Available online at www.sciencedirect.com
ScienceDirect
Fungal Genetics and Biology 45 (2008) 581–596
FUNGAL GENETICS AND BIOLOGY
www.elsevier.com/locate/yfgbi

Technological Advancement

Real-time PCR and microscopy: Are the two methods measuring the same unit of arbuscular mycorrhizal fungal abundance?

Hannes A. Gamper [a,*], J. Peter W. Young [a], David L. Jones [b], Angela Hodge [a]

[a] University of York, Department of Biology, P.O. box 373, Heslington, York YO10 5YW, UK
[b] Bangor University, Environment Centre Wales, Bangor, Gwynedd, LL57 2UW, UK

Received 13 September 2007; accepted 18 September 2007
Available online 25 September 2007

Abstract

To enable quantification of mycelial abundance in mixed-species environments, eight new TaqMan® real-time PCR assays were developed for five arbuscular mycorrhizal fungal (AMF, *Glomeromycota*) taxa. The assays targeted genes encoding 18S rRNA or actin, and were tested on DNA from cloned gene fragments, from spores, mycelia, and from root-free soil, and on reverse-transcribed rRNA templates from entire mycelia and from colonized roots. The assays showed high specificity, sensitivity, and reproducibility, enabling reliable quantitation over broad ranges of template molecules. From cultured mycelia, DNA and RNA measures both correlated with spore number rather than extraradical hyphal length, and epifluorescence microscopy identified pronounced heterogeneity in vitality and nuclear distribution in hyphae. Root colonization was also spatially heterogeneous, as shown by a mixing experiment with root fragments of different length. Therefore, although real-time PCR can reproducibly and accurately quantify AMF nucleic acids, these are poorly correlated with visual measures because of spatial heterogeneity.

Keywords: *Glomus*; *Glomeromycota*; Hyphae; Multi- and low copy genes; Population size; Quantification; Root colonization; *Scutellospora*; Specific primers; Spores

1. Introduction

Arbuscular mycorrhizal fungi (AMF, *Glomeromycota*), a ubiquitous group of obligate biotrophic soil fungi, are associated with the majority of land plants and are involved in mineral nutrition and stress alleviation of their symbiotic plant partners (Smith and Read, 1997). However, they are notoriously difficult to identify and quantify because of a lack of diagnostic features during their intra- and extraradical hyphal growth. Consequently, little is known about their total population size, the extraradical hyphal abundance of individual taxa, interactions with other organisms, and quantitative host plant preferences in their natural environment (Pivato et al., 2007; van der Heijden and Sanders, 2002). Molecular tools are largely still lacking for investigations on these fundamental topics in mycorrhizal ecology, since they have to be specific and quantitative at the same time and technological developments have only recently led to approaches, which combine these two goals.

Traditionally, quantification of AMF has relied on the microscopic assessment of spore (Daniels and Skipper, 1982; Gerdemann, 1963; Oehl et al., 2005) and hyphal (Jakobsen et al., 1992; Sylvia, 1992) densities in soils and of root colonization levels (Giovannetti and Mosse, 1980; Kormanik and McGraw, 1982; McGonigle et al., 1990; Phillips and Hayman, 1970; Plenchette and Morel, 1996; Vierheilig et al., 2005), or on the biochemical determination of AMF indicator fatty acids (Olsson et al., 2003; Olsson, 1999) or chitin (Bethlenfalvay and Ames, 1987; Frey

* Corresponding author. Present address: Netherlands Institute of Ecology (NIOO-KNAW)—Centre for Terrestrial Ecology, Department of Terrestrial Microbial Ecology, Boterhoeksestraat 48, P.O. box 40, 6666 ZG Heteren, The Netherlands. Fax: +31 (0) 264723227.
E-mail address: h.gamper@nioo.knaw.nl (H.A. Gamper).

1087-1845/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2007.09.007

et al., 1994; Schmitz et al., 1991). Although, with expertise and considerable effort, studies on spores can potentially resolve most of the taxa present (Bever et al., 2001), such studies only provide information about the resting phase of the fungus. Microscopy and biochemical analysis can be used to quantify the physiologically and biologically active hyphal growing phases, but they both frequently suffer from a near complete lack of taxonomic resolution and interference from other fungi. Consequently, until now, hyphal abundance of individual taxa in the more active part of the AMF life cycle, represented by mycelia, has only been studied under strictly controlled conditions. Conventional methods of molecular ecology, although taxonomically highly resolving, are only semi-quantitative and when carried out at the end-point of the whole PCR process, often also after nested amplification, can be impaired by PCR biases (Speksnijder et al., 2001; von Wintzingerode et al., 1997; Wagner et al., 1994). Band intensities after electrophoretic separation of PCR amplicons or relative representation of a certain sequence fragment in a clone library of transformed bacteria can both be affected by differences among samples in the degree of PCR inhibition or template competition. Therefore, these traditional genetic quantification techniques can be inaccurate in reflecting the abundance of different organisms in a DNA sample. By overcoming the above shortcomings and enabling identification and quantification of specific organisms from complex mixtures of DNA templates, quantitative real-time PCR (qPCR) technology (Heid et al., 1996; Holland et al., 1991; Monis and Giglio, 2006) is particularly promising for application in AMF molecular ecology, which often deals with complex DNA templates from the environment. It has the potential for simultaneous specific and quantitative investigations on particular taxa of AMF in roots and soils, which are both commonly colonized simultaneously by several taxa. Real-time PCR is already widely used as a routine genetic quantification technique in medicine (Espy et al., 2006; Hodgson et al., 2002), bacterial microbiology (Saleh-Lakha et al., 2005), and fungal pathogen diagnostics (Schena et al., 2004).

Modern qPCR technology employs either non-specific fluorogenic DNA-binding dyes, such as SYBR® Green I, or sequence-specific, fluorogenic hybridization probes, such as TaqMan® or MGB® hydrolysis probes (Klein, 2002). Increase in fluorescence over the course of PCR reflects the number of newly generated DNA amplicons and can be used to estimate DNA contents in samples. There is a negative linear mathematical relationship between the threshold amplification cycle (i.e. the point at which fluorescence rises appreciably above the background fluorescence) and the logarithm of the number of initial DNA template molecules. This relationship exists as a consequence of exponential PCR amplification at least at early stages of the whole reaction, when PCR inhibitors are less important, reagents not limiting, and product reannealing is not yet significant. The DNA content in unknown samples is estimated by interpolating standard calibration curves derived from a dilution series of a reference sample of the target taxon whose DNA content has been quantified by other means. Calibration curves, also known as standard curves, relate the cycle threshold values to the logarithms of the respective initial target DNA abundances in the serial dilutions used to draw them. The main advantages of real-time over traditional endpoint quantitative PCR are its increased accuracy, as a consequence of avoiding high PCR cycle numbers after initial amplification, and reduced handling times, because amplification and detection are combined.

This study aimed at the development of specific qPCR assays that could ultimately be used to quantify specific AMF taxa under environmental conditions. Particular weight was given to a thorough validation of the qPCR approach against established methods of AMF quantification, and an assessment of the influence of spores versus hyphae on the genetic quantification of AMF mycelium. Various, increasingly biologically complex AMF materials from controlled growth conditions were used to compare genetic quantitation with more traditional microscopic quantification.

2. Materials and methods

2.1. Taxonomic reference cultures for assay development

Reference cultures of taxonomically identified AMF isolates were propagated in open pot cultures of *Plantago lanceolata* L. in a 1:1 (v/v) mixture of coarse silica sand and TerraGreen® (an expanded clay soil conditioner, TDS043 GRAN, Oil-Dri Ltd, Wisbech, UK) for appropriate time to obtain newly formed spores from the various species. The inocula of the reference cultures originated from living culture collections (BEG, http://www.kent.ac.uk/bio/beg/; INVAM, http://invam.caf.wvu.edu/), a personal collection of single spore isolates (identifier CH-FACE), or were obtained from Dr. Jan Jansa (identifier ID, Institute of Plant Sciences, Swiss Federal Institute of Technology, Zurich, Switzerland), unless otherwise stated: *Acaulospora delicata* Walker, Pfeiffer & Bloss (ID1094), *A. foveata* Trappe & Janos (CR401), *A. laevis* Gerdemann & Trappe, *A. mellea* Spain & Schenck (ID51), *A. scrobiculata* Trappe [ID288, UY991 (University of York)], *Diversispora* Walker & Schüßler sp. (CH-FACE #83, #234, #272, #410), *Glomus caledonium* (Nicol. & Gerd.) Trappe & Gerd. (BEG20, CH-FACE #526, ID658), *G. claroideum* Schenk & Smith (BEG14, BEG23, BEG31, CH-FACE #52, #282, #412, UK210B), *G. clarum* Nicol. & Schenck (BEG142), *G. coronatum* Giovannetti (BEG22), *G. diaphanum* Morton & Walker (CH-FACE #107, #589), *G. eburneum* Kenn., Stutz & Morton (AZ420A), *G. geosporum* (Nicol. & Gerd.) Walker (BEG11), *G. hoi* Berch & Trappe [BEG48, UY110 (University of York)], *G. intraradices* Schenk & Smith [BB-E (Biorize, Dijon, France), BEG75, BEG144, CH-FACE #35LlNiv, 128, #488], *G. lamellosum* Dalpé, Koske & Tews (ID1096), *G. mosseae* (Nicol. &