Methods

Expression of the fluorescence markers DsRed and GFP fused to a nuclear localization signal in the arbuscular mycorrhizal fungus *Glomus intraradices*

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Summary

• Here, arbuscular mycorrhizal (AM) fungi were monitored *in vivo* introducing the fluorescent reporters DsRed and GFP (green fluorescent protein) in *Glomus intraradices* using a biolistic approach and *Agrobacterium tumefaciens*-mediated transformation.

• Both reporter genes were fused to the nuclear localization signal of the *Aspergillus nidulans* transcription factor *StuA* to target fluorescence to nuclei. Expression of DsRed was driven by two *Glomus mosseae* promoters highly expressed during early symbiosis, *GmPMA1* and *GmFOX2*, while expression of GFP was driven by the *A. nidulans gpd* promoter.

• All promoters worked in *G. intraradices* as well as in *A. nidulans*. Red and green fluorescence was localized to nuclei of *G. intraradices* spores and hyphae 3 d after bombardment. However, expression was transient. The efficiency of the *Agrobacterium*-mediated transformation was very low.

• These results indicate that the biolistic method allows the expression of foreign DNA into *G. intraradices* with high frequency, but it is insufficient to render stable transformants. DsRed vs GFP is a more appropriate living reporter to be used in *G. intraradices* because of the lower autofluorescence in the red channel but targeted to the nucleus both marker genes can be visualized. This is the first report of fluorescent marker expression in an AM fungus driven by arbuscular mycorrhizal promoters.

Key words: *Agrobacterium tumefaciens*, bombardment, DsRed, green fluorescent protein (GFP), *Glomus intraradices*, *Glomus mosseae*, mycorrhizal fungi, transformation.

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Introduction

Transformation is an essential tool for modern genetic research that leads to the identification of genes and their function by random or targeted integration of DNA into the genome. It also permits the expression of reporter genes that allow the monitoring of growth and development of a given organism when interacting with its host. This is particularly important in the case of pathogenic and mutualistic fungi because those noninvasive technologies allow time-lapse recording of the interaction and offer a very dynamic view of complex interactions such as, for example, the arbuscular

mycorrhizal (AM) symbiosis. Although several transformation systems are available for filamentous fungi not all are suitable for a given organism (e.g. polyethylene glycol (PEG)/$CaCl_2$-mediated transformation of protoplasts or electroporation). Meanwhile different techniques originally developed for plant transformation are used successfully for filamentous fungi such as *Agrobacterium tumefaciens*-mediated transformation (ATMT) or the biolistic transformation, which represent alternatives to conventional transformation methods. The biolistic transformation, or particle bombardment, was first developed for plant cells and tissues by Sanford *et al.* (1987) and uses helium pressure to introduce DNA-coated microparticles with high velocity into target cells. Because of the easy handling this technique is widely used for transient and stable transformation approaches for such diverse organisms as bacteria (Shark *et al.*, 1991), fungi (Armaleo *et al.*, 1990; Lorito *et al.*, 1993; Fungaro *et al.*, 1995; Herzog *et al.*, 1996) and plant (Klein *et al.*, 1988) or animal cells (Johnston, 1990). However, the transformation frequency is affected by a large number of variable parameters such as particle size, bombardment pressure and targeted cell type, which have to be optimized for each organism (Heiser, 1992).

The use of *A. tumefaciens* as a vector to introduce DNA into fungi was first tested by Bundock *et al.* (1995) in *Saccharomyces cerevisiae*. They showed that these bacteria, which are known to induce tumours in plants by delivering T-DNA from the Ti plasmid to the plant nucleus and are widely used in modern molecular biology to transform plants, were also able to deliver the T-DNA in a similar manner to different eukaryotic cells, including yeast cells, several filamentous fungi and mammalian cells (Bundock *et al.*, 1995; de Groot *et al.*, 1998; Kunik *et al.*, 2001). This discovery opened new avenues for the transformation of fungi that have been shown so far to be resistant to transformation or not cultivable (Grimaldi *et al.*, 2005).

The AM symbiosis is formed by fungi from the phylum Glomeromycota and roots of most land plants (Schüßler *et al.*, 2001), thereby representing the evolutionary oldest and most significant plant symbiosis (Smith & Read, 1997; Redecker *et al.*, 2000). In contrast to the plant partner, AM fungi are obligate symbionts, which limits the cultivation of these fungi to the presence of an intact plant or root organ cultures. The only plant-independent phase of AM fungi is the spore. After the so-called intraradical colonization of the root cortex, an extraradical mycelium, supplied with nutrients from its nursing root, develops multiple chlamydospores at its end. These large spores contain thousands of nuclei (Bécard & Pfeffer, 1993) and enough energy to germinate many times in search for a compatible root (Mosse, 1988; Logi *et al.*, 1998). Arbuscular mycorrhizal fungi are thought to be asexual and therefore no classical genetic crossings can be performed. Hence, despite the significant role that AM fungi play in the maintenance of the terrestrial ecosystem (Smith & Read, 1997) they have not been amenable to detailed genetic studies. Molecular genetics analyses performed with the help of polymerase chain reaction (PCR) methods have revealed such a high level of polymorphism of the rRNA genes and for some coding genes within a single spore that it is difficult to reconcile the idea of concerted evolution and multinucleated spores in the absence of recombination. This has originated the yet unresolved question of whether the genetic variability lies within a single nucleus or it is caused by heterokaryosis (for review see Pawlowska, 2005). Transformation techniques might help to elucidate this and other important questions of the biology of AM fungi. However, only one successful event of transient transformation of an AM fungus carried out by particle bombardment has been previously shown (Forbes *et al.*, 1998) and not only AM fungi seem to be recalcitrant to transformation. Thus, only one report exists for transient transformation of an ectomycorrhizal ascomycete (*Tuber borchii*, obtained through *A. tumefaciens*-mediated transformation (ATMT); Grimaldi *et al.*, 2005) and although protoplast transformation of some ectomycorrhizal fungi was long ago shown to be possible (Barret *et al.*, 1990; Marmeisse *et al.*, 1992), the low efficiency of this and other methods has made the use of ATMT the technique of choice in the last years (Pardo *et al.*, 2002; Combier *et al.*, 2003; Martino *et al.*, 2007).

We are convinced that the use of novel molecular methodologies will allow the establishment of appropriate protocols to tag and to inactivate particular AM fungal genes and investigate their role in the symbiosis. In this work we present our results concerning the use of two fluorescent markers DsRed and green fluorescent protein (GFP) fused to a nuclear localization signal to tag nuclei in the AM fungus *Glomus intraradices*. This is the first report where living reporter genes driven by AM promoters as well as by an *Aspergillus nidulans* promoter are expressed in an AM fungus. We believe this work will help to develop methodologies for *in vivo* monitoring of AM fungi during symbiotic development.

Materials and Methods

Biological material

The AM fungus *G. intraradices* (Schenk and Smith) (DAOM 181602) was cultivated in the *in vitro* system described by Bécard & Fortin (1988) with *Agrobacterium rhizogenes* transformed carrot roots in bicompartmental Petri plates as described by St-Arnaud *et al.* (1996). Spores from 4- wk-old cultures were cold stratified for 2 wk and then collected by a sieving system with a decreasing mesh width of 1 mm, 125 μm and 40 μm. To remove old hyphae and carrot roots spores were treated several times with a stirring appliance. Spores were surface sterilized by overnight incubation in antibiotic solution (100 μg ml^{-1} gentamycin and 200 μg ml^{-1} streptomycin) and additional 10 min in 2% ChloraminT solution (C-9887; Sigma-Aldrich, Taufkirchen, Germany). Extraradical mycelium for ATMT was grown in modified

liquid M media (Bécard & Fortin, 1988). For this, the solid medium of the distal compartment of 2-wk-old cultures was replaced with liquid M media (8 ml) (Bécard & Fortin, 1988) and the plates were incubated at 27°C for 10 d. To facilitate the growth of the hyphae a small grid was scratched on the bottom the plate with a sterile scalpel.

Agrobacterium tumefaciens strain AGL1 (genotype AGL0 recA::bla pTiBo542deltaT Mop+ CbR) (Lazo *et al.* 1991) was grown in MYA medium (yeast extract 4 g l^{-1}, NaCl 5 g l^{-1}, mannitol 8 g l^{-1}, ammonium sulphate 2 g l^{-1}, casamino acids 0.5 g l^{-1}, Agar-Agar 20 g l^{-1}) containing 100 mg l^{-1} ampicillin. Binary vectors were introduced in this strain by electroporation and then selected in MYA medium containing 100 mg l^{-1} kanamycin.

Plasmid construction and functional expression in *A. nidulans*

In order to transform spores and extraradical mycelium of *G. intraradices* by *A. tumefaciens*, we constructed a binary vector containing a promoter-reporter cassette using the backbone of the binary vector pPK2 (Covert *et al.*, 2001). A 2.2 kb *Bam*HI (blunt)–*Kpn*I fragment containing the GFP gene fused to the *stuA* nuclear localization gene under the control of the *A. nidulans gpd* promoter, isolated from the vector pRS31 (Suelmann *et al.*, 1997). This fragment was inserted in the *EcoR*V–*Kpn*I sites of the pPK2 vector to render the vector pNRGFP.

For biolistic transformation promoter–reporter cassettes were constructed using two promoter sequences, *GmPMA1* and *GmFOX2*, from the AM fungus *G. mosseae*. *GmFOX2*, encodes a highly conserved multifunctional protein for peroxisomal β-oxidation (Requena *et al.*, 1999), while *GmPMA1* encodes a plasma membrane H^+-ATPase (Requena *et al.*, 2003). Both genes are highly expressed during the presymbiotic growth of the fungus.

The *GmPMA1* promoter sequence was obtained by PCR on genomic DNA using the primers PROHF1 5′-GAATTCGGATCTGAATTTGAGG-3′ and PROHR1 5′-GAAGGTCTGTGCACTATGGG-3′ that amplify an 850 bp fragment 173 bp upstream of the ATG. This fragment was cloned into the vector pCR2.1-TOPO (Invitrogen, Karlsruhe, Germany) and from there excised as a *Kpn*I–*Xba*I fragment and cloned into the same sites in pNRGFP, to create the vector pNHATP-GFP. The cassette DsRed-stuA was PCR amplified from the vector pJH19 (Toews *et al.*, 2004) using the primers dsRED-for 5′-GAGGATCCCCCGGGCTGCAGGAAT-3′ and dsREDstuA-rev 5′-GCCCCCCCTCGAGTCATTGCTGCA-3′ and subcloned into the vector pCR2.1-TOPO (Invitrogen). From there it was excised with *Bam*HI and cloned into the *Bam*HI site of pNHATP-GFP to render the vector pNHATPDsredstuA.

The promoter of *GmFOX2* was amplified from genomic DNA using the primers PROXF1 5′-GGAAGAGGAATTTGATGATGG-3′ and PROXR1 5′-GTCCTCAACACAACGTTTCAACG-3′ that amplify a 1kb fragment 250 bp upstream of the ATG. To create the vector pNRPK2PFOX, this promoter was cloned into pPK2 (Covert *et al.*, 2001) as a *Kpn*I–*EcoR*V fragment after subcloning into pCR2.1-TOPO (Invitrogen). A *Bam*HI fragment containing the DsRed-stuA cassette (see earlier) was inserted into pNRPK2PFOX to create the vector pNHFOXdsREDstuA.

Start of transcription in both promoter regions was determined using the computer program NEURAL NETWORK PROMOTER PREDICTION (http://www.fruitfly.org/seq_tools/promoter.html).

All three binary vectors were introduced into the GR5 strain of *A. nidulans* (pyrG89, wA3, pyroA4) (Waring *et al.*, 1989) by PEG/$CaCl_2$-mediated transformation as described by Yelton *et al.* (1984). GR5 was cotransformed with the plasmid pAB4-1 1 (van Hartingsvelt *et al.*, 1987) containing the selection marker *pyrG* from *Aspergillus niger* (Wilson *et al.*, 1988). Transformants were selected in MM (Hill & Käfer, 2001) containing pyridoxine and then further screened for GFP or DsRed fluorescence under the fluorescence microscope.

A. tumefaciens-mediated transformation

Agrobacterium tumefaciens-mediated transformation was performed basically as described by Bundock *et al.* (1995) with the following modifications. The AGL1 strain, transformed with the different binary vectors constructed in this work, was induced with 200 μM acetosyringone (Sigma-Aldrich) for 6 h before cocultivation with spores or extraradical hyphae of *G. intraradices*. Surface-sterilized spores were used either directly or after germination for transformation experiments. About 3000 spores were inoculated with 200 μl induced AGL1 on solid IM plates for 2–5 d at 22°C. Alternatively, spores were coincubated with 2 ml of induced agrobacteria in a rotary shaker for 2–5 d at 22°C. After that time, spores were collected washed and surface sterilized as above but adding 400 mg l^{-1} augmentin (Hexal) to the antibiotic solution to kill the agrobacteria. Spores were then plated on water-agar containing 50 mg l^{-1} augmentin and analysed for red fluorescence periodically after 2 d.

For the ATMT of extraradical mycelium, liquid M medium was removed after 10 d and 1 ml of induced AGL1 transformed with the plasmid pNRGFP was applied to the hyphal compartment. Cocultivation was carried out for 2 d at 27°C and after that the mycelium was washed three times with M medium and then incubated 24 h in M medium with the addition of 300 mg l^{-1} of Cefoxitin (InfectoPharm, Heppenheim, Germany). As negative control extraradical mycelium was cultivated in IM without agrobacteria. After that time, hyphae were excised and observed under the fluorescence microscope.

Biolistic transformation

Surface-sterilized *G. intraradices* spores were placed on 3 cm^2 cellophane pieces on water agar plates and incubated at 27°C.

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Table 1 Parameters used for biolistic transformation of *Glomus intraradices* spores

Parameters	Tested options
Genegun II (Finer *et al*., 1992)	
Chamber vacuum	685.8 mmHg (91.4 Kpa)
Material and size of microparticles	Gold, 0.6 μm
Amount of DNA per shot	1, 3, 5 μg circular
Pressure of helium	22 psi (1.5 bar)
	44 psi (3.0 bar)
	58 psi (4.0 bar)
	87 psi (6.0 bar)
Target distance	3, 6, 8 cm
Gengun I (Bio-Rad PDS-1100/He)	
Chamber vacuum	685.8 mmHg (91.4 Kpa)
Material and size of microparticles	Gold, 0.6 μm
Amount of DNA per shot	1, 3, 5 μg circular
Pressure of helium	900 psi (62 bar)
	1100 psi (90 bar)
	2000 psi (138 bar)
Target distance	3, 6 cm

Spores were used for bombardment either directly or after germination (3 d). Biolistic transformation was performed with two different gene delivery systems. Genegun I corresponded to the PDS/1000He system from Bio-Rad (Hercules, CA, USA). Genegun II was constructed according to Finer *et al.* (1992). Gengun I was used with 900, 1100 or 2000 psi (approx. 62, 76 and 138 bar, respectively) rupture disks, microcarriers and stopping screens from Bio-Rad. For Genegun II reusable plastic macrocarriers (13 mm Plastic Swinney Filter Holder; PALL Gelman Laboratory) and nylon meshes (PA-450/45 Nybolt 1020 mm; PALL Gelman Laboratory, Portsmouth, UK) for scattering of the gold particles were used. Gold particles (0.6 μm; Bio-Rad) were prepared and coated with DNA as followed: 60 mg gold particles were washed with 1 ml 100% ethanol, centrifuged for 1 s at 1400 ***g*** and resuspended in 500 μl 50% glycerol. For one bombardment 12.5 μl gold particles, 1–5 μg of circular plasmid DNA, 12.5 μl 2.5 M $CaCl_2$ and 5 μl 0.1 M spermidine-free base (Sigma-Aldrich) were mixed together. The suspension was vortexed briefly after addition of each component to the gold particles. Finally the DNA-covered gold particles were washed with 120 μl 100% ethanol, resuspended in 37.5 μl 100% ethanol and loaded in 10-μl steps on the macrocarrier. Several bombardment parameters, summarized in Table 1, were tested to optimize conditions for DNA delivery, including different shooting pressures, DNA concentrations and target distance. Spores were bombarded directly on water-agar plates and then further incubated at 27°C incubator. Control plates were bombarded with uncovered gold particles (without plasmid DNA). Spores were checked to contain gold particles within the spores by using a three-dimensional reconstruction of the spores in the microscope using the Z-stack/apotome system.

Fluorescence microscopy

Fluorescence microscopy was performed with an AxioImager Z1 fluorescence microscope (Carl Zeiss, Göttingen, Germany) and filter sets 38HE for GFP and 43HE for DsRed detection. Screening of transformed spores was achieved by removing small samples from the agar plates in 1-d intervals. Extraradical mycelium was also prepared on objective slides for fluorescence analysis. Images were captured using an AxioCam MRm camera and the AxioVision Release 4.2 software (Carl Zeiss).

Results

Autofluorescence of spores and hyphae of *G. intraradices*

Spores and extraradical hyphae of *G. intraradices* showed a very high autofluorescence when exposed to blue light at 470/40 excitation (Filter 38HE; Zeiss) for GFP detection. The older the hyphae, the greater was the autofluorescence. We also noted that the intactness of the cytoplasm and the cytoplasmic streaming influenced the degree of autofluorescence. Thus, empty hyphae showed no autofluorescence in the empty compartment while cell walls were still very fluorescent. By contrast, hyphae in the process of dying often showed very fluorescent accumulations in the cytoplasm. Exposure of *G. intraradices* to green light 550/25 excitation (Filter 43HE; Zeiss) for DsRed detection showed much less autofluorescence and this was mainly, if not exclusively, reduced to the cell wall of the spores. Nevertheless, hyphae that were dying also had red fluorescent accumulations in their cytoplasm (Fig. 1).

Plasmid construction and functional expression in *A. nidulans*

To facilitate the screening and to avoid diffuse fluorescent signals, we fused the reporter genes DsRed and GFP to the nuclear localization signal of the transcription factor StuA from *A. nidulans*. The fluorescent signal should be then targeted to nuclei. DsRed expression was driven by two AM promoters from *G. mosseae* highly expressed during presymbiosis (Requena *et al.*, 1999, 2003). The GFP expression was driven by the *A. nidulans gpd* promoter. To test whether the constructs were functional and the signal exclusively localized to nuclei, we transformed them in the *A. nidulans* strain GR5. As expected, transformants expressing GFP under the control of the *gpd* promoter (constructs pNRGFP and pNHATPDsRedstuA) showed fluorescent green signal localized to nuclei (Fig. 2a,c). Interestingly *G. mosseae* promoters were also functional in *A. nidulans* and expressed the DsRed-stuA protein that was properly localized to the nuclei in both cases (constructs pNHFOXDsRedstuA and pNHATPDsRedstuA) (Fig. 2b,c).

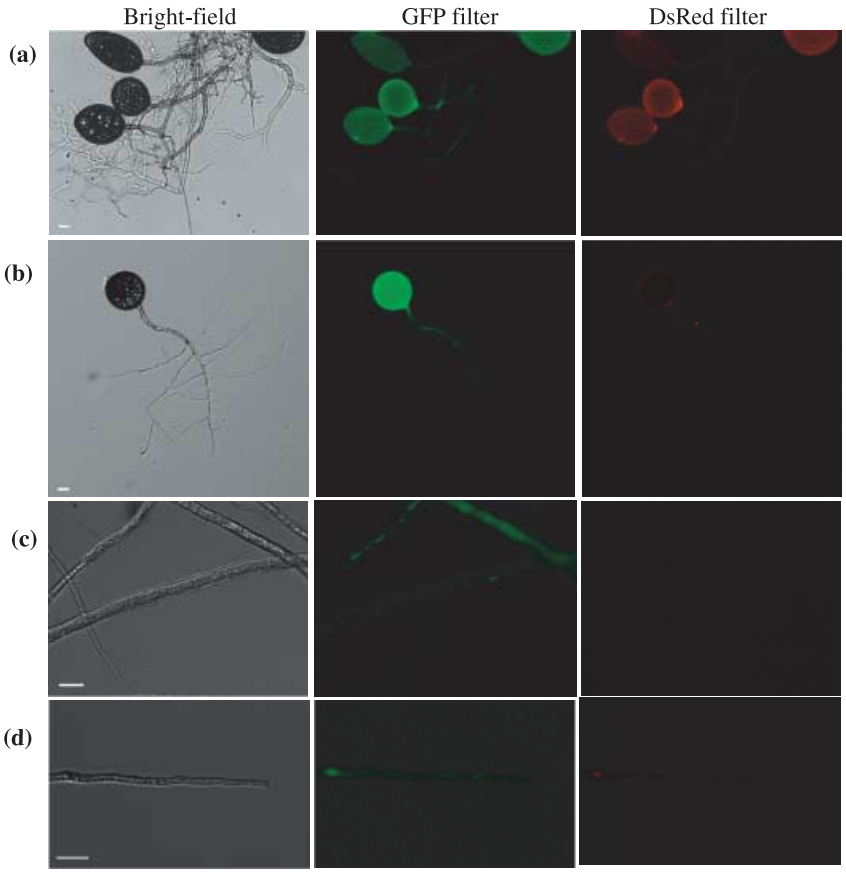

Fig. 1 Autofluorescence of *Glomus intraradices* spores and hyphae under green and red light excitation. (a,b) Bright-field and fluorescence microscopic images of untransformed (a) ungerminated or (b) germinated spores of *G. intraradices* showing strong autofluorescence with filters for green fluorescent protein (GFP) and DsRed. (c,d) Bright-field and fluorescence microscopic images of untransformed *G. intraradices* hyphae. Autofluorescence in the DsRed channel is almost absent in (c) old as well as in (d) young hyphae. Bars, 20 μm.

Agrobacterium-mediated *G. intraradices* transformation

Glomus intraradices spores cocultivated with *A. tumefaciens* AGL1 containing each of the three plasmids from this study showed no signs of specific fluorescence when exposed to either blue or green light. Several parameters were changed to try to improve the *A. tumefaciens* performance, including time of cocultivation, temperature and amount of acetosyringone, without success. By contrast, *A. tumefaciens* was able to transform extraradical hyphae of *G. intraradices*, albeit not very efficiently (1 event every 10 times). Hyphae that had been cocultivated for 2 d with the *A. tumefaciens* strain AGL1 carrying the construct pNRGFP were detached from their nursing roots in the bicompartmental system and analysed under the fluorescent microscope. Only hyphae with active cytoplasm streaming were taken into account. As described earlier, *G. intraradices* extraradical hyphae show an extremely high degree of autofluorescence, which made the analysis extremely time-consuming. Nevertheless, it could be observed that among the high green fluorescent background a few hyphae had GFP labelling in elongated cellular compartments (data not shown) that resembled some of the elongated nuclei often found in 4,6-diamidino-2-phenylindole (DAPI)-stained hyphae (own pers. ob.; Bago *et al.*, 1998).

Biolistic transformation of *G. intraradices*

Given the high level of autofluorescence observed in the green channel we decided to work only with constructs containing DsRed as reporter gene, alone or in combination with GFP. After bombardment, spores were returned to the incubator and examined periodically for red fluorescence under the microscope. Specific red fluorescence could mainly be observed 3 or 4 d after shooting. After longer incubation times fluorescence was no longer visible (Table 2). Red fluorescence was always localized to small dots within the spores that we presume correspond to nuclei (Fig. 3a,b). However, a colocalization using DAPI or Hoechst dye (Invitrogen) as marker for DNA was not possible without crashing the spores, as both stains do not penetrate intact spores of *G. intraradices* unless incubated overnight and then there is no longer red or green fluorescence. However, overnight DAPI staining of untransformed spores showed a size and distribution of nuclei similar to the red fluorescence observed after transformation (Fig. 3c). Measurements of the nuclear diameter in transformed spores and in DAPI spores showed the same average size (1.7 μm).

Pressure did not play a decisive role in the ability of gold particles to penetrate the spores, as different pressures (44 psi (3.0 bar), 900 psi and 1100 psi) were able to generate transformed spores (Figs 3a and 4a,b). Helium pressures of

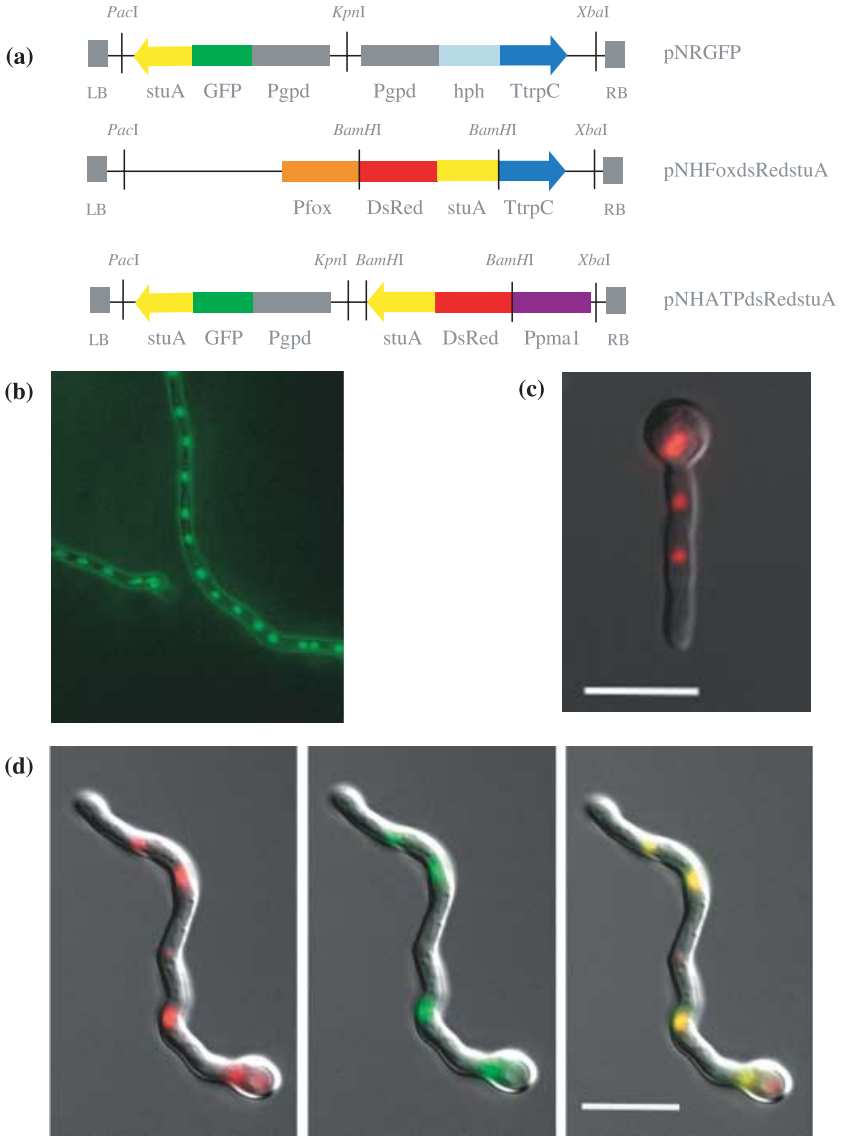

Fig. 2 Schematic representation of binary vectors used in this study and expression of these constructs in *Aspergillus nidulans* strain GR5. (a) T-DNAs of transformation vectors showing relative position of promoter sequences, reporter genes and the relevant restriction enzyme sites. All plasmids were constructed using the binary vector pPK2 as a backbone (Covert *et al*., 2001). (b) Localization of green fluorescent protein (GFP) in nuclei of *A. nidulans* hyphae after transformation with the binary vector pNRGFP. (c) Merged bright-field and red channel image of an *A. nidulans* germling transformed with pNHFoxDsredstuA. DsRed is visible in the nuclei indicating activity of *GmFOX2* promoter in *A. nidulans*. (d) Germling of *A. nidulans* transformed with pNHATPDsredstuA showing reporter gene localization in nuclei. DsRed expression is driven by the *Glomus mosseae GmPMA1* (red nuclei), while GFP expression (green nuclei) is driven by the *gpd* promoter of *A. nidulans*. Merged images of DsRed and GFP results in yellow nuclei. Bars, 10 μm.

2000 psi damaged the spores. Better results were obtained when target tissue was located 6 cm away of the gun rather than at 3 cm (data not shown). The different DNA concentration tested 1, 3 and 5 μg did not show major differences regarding transformation efficiency but it did the construct transformed. Thus, an average of 8300 transformed spores per 10^7 spores was obtained when using both germinated and ungerminated spores as target and the plasmid pNHFOXDsRedstuA. However, an average of 1700 transformed spores per 10^7 spores was obtained when using the plasmid pNHATPdsRedstuA. Although more transformants were obtained when using constructs with the *GmFOX2* promoter, the red fluorescence driven by the *GmPMA1* promoter was stronger and needed a shorter exposure time for visualization, which resulted in a better signal to noise ratio (Fig. 4a). The use of the pNHATPdsRedstuA construct also drives expression of GFP under the control of the *gpd* promoter and allows GFP localization in the cell nucleus owing to the StuA localization signal. Observations under the green and red light showed that all nuclei labelled with DsRed were also labelled with GFP (Fig. 4b). After the careful crushing and DAPI staining of transformed spores it could be clearly shown that those fluorescent spots corresponded to nuclei (Fig. 4c). This was even clearer in hyphae growing out of transformed spores, where transformed nuclei were labelled with both DsRed and GFP, and could be identified easily stained with DAPI (Fig. 5).

Discussion

The symbiosis between plant roots and AM fungi is a paradigm for mutualistic interactions between plant and fungi. Many aspects concerning this symbiosis have been elucidated in the

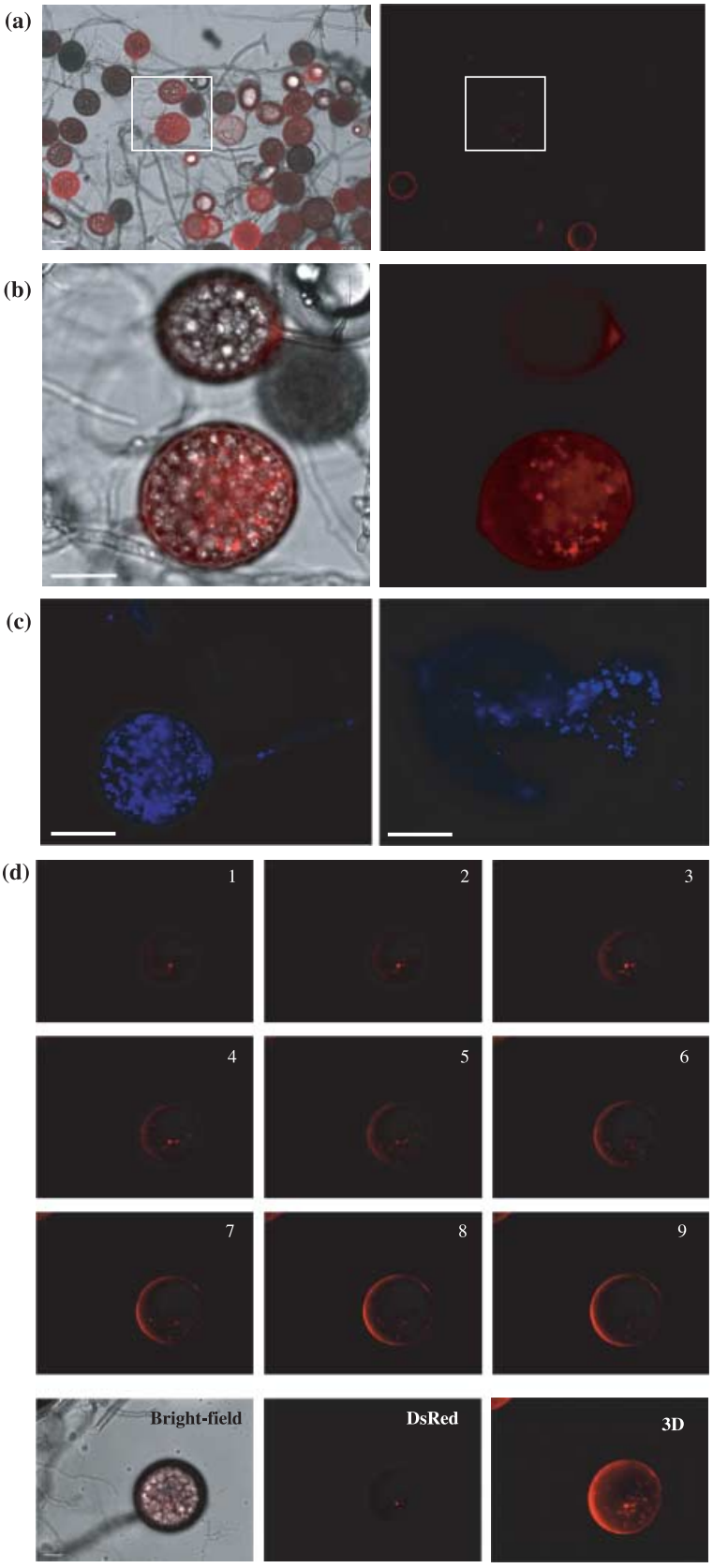

Fig. 3 *Glomus intraradices* spores expressing DsRed under the control of the *GmFOX2* promoter after transformation with pNHFoxDsredstuA by particle bombardment. (a) Merged brightfield-DsRed and DsRed images showing ungerminated spores bombarded on water-agar plates with a pressure of 44 psi (3.0 bar). DsRed specific fluorescence was localized in small fluorescent dots within spores, possibly nuclei. Different levels of autofluorescence could be also observed among spores of the same sample. (b) Magnification of insets in (a) showing a transformed spore with typical fluorescent dots after transformation. Note the absence of fluorescence dots in the untransformed spore located above. (c) Two untransformed spores stained overnight with DAPI. Single nuclei diameter were measured and the average size obtained was 1.7 μm (d) Three-dimensional reconstruction of a *G. intraradices* transformed spore at 44 psi using the Z-stack/apotome system showing homogeneously distributed red dots. Images from 1 to 9 are different planes located at 2-μm intervals. The three-dimensional reconstruction of all planes is shown below together with a two-dimensional picture of the same spore. Bars, 20 μm.

Table 2 Transformation efficiency of *Glomus intraradices* spores transformed by particle bombardment with pNHATPDsRedstuA

Pressure	Time after bombardment (d)	Number of spores	Number of transformed spores	Average number of transformants per 10^7 spores
900 psi (62 bar)	2	15 132	1	811 ± 212
		10 404	1	
	3	9 108	3	3105 ± 268
		10 292	3	
	4	7 331	1	1092 ± 386
		12 216	1	
1100 psi (76 bar)	2	12 815	3	2725 ± 514
		8 064	2	
		7 692	2	
		5 749	2	
	3	11 981	4	2230 ± 790
		8 892	2	
		4 559	1	
		7 056	1	
	4	12 652	1	790 ± nd
	5	11 089	1	902 ± nd

nd, Not determined.

last decade with the development of molecular and genetic approaches. In particular, the cloning of plant genes whose mutation impairs mycorrhiza formation has provided a very detailed map of the signalling pathways guiding the process (for reviewed see Oldroyd & Downie, 2006). An equivalent advance is now required at the fungal side to obtain a complete picture of the symbiosis. However, the resistance of AM fungi to classical genetic methods such as mutations and recombination hinders this advance. A transformation system is therefore essential in order to inactivate fungal genes identified by transcriptional profiling methods suspect of playing major roles during mycorrhization and to monitor protein subcellular localization. The first attempt of AM fungal transformation was carried out in the laboratory of Harrier (Forbes *et al.*, 1998). They showed that AM spores of *Gigaspora rosea* expressed transiently the β-glucuronidase gene (GUS) under the control of the *A. nidulans* promoter *gpd*. They obtained a 40–50% of transformation success when spores were bombarded with gold particles at *c.* 1500 psi (103.5 bar). GUS is a very useful marker that is used regularly in many organisms because of its easy handling and visualization (Jefferson, 1989; Roberts *et al.*, 1989; Schmitz *et al.*, 1990; Thrane *et al.*, 1995). However, to determine GUS activity the material has to be sacrificed and cannot be used for *in vivo* approaches. We wanted to go one step further and test the convenience of living reporters such as GFP or DsRed to monitor expression in living cells of AM fungi. We decided to use the model fungus *G. intraradices* because of its easiness of cultivation under *in vitro* conditions with *A. rhizogenes*-transformed carrot roots (Bécard & Fortin, 1988). Very few AM fungi have been shown to be suitable for cultivation in this system and *G. intraradices* grows very vigorously and sporulates profusely. This fact, together with its small genome size (Hijri & Sanders, 2004), has made *G. intraradices* a model for molecular studies in AM fungi and resulted in its choice for genome sequencing by the DOE Joint Genome Institute (http://www.jgi.doe.gov/).

We first analysed the autofluorescence of *G. intraradices* when exposed to fluorescent light. It was already known that some AM fungi show extreme autofluorescence when colonizing root plants (Ames *et al.*, 1982; Gange *et al.*, 1999; Vierheilig *et al.*, 2001; Dreyer *et al.*, 2006) what for some purposes is an ideal circumstance (Genre *et al.*, 2005). However, under asymbiotic conditions differences in fluorescence are reported for different AM fungi, possibly owing to different composition of the cell wall (Dreyer *et al.*, 2006). We observed that autofluorescence in the green channel is in general much higher than in the red channel and therefore screening of a specific GFP signal is much more tedious. However, care must be taken in all cases regarding the physiological status of the fungus and a thorough light microscopic analysis is required for each piece of mycelium analysed.

It has been reported several times that heterologous promoters often do fail to drive expression of reporter genes in phylogenetically different fungi (Casselton & de la Fuente Herce, 1989). Given that very few promoter sequences from AM fungi exist in the public databases, and to our knowledge none from *G. intraradices*, we chose to use two promoter sequences isolated in our laboratory from the AM fungus *G. mosseae* as well as the *gpd* promoter sequence from *A. nidulans* often used to express proteins in many different fungi. The AM promoters used in this work belong to two genes whose expression is very high during the presymbiotic growth (Requena *et al.*, 1999, 2003). Both genes are phylogenetically

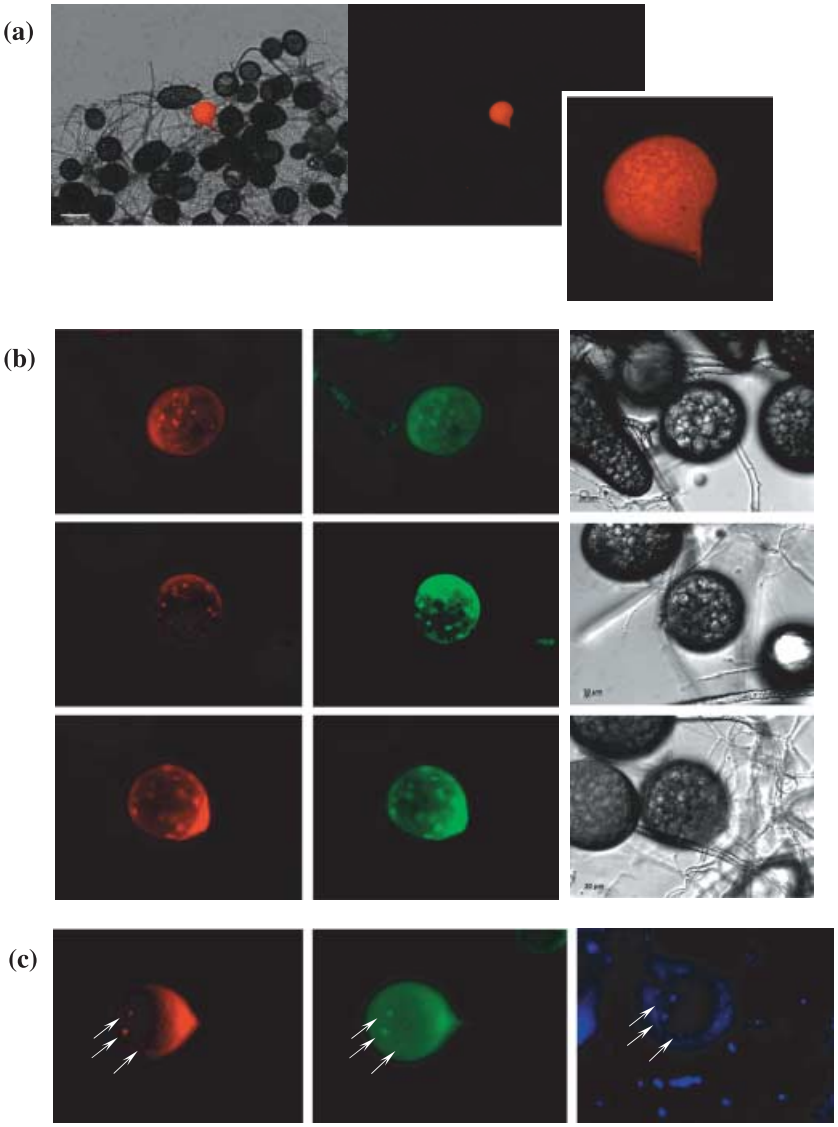

Fig. 4 *Glomus intraradices* spores transformed with the pNHATPDsredstuA construct expressing DsRed under the control of the AM promoter *GmPMA1*. (a) Spores were bombarded at 900 psi (62 bar). Note the strong DsRed fluorescence of transformed spores that allows very good discrimination of transformed vs untransformed spores. Inset shows magnification of the transformed spore displaying DsRed fluorescence located to discrete dots. (b) Three example of transformed spores (1100 psi (76 bar)) expressing dsRed and green fluorescent protein (GFP) in nuclei under the control of the *G. mosseae* PMA1 and *Aspergillus nidulans* gpd promoters. (c) Transformed spore (1100 psi) showing dsRed and GFP labelled nuclei. After microscopic observation the spore was slightly crushed and stained with 4,6-diamidino-2-phenylindole (DAPI). Arrows show transformed nuclei.

very conserved and have homologues in the filamentous ascomycete *A. nidulans*. When transformed into *A. nidulans* both promoter reporter constructs were able to drive expression of the red fluorescent protein DsRed and this was targeted to the nuclei as predicted. This was a good indication that the constructs, if transformed into *G. intraradices,* would also work. Indeed this was the case and red fluorescent spores were obtained when bombarded with both constructs, showing that these *G. mosseae* promoters also work in *G. intraradices*. Red fluorescence was located to bright dots in the spores that corresponded in size and distribution to nuclei. Three-dimensional reconstruction of transformed spores showed that the dots were homogeneously distributed. Colocalization of GFP and dsRED as well as of DAPI staining showed unequivocally that these spots corresponded to nuclei, showing that the nuclear localization target of the *stuA* gene from *A. nidulans* also works in AM fungi. This is not so surprising since a putative homologue of the transcription factor StuA exists in *G. intraradices* (Ouziad *et al.*, 2005). When comparing both DsRed constructs we observed that expression of red fluorescence under the control of the *GmFOX2* promoter was weaker than when driven by GmPMA1 in *G. intraradices*, while we did not see obvious differences when both constructs were expressed in *A. nidulans*. This might reflect the different expression levels of both promoters in AM fungi during the presymbiotic stage, and why these promoters possibly only work in *A. nidulans* as minimal promoters. However, transformation efficiency was slightly higher when using the construct with the *GmFOX2* promoter.

Although we were able to introduce and express foreign DNA in an AM fungus by bombardment, we observed that this expression was on all occasions only transient and disappeared quite rapidly. This has been reported often when using biolistics to transform DNA into an organism, including AM fungi

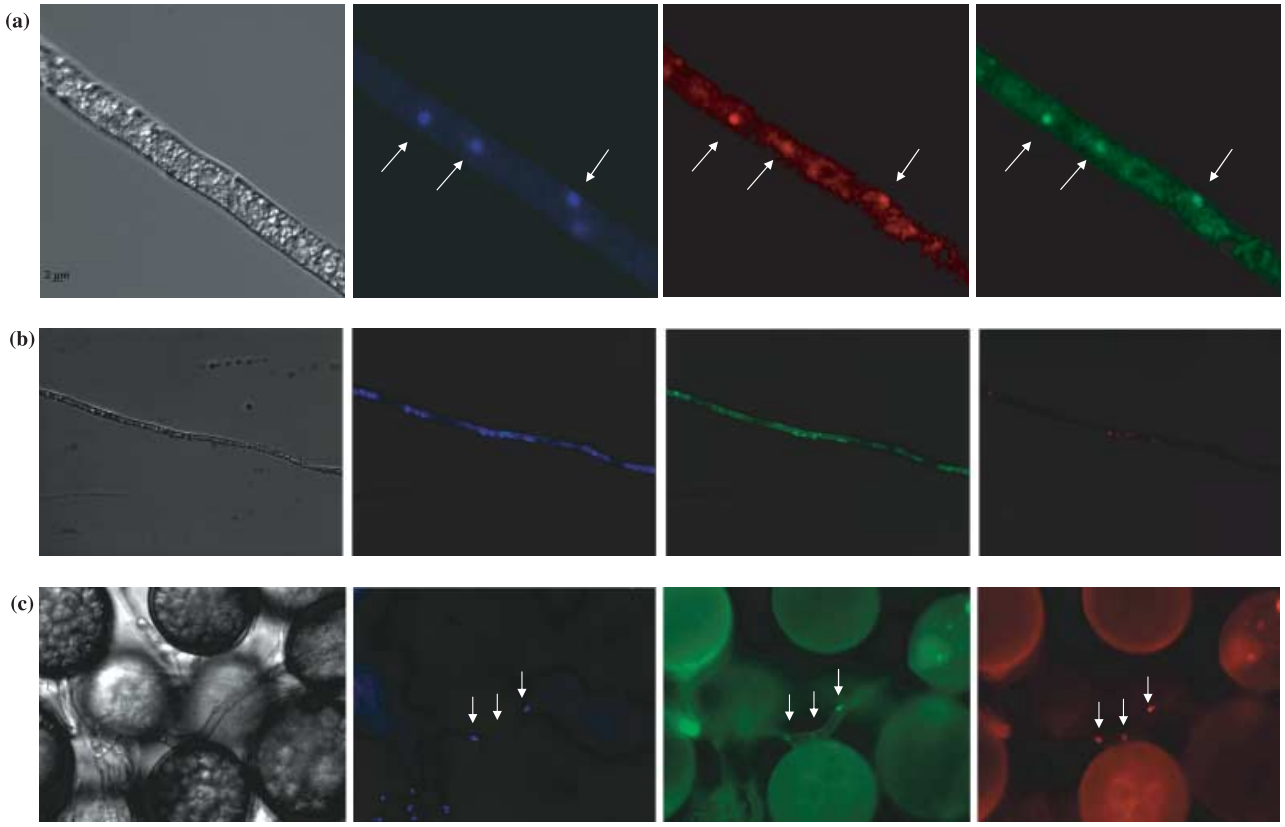

Fig. 5 Growing hyphae from *Glomus intraradices* spores showing red and green fluorescence (bottom panel) nuclei after bombardment (1100 psi (76 bar)) with the dual promoter construct pNHATPDsRedstuA. In the upper right panel nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI), and upper left the same hypha in Normarski. (a) Transformed nuclei in a hypha growing out of a spore that was not germinated at the time of bombardment. Arrows indicate position of transformed nuclei. (b) Transformed nuclei in a hypha from a spore that had already germinated at the time of bombardment. (c) Transformed nuclei in a hypha growing out of a spore that had not germinated at the time of bombardment. Arrows indicate transformed nuclei. Observe in the left corner of the DAPI panel other nuclei not transformed from different hyphae.

(Forbes *et al.*, 1998). The DNA is not integrated into the genome and the expression of the gene of interest is very limited (Schillberg *et al.*, 2000). In contrast to bombardment, ATMT has been reported to be more stable. This is possibly because *A. tumefaciens* inserts the DNA in the genome and usually as a single copy. Therefore, a higher level of mitotic stability of transformants has been reported. However, our data show that AM fungi are not that as amenable to transformation with *A. tumefaciens* as other filamentous fungi, and only rare transformation events were observed when transforming extraradical mycelium and none when using spores. This could be because of the thickness of the AM fungal cell wall that in some cases can reach up to 13 μm, as described for *Gigaspora margarita* (Sward *et al.*, 1981), or as has been suggested for other fungi, or because of differences in the cell wall composition (Michielse *et al.*, 2004). Experiments are underway to overcome these problems.

In summary, in this work we present the first report of the expression of fluorescent proteins in an AM fungus driven by AM promoters. This might be a first step to track AM fungi *in vivo* during symbiotic development and to establish a stable transformation system. Other avenues must be explored such as the use of resistance markers to help the screening of the transformants as well as modified protocols to improve *A. tumefaciens* ability to transform *G. intraradices*. The fact that not all nuclei show fluorescence in a transformed spore, but that all nuclei labelled with dsRed when transformed with the construct pNHATPdsRedstuA were also labelled with GFP shows that each nucleus controls its own section of cytoplasm. Thus, when transformed, they export the mRNA to their cytoplasm and the protein with the localization signal returns back to the same nucleus. This is an important finding because it might explain how the AM coenocytic mycelium controls different developmental processes in specific sections of the fungal colony.

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