of plant roots. American Society of Plant Physiologists, Rockville, Md, USA, pp 496–497

Mandal RK (1984) The organization and transcription of eukaryotic ribosomal RNA genes. Nucleic Acids Res 31:115–160

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA (1990) A new method that gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol 115:495–501

Requena N, Füller P, Franken P (1999) Molecular characterization of GmFOX2, an evolutionarily highly conserved gene from the mycorrhizal fungus *Glomus mosseae*, down-regulated during interaction with rhizobacteria. Mol Plant Microbe Interact 12:934–942

Rogers S, Bendich AJ (1987) Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. Plant Mol Biol 9:509–520

Roussel H, vanTuinen D, Franken P, Gianinazzi S, Gianinazzi-Pearson V (2001) Signalling between arbuscular mycorrhizal fungi and plants: identification of a gene expressed during early interactions by differential RNA display analysis. Plant Soil 232:13–19

Samartzidou H, Widger AR (1998) Transcriptional and posttranscriptional control of mRNA from *Irta*, a light-repressed transcript in *Synechococcus* sp. PCC 7002. Plant Physiol 117:225–234

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. USA

Sanders IR, Alt M, Groppe K, Boller T, Wiemken A (1995) Identification of ribosomal DNA polymorphisms in spores of the Glomales: application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. New Phytol 130:419–427

Sawaki H, Saito M (2001) Expressed genes in the extraradical hyphae of an arbuscular mycorrhizal fungus, *Glomus intraradices*, in the symbiotic phase. FEMS Microbiol Lett 195:109–113

Simon L (1996) Phylogeny of the Glomales: deciphering the past to understand the present. New Phytol 133:95–101

Simon L, Lalonde M, Bruns TD (1992) Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. Appl Environ Microbiol 58:291–295

Simon L, Bousquet J, Lévesque RC, Lalonde M (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363:67–69

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic, San Diego

Toth R, Miller RM, Jarstfer Ag, Alexander A, Bennet EL (1991) The calculation of intraradical fungal biomass from percent colonisation in vesicular-arbuscular mycorrhizae. Mycologia 83:553–558

Ueda K, Mikata K (1997) Detection of heterogeneity of 18S rRNA inter-genes and mutation arising during PCR amplification. Inst Ferment Res Commun 18:20–25

Van Buuren ML, Maldonado-Mendoza IE, Trieu AT, Blaylock LA, Harrison MJ (1999) Novel genes induced during an arbuscular mycorrhizal (AM) symbiosis between *M. truncatula* and *G. versiforme*. Mol Plant-Microbe Interact 12:171–181

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols, a guide to methods and applications. Academic, San Diego, pp 315–322