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SHORT COMMUNICATIONS

Rapid determination of fungal colonization and arbuscule formation in roots of *Medicago truncatula* using real-time (RT) PCR

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Summary

The quantifications of root colonization and symbiotic activity in the arbuscular mycorrhizal (AM) association of *Medicago truncatula* and *Glomus intraradices* were performed by quantitative polymerase chain reaction (real-time PCR). A strong correlation between fungal colonization of the root system and the amounts of fungal rDNA and rRNA were shown. In contrast, the transcript levels of the AM-specific phosphate transporter 4 from *M. truncatula* (*MtPT4*) correlate with arbuscule formation rather than with fungal colonization. These results suggest (i) that real-time PCR assay is a rapid, useful, and accurate method for the determination of arbuscular mycorrhizal features, (ii) that the amount of fungal rDNA or rRNA is a good parameter to estimate fungal colonization, and (iii) that it is necessary to evaluate the amount of other transcripts—like the *MtPT4* transcript—to obtain additional information about the symbiotic state of the colonized root system.

Introduction

About 80% of all vascular land plants are able to form a mutualistic symbiosis with arbuscular mycorrhizal fungi (Newman and Reddell, 1987). The fungi provide plants with access to mineral nutrients (most notably phosphate) and in return the plants deliver carbohydrates to the fungi. During the complex process of arbuscular mycorrhiza (AM) development, a co-ordinate differentiation of both symbionts is required. In an AM association, fungus and plant coexist in intimate contact as the fungus grows within the cortex of the root. However, morphological identification of

Abbreviations: AM, Arbuscular mycorrhiza; ITS, Internal transcribed spacer; PCR, Polymerase chain reaction; rDNA, Region of DNA encoding ribosomal RNA; RT, Reverse transcription

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AM fungi is only possible for spores but not for symbiotic structures (Morton and Bentivenga, 1994). Therefore, the species composition of active AM populations within roots can only be assessed by molecular methods.

There are two main methods used to quantify the colonization of roots by AM fungi. The first one is based on the quantification of fungal metabolites as sterols or chitin (Frey et al., 1994). The second method relies on the staining of roots followed by microscopical evaluation, but this method is time consuming and labour intensive (McConigle et al., 1990). Recently, the polymerase chain reaction (PCR) became a popular and powerful tool for the identification and quantification of AM fungi. Primer specificity for a certain AM fungus is based on unique sequences from regions of fungal DNA coding for rRNA (rDNA) (Simon et al., 1992). In this way, species-specific primers have been developed for *Glomus intraradices*, *Glomus mosseae* (Lanfranco et al., 1995; Redecker, 2000), and *Scutellospora castanea* (Zeze et al., 1996). Those primers were also used for the quantification of root colonization by conventional PCR (Edwards et al., 1997; Simon et al., 1992). Whereas this method is not ideally suited for quantitative assertions, real-time PCR has made it possible to accurately quantify nucleic acids. Results can be obtained quickly and can be subjected to statistical analysis. In 2004, the first report of real-time PCR application for the quantification of AM fungi in colonized roots was published (Alkan et al., 2004). The authors used the SYBR Green fluorescent dye and PCR primers specific for the *G. intraradices* rDNA for absolute quantification of the AM fungus.

Several investigations (Gianinazzi-Pearson et al., 2000; Harrison et al., 2002; Krajinski et al., 2000, 2002; Lanfranco et al., 1999) focused on discovering and characterizing AM-specific genes in plants. The recently cloned and characterized phosphate transporter 4 from *Medicago truncatula* (*MtPT4*) (Harrison et al., 2002) shows a highly specific expression in roots colonized by AM fungi. Expression of this gene takes place exclusively in cells containing arbuscules. Therefore, quantification of the *MtPT4* transcript using real-time RT PCR could be a useful molecular approach to evaluate the functionality of the mycorrhizal symbiosis.

The aim of this study was to establish a quantitative (RT) real-time PCR protocol, using TaqMan™ probes, which would allow one to quantify the amount of *G. intraradices* and in parallel to estimate the level of arbuscule formation within mycorrhizal roots from *M. truncatula*.

Materials and methods

Plant material and inoculation with arbuscular mycorrhizal fungus

M. truncatula (L.) Gaertn. var. Jemalong was grown in a greenhouse in pots filled with expanded clay (Lecaton, 2–5 mm particle size; Fibo Exclay Deutschland, Pinneberg). Seven-day-old seedlings (5 plants per 10 cm pot) were inoculated with the arbuscular mycorrhizal fungus *G. intraradices* Schenck & Smith by the application of propagates in expanded clay (isolate 49, provided by H. von Alten, University Hanover, Germany). Further details of plant growth conditions have been described previously (Maier et al., 1995). All experiments (three independent measurements each) were done twice. For all individual measurements at least 20 plants were pooled. The percentage of mycorrhizal inoculum was 15% for the first set of experiments and 5%, 15%, and 50% for the second set of experiments, respectively. Roots were harvested 14, 24, 31, 37, 45, 51, and 58 days after inoculation for the first set of experiments and 35 days after inoculation for the second set. The approximate percentage of root length colonization was estimated by evaluating mycorrhizal structures from all roots of five randomly selected plants for each time point after staining with ink according to Vierheilig et al. (1998).

Real-time (RT) PCR

Plant material (100 mg fresh weight) was homogenized in liquid nitrogen. Total root RNA and DNA were extracted using the Plant RNeasy Extraction Kit (Qiagen, Hilden; Germany) and the Plant DNeasy Extraction Kit (Qiagen), respectively. cDNA synthesis was performed using Superscript II™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, USA) starting with 1 μg of total RNA in a total volume of 10 μl with oligo (dT)15 primers and 5′RACE Anchor Primer (Invitrogen) at 42 °C for 60 min.

TaqMan™ probes and primers were designed using the Primer Express™ software (Applied Biosystems, Foster City, USA). Purified PCR primers were purchased from Applied Biosystems (Assays-by-Design (SM) Service) and contained a 6-FAM™ reporter dye connected to the 5′ end, and the non-fluorescent quencher (NFQ) connected to the 3′ end. A fragment from the *MtPT4* transcript was amplified using the primers MTPT4-162F (5′-CATCTTTGCCGGATTGGTTTCAA-3′) and MTPT4-162R (5′-GGCT GTGTTGAAAAATTGGGTCTT-3′). A

fragment from *G. intraradices* rDNA comprising regions from the 5.8S gene and the ITS was amplified using primers GLOM-RNSF (5′-GTAT GCCTGTTTGAGGGTCAGTATT-3′) and GLOM-RNSR (5′-AAACTCCGGAACGTCACTAAA GAG-3′). The primers EF1-KLMF (5′-GCACCAGTGCTCGATTGC-3′) and EF1-KLMR (5′-TCGCCTGTCAATCTTGGTAACAA-3′) were used to amplify a fragment corresponding to the cDNA coding for the elongation factor 1. This set of primers was used previously as a constitutive expression control (Doll et al., 2003). Real-time PCR was carried out using the ABI Prism™ 7000 sequence detection system, optical caps, and optical tubes (Applied Biosystems). PCR amplification mixtures (20 μl) contained 20 ng template cDNA or 50 ng sample DNA, 2 × TaqMan™ Master Mix buffer (10 μl) (Applied Biosystems), and 20 × TaqMan™ probe with primers (1 μl). The cycling conditions were chosen according to the instructions from the manufacturer. They comprised 10 min polymerase activation at 95 °C and 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Each assay was performed in triplicate. Data evaluation was carried out using the ABI Prism sequence detection software. The threshold cycle number (C_t) was determined according to instructions from the manufacturer. The differences in C_t values between EF1 and the experimental amplicons (ΔC_t) were normalized to the lowest ΔC_t value ($\Delta\Delta C_t$). Relative numbers of transcripts or gene copies were calculated using the formula $2^{-\Delta\Delta Ct}$, setting the number of the sample with the lowest ΔC_t-value as 100%.

The specificity of the selected primers was checked by conventional RT-PCR. The PCR reaction contained $\frac{1}{5}$ of the reverse transcription reaction, 0.2 mM 2′-deoxynucleoside 5′-triphosphates (dNTPs), 40 pmol primers, 1 × PCR reaction buffer (10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) and 1 unit of *Taq* polymerase (Invitrogen) in a total volume of 50 μl. The PCR was performed as follows: 95 °C for 2 min, 35 cycles (denaturation at 95 °C for 1 min, annealing at 59 °C for 1 min, elongation at 72 °C for 2 min), and termination at 72 °C for 10 min.

Results and discussion

In the first set of experiments, levels of mycorrhization as well as levels of *G. intraradices* rDNA, rRNA and the transcript of the *M. truncatula* PT4 gene were positively correlated with the time of growth and inoculation of the plants (Fig. 1). In particular, there was a linear relationship between the mycorrhization rates determined by visual observation and the levels of *G. intraradices* rDNA (correlation coefficient $r^2 = 0.59$) and rRNA ($r^2 = 0.76$). As judged by conventional RT-PCR, the amplicons chosen for quantification were produced with the predicted size. They could only be obtained when starting with DNA or cDNA-templates from mycorrhizal plants and not from non-mycorrhizal plants. The only exception from the linear correlation between inoculation time, mycorrhizal colonization, and the levels of the various amplicons was the case of the *M. truncatula* PT4 transcript. The percentage of mycorrhizal colonization and transcript level for *MtPT4* correlated clearly ($r^2 = 0.74$) until day 51. At day 58 the amount of this transcript decreased dramatically in roots showing a very high colonization rate (Fig. 1C) but low amounts of arbuscules (not shown). This effect is much stronger when compared to the small decrease of rRNA levels at day 58, which is rather due to statistical variations (Fig. 1). In this case, the colonization rate measured by microscopical staining or by quantification of fungal rDNA or rRNA did not reflect the actual symbiotic activity. Our second set of experiments provides further evidence regarding this statement (Fig. 2). Regardless of the initial percentage of inoculum, colonization rate and the amounts of fungal rDNA and rRNA reached high values. The transcript levels for the *M. truncatula* PT4 gene, however, did not correlate with these measurements, but clearly correlated with the percentage of initial inoculum (Fig. 2A) and the density of arbuscules within roots (Fig. 2B).

Previous investigations described possibilities to estimate the proportion of plant and fungal RNA by northern blot analysis and ribonuclease assay. According to these results, the amount of fungal rRNA correlated with the level of root mycorrhization (Diaz et al., 1997; Maldonado-Mendosa et al., 2002). According to our experiments the quantification of fungal rRNA and rDNA by real-time PCR shows indeed a linear correlation with the level of mycorrhization (Fig. 1B). The application of rRNA as template seems to be more suitable due to the better correlation with colonization and due to practical reasons. Using PCR primer specific for the various AM fungi as described by Redecker (2000) or Renker et al. (2003), similar experiments could be possible under field conditions.

Moreover, our data clearly show that the level of mycorrhizal colonization—measured by microscopical analysis of stained roots or by quantification of fungal rDNA or rRNA—does not necessarily correspond to the degree of symbiotic activity. At least under strong phosphate deficiency, the *M. trunca-*

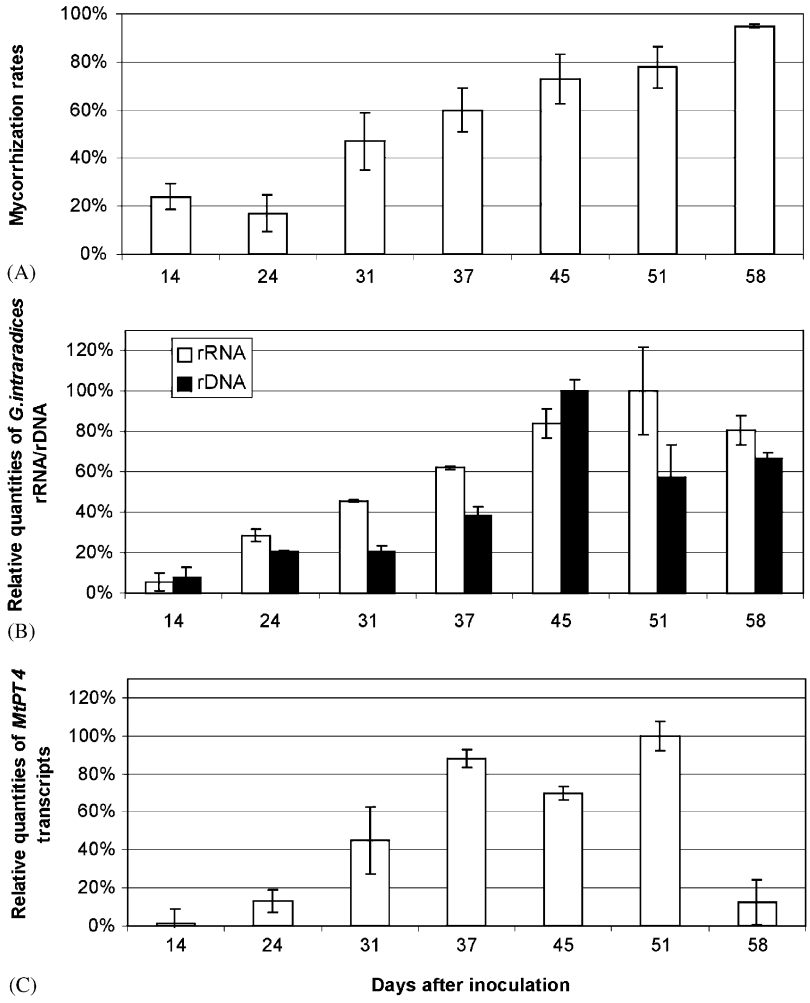

Figure 1. Visual estimation of mycorrhization rate and real-time (RT) PCR analysis of colonized *M. truncatula* roots. Standard deviations were calculated from two independent experiments performed with three replicates each. Roots were inoculated with G. *intraradices* at day 7. The percentage of fungal inoculum was 15%. (A) Percentage of root length colonization of roots harvested at the indicated time points after inoculation. (B) Relative quantities of *G. intraradices* rDNA/rRNA. (C) Relative quantities of *MtPT4* transcripts.

tula PT4 transcript is especially suitable to represent this activity because it is directly linked to one key feature of the mycorrhizal symbiosis, namely the transport of phosphate (Harrison et al., 1997, 2002). In addition, according to electronic northern analysis, it is among the transcripts most strongly induced upon mycorrhizal colonization. In future experiments, the quantification of this transcript under field conditions will allow us to estimate the correlation between external phosphate supply, phosphate transport by AM fungi, and root colonization.

In summary, we conclude that real-time PCR is a rapid, accurate, and quantitative method for the evaluation of AM features. The minimal amount of plant and fungal material required is extremely low. Our primer sets allow direct estimation of the root colonization values (GLOM-RNSF and GLOM-RNSR primers using DNA or cDNA templates) and of transcript levels for the arbuscule-specific phosphate transporter *MtPT4* (MTPT4-162F and MTPT4-162R primers). The expression of this gene represents one functional key feature of the AM symbiosis and provides important information about the symbiotic state of the colonized root system.

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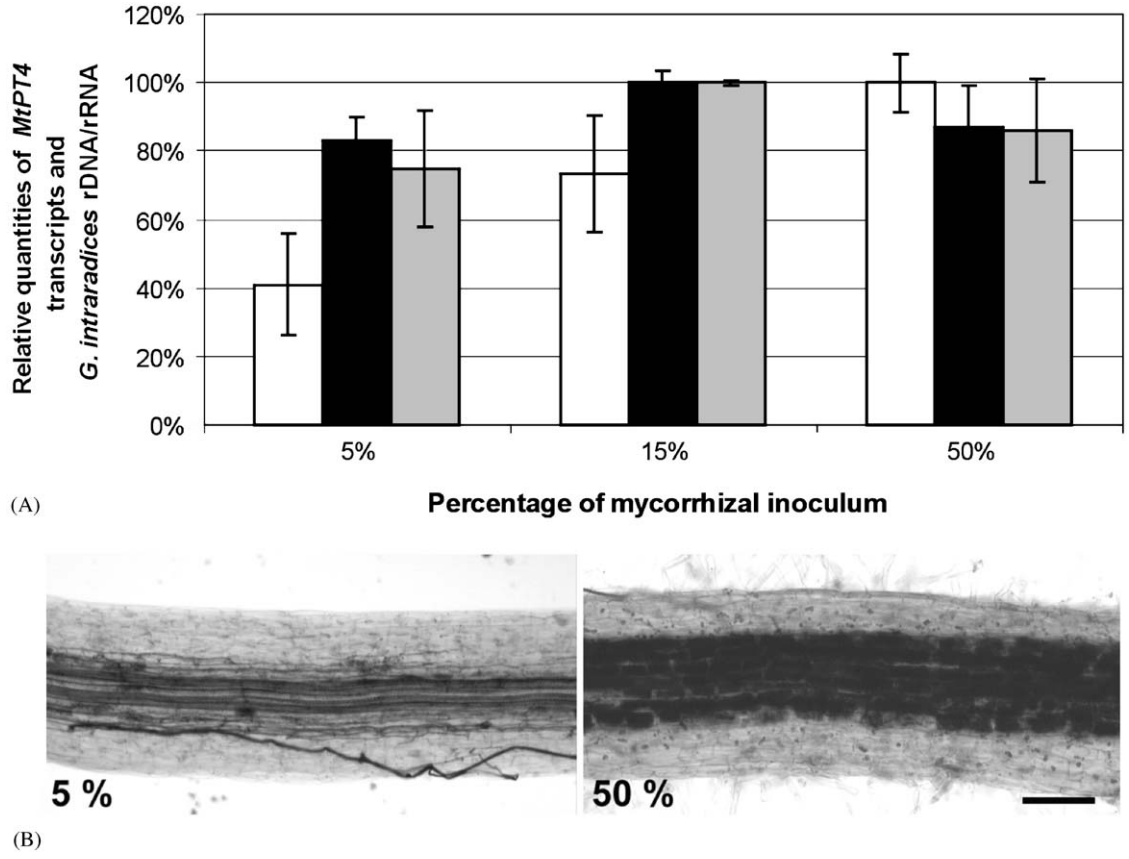

Figure 2. Real-time (RT) PCR analysis and estimation of arbuscule formation in *M. truncatula* roots inoculated with different amounts of fungal inoculum. Standard deviations were calculated from two independent experiments performed with three replicates each. Roots were inoculated with *G. intraradices* at day 7 and harvested 35 days post-inoculation. Percentage of root length colonization was 73%, 74%, and 73%, respectively. (A) Relative quantities of *MtPT4* transcripts (□), *G. intraradices* rDNA (■) and rRNA (▒). (B) Ink stained mycorrhizal roots showing different density of arbuscules after inoculation with different amounts of fungal inoculum. Bar represents 100 μm for both micrographs.

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