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The intracellular delivery of TAT-aequorin reveals calcium-mediated sensing of environmental and symbiotic signals by the arbuscular mycorrhizal fungus Gigaspora margarita

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Summary

Arbuscular mycorrhiza (AM) is an ecologically relevant symbiosis between most land plants and Glomeromycota fungi. The peculiar traits of AM fungi have so far limited traditional approaches such as genetic transformation. The aim of this work was to investigate whether the protein transduction domain of the HIV-1 transactivator of transcription (TAT) protein, previously shown to act as a potent nanocarrier for macromolecule delivery in both animal and plant cells, may translocate protein cargoes into AM fungi.
We evaluated the internalization into germinated spores of Gigaspora margarita of two recombinant TAT fusion proteins consisting of either a fluorescent (GFP) or a luminescent (aequorin) reporter linked to the TAT peptide.
Both TAT-fused proteins were found to enter AM fungal mycelia after a short incubation period (5–10 min). Ca2+ measurements in G. margarita mycelia pre-incubated with TAT-aequorin demonstrated the occurrence of changes in the intracellular free Ca2+ concentration in response to relevant stimuli, such as touch, cold, salinity, and strigolactones, symbiosis-related plant signals.
These data indicate that the cell-penetrating properties of the TAT peptide can be used as an effective strategy for intracellularly delivering proteins of interest and shed new light on Ca2+ homeostasis and signalling in AM fungi.

Introduction

Arbuscular mycorrhizal (AM) fungi form an old and widespread mutualistic symbiosis with the vast majority of land plants, in which the fungi supply mineral nutrients, particularly phosphorus, to the plant, receiving in turn carbohydrates from the photosynthetic host. In view of its relevance for ecology and increasing demand for sustainable agriculture, the AM symbiosis has been the subject of a myriad of studies in the past decades, mainly focused on the cytological, biochemical and molecular features of this intimate plant–fungal beneficial association (see Gutjahr & Parniske, 2013 for a review). One of the most investigated features in the early stages of the establishment of the AM symbiosis is the calcium-mediated signalling pathway that is triggered in the host plant by AM fungal diffusible signals. AM germinated spore exudates were found to trigger a transient elevation in cytosolic Ca2+ in soybean (Glycine max) cell cultures (Navazio et al., 2007). Moreover, repeated oscillations in perinuclear and nuclear Ca2+ concentration (a phenomenon called Ca2+ spiking) were evoked in response to AM fungi in host plants (Kosuta et al., 2008; Chabaud et al., 2011; Sieberer et al., 2012). The chemical nature of the symbiotic signals released by AM fungi (the so-called 'Myc factors') has recently been determined (Maillet et al., 2011; Genre et al., 2013). Whereas the role of Ca2+ as a crucial intracellular messenger in the plant symbiotic signalling pathway induced by AM fungi has been firmly established, much less information is available on the

fungal side. It has recently been demonstrated that fungal genes related to Ca2+ homeostasis and signalling are up-regulated during AM symbiosis development (Liu et al., 2013; Tisserant et al., 2013). Nevertheless, whether Ca2+ is involved in the perception of plant symbiotic signals by AM fungi, thus playing a role in the microsymbiont comparable to that played in the plant counterpart, is not yet known. Similarly, no information is available on Ca2+-based sensing systems in AM fungi for the detection of environmental cues. Measurements of Ca2+ dynamics in AM fungi using either fluorescent Ca2+ indicators or genetically encoded Ca2+ probes have so far been hampered by the biological traits of AM fungi, such as the complex structure of the cell wall, mainly containing multilayered chitin and glucans, which impedes the intracellular delivery of Ca2+-sensitive dyes; in addition, the aseptate and coenocytic nature of the hyphae, containing hundreds of nuclei sharing the same cytoplasm, renders the isolation of protoplasts unfeasible and classical methods of genetic transformation difficult to perform (Harrier & Millam, 2001; Bonfante & Genre, 2010; Sanders & Croll, 2010; Lanfranco & Young, 2012). Although stable transformants of AM fungi have not yet been obtained, transient expression of exogenous genes has been achieved using a biolistic approach (Helber & Requena, 2008).

As an alternative to conventional genetic transformation methods, a strategy based on the intracellular delivery of proteins mediated by the so-called cell-penetrating peptides (CPPs) (Chugh et al., 2010; Sawant & Torchilin, 2010; Bechara & Sagan, 2013) was applied to AM fungi in this work. The TAT peptide, derived from the protein transduction domain of the human immunodeficiency virus type 1 (HIV-1) transactivator of transcription (TAT) protein, has previously been demonstrated to function as an efficient nanocarrier for the intracellular delivery of cargoes of interest in both animal and plant cells (Gump & Dowdy, 2007; Chugh et al., 2010). After verifying the successful internalization of the fluorescent marker GFP into the hyphal network of the AM fungus Gigaspora margarita, we used the TAT-mediated delivery of the bioluminescent Ca2+ reporter aequorin as an innovative tool to analyse Ca2+-based sensing mechanisms of AM fungi. Our results reveal that AM fungi perceive different abiotic stimuli as well as strigolactones, symbiosis-specific plant host signals, via changes in intracellular Ca2+ concentrations. The data obtained suggest that the TAT peptide may be used as a promising delivery vehicle for a more detailed study of the still enigmatic biology of AM fungi.

Materials and Methods

Fungal material

Spores of Gigaspora margarita (BEG 34; International Bank for the Glomeromycota, University of Kent, UK) were surface-sterilized with 3% (w/v) chloramine-T and 0.03% (w/v) streptomycin sulfate, then rinsed several times with sterile distilled water and stored at 4°C. To induce germination, batches of 100 spores were placed in 1 ml of sterile distilled water and incubated at 30°C in the dark for 7 d (germination rate > 90%).

DNA constructs and isolation of recombinant proteins

The plasmids encoding the N-terminal His-tagged GFP, TAT-GFP and TAT-aequorin have been previously described (Peroni et al., 2007; Zonin et al., 2011). To generate the His-tagged aequorin protein, the sequence encoding aequorin from plasmid pTAT-aequorin (Zonin et al., 2011) was amplified by PCR using the following primers: 5′-CTCTAGAATGAAGCTTTATGATGTTCCTGA-3′ and 5′-AGGAATTCTTAGGGGACAGCTGGACCGTA-3′. After digestion with XbaI and EcoRI, the amplicon was cloned between NheI and EcoRI restriction sites of plasmid pRSET-Dpl (Cereghetti et al., 2004). For expression of recombinant proteins, the plasmids were inserted by transformation into BL21 DE3 (Lys E) Escherichia coli bacterial cells. Protein induction was started by adding 0.5 mM isopropyl-β-d-1-thio-galactopyranoside (IPTG) to 2 l of BL21 suspension culture (LB medium supplemented with 50 μg ml−1 ampicillin) in the exponential phase of growth (optical density at 600 nm = 0.6). After overnight incubation at room temperature, bacterial cells were harvested by centrifugation at 3000 g for 15 min at 4°C. For aequorin and TAT-aequorin, the pellet was resuspended in solubilization buffer (7.5 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 8.0, 2.5 mM imidazole, 6 M urea, 0.5 M NaCl, and 20% glycerol) and disrupted by sonication. The lysate was clarified by centrifugation (11 000 g for 35 min at 4°C) and loaded onto a 3-ml Ni-NTA agarose column (Qiagen, Hilden, Germany). After washing with solubilization buffer without urea, the recombinant proteins were eluted with 0.37 M imidazole. Finally, the recovered protein was extensive dialysed against 7.5 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 7.4, 0.2 M NaCl, and 20% glycerol and flash-frozen in aliquots at −80°C.
GFP and TAT-GFP proteins were purified as described by Battistutta et al. (2000) and Peroni et al. (2007). Protein concentration was estimated from the calculated extinction coefficient (M−1 cm−1) at 280 nm (Abs 0.1%, g l−1) of 2.62 for aequorin, 1.82 for TAT-aequorin, 5.07 for GFP, 0.774 for TAT-GFP.

TAT-mediated protein delivery

For TAT-mediated delivery, recombinant TAT-fusion proteins were directly added to G. margarita germinated spores. Incubation with TAT-GFP was performed at 3 μM for 10 min. Incubation with TAT-aequorin was carried out at 30 μM for different time intervals, ranging from 5 min to 24 h. After incubation with the TAT fusion proteins, fungal mycelia were extensively washed (five washes with 1 ml of sterile distilled H2O) before fluorescence microscopy analyses (for TAT-GFP) or luminescence detection (for TAT-aequorin). In control experiments, germinated spores of G. margarita were incubated with either 3 μM GFP or 30 μM aequorin (not fused to the TAT peptide).

Fluorescence microscopy

Fluorescence was analysed in living G. margarita mycelia using a Leica DMI4000 B inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany), and 35-mm μ-dishes with a thin bottom (ibidi GmbH, Martinsried, Germany). A filter set for GFP detection (excitation at 450/490 nm; emission at 500/550 nm) was used. Images were acquired with a Leica DFC300 FX digital camera, using the Leica Application Suite (las) software (Leica Microsystems, Heerbrugg, Switzerland).

In some experiments, after incubation with TAT-GFP, the samples were washed with phosphate-buffered saline (PBS) and 0.02% (w/v) EDTA and subsequently incubated with 0.05% (w/v) trypsin for 5 min. Microscopy observations were carried out after two washes in PBS and one wash in H2O.

Protein extraction, SDS-PAGE and western blot analysis

To prepare protein crude extracts, germinated spores of G. margarita (batches of 100) were lysed with 200 µl of ice-cold Cell Disruption Buffer from the PARIS™ kit (Ambion by Life Technologies, Carlsbad, CA, USA) supplemented with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 1 µM leupeptin) in three cycles of 30 s each using a motorized pestle (Sigma-Aldrich). Samples were subsequently subjected to three cycles of 30 s of sonication (3510 Branson, Danbury, CT, USA) followed each time by 30 s on ice. After centrifugation at 1000 g for 15 min at 4°C, the protein concentration in the supernatant was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Proteins were separated by SDS-PAGE (12% polyacrylamide), transferred onto PVDF membranes (Immobilon-P; Millipore, Billerica, MA, USA) and subsequently processed for immunoblotting, as previously described (Zonin et al., 2011). A monoclonal anti-polyhistidine antibody (Sigma-Aldrich) was used at a 1 : 1500 dilution.

***In vitro* apoaequorin reconstitution**

Germinated spores of G. margarita (batches of 100 spores, germinated for 7 d) were incubated with 30 µM TAT-aequorin for 2 h. After extensive washing (five washes with 1 ml of sterile distilled H2O), total protein extracts were isolated using the apoaequorin reconstitution buffer (150 mM Tris-HCl and 4 mM EGTA, pH 8.0, supplemented with 0.8 mM phenylmethylsulfonyl fluoride). Protein samples were subsequently incubated with 1 mM β-mercaptoethanol and 5 µM coelenterazine (Molecular Probes by Life Technologies) for 4 h in the dark at 4°C. Luminescence emitted by 25-µg protein samples was recorded after the addition of 100 mM CaCl2 and integrated for a 200-s time interval.

Calcium measurements in germinated spores of *G. margarita*

Germinated spores of G. margarita (batches of 100 spores, germinated for 6 d) were incubated for 4 h with 5 mg ml−1 Australys Zimolysine (Oliver Ogar, Montebello Vicentino, Italy) in 1.2 M MgSO4 and 10 mM sodium phosphate, pH 5.8. This enzymatic mixture is characterized by a high β-glucanase activity and secondary activities such as that of chitinase. After extensive washing, the fungal samples were incubated overnight in the dark with 5 µM coelenterazine, washed again and incubated with 30 µM TAT-aequorin for 1–2 h. After further washing, Ca2+ measurements were carried out in samples of 200 germinated spores using a purpose-built luminometer (Electron Tubes Ltd, Uxbridge, UK) containing a 9893/350A photomultiplier (Thorn EMI, Middlesex, UK). Fungal samples were challenged with abiotic and biotic stimuli by injection of an equal volume (100 µl) of two-fold concentrated solutions with a light-tight syringe. Two synthetic strigolactone analogues were used: GR24, which was purchased from Chiralix (Nijmegen, the Netherlands), and EGO5 (Prandi et al., 2011), which was kindly provided by Cristina Prandi (Turin, Italy). Stock solutions of GR24 (10−3 M) and MEB10 (10−2 M) were

prepared in acetone and control samples were treated with the same percentage of the solvent (maximal concentration 0.5%). Mechanical perturbation was applied by injection of an equal volume (100 µl) of H2O at room temperature, and cold shock was applied by adding 200 µl of ice-cold H2O directly to germinated spores of G. margarita . At the end of the experiments, the remaining aequorin pool was discharged by injection of 100 µl of 1 M CaCl2, 30% ethanol, and 4% Igepal CA-630 (Sigma-Aldrich). The light signal was collected and converted off-line into Ca2+ concentration values using a computer algorithm based on the Ca2+ response curve of aequorin (Brini et al., 1995).

Fungal viability assay

Viability of the fungal mycelium was monitored using the LIVE/DEAD FungaLight™ Yeast Viability kit (Invitrogen by Life Technologies), following the manufacturer's instructions. The assay utilizes a mixture of the green-fluorescent nucleic acid stain SYTO 9 (excitation/emission maxima 480/500 nm) and the red-fluorescent nucleic acid stain propidium iodide (excitation/emission maxima 490/635). Fungi with intact cell membranes fluoresce green, whereas fungi with damaged membranes fluoresce red. As an alcohol-killed control, samples of germinated spores of G. margarita were incubated for 1 h with 70% isopropanol.

Results

The TAT peptide can import the green fluorescent protein into AM fungal hyphae

In order to start evaluating whether the protein transduction domain (PTD) of the TAT protein from the HIV-1 virus can be used as a nanocarrier to import proteins of interest into AM fungi, a TAT-GFP recombinant fusion protein, composed of the 11 amino acids of the TAT peptide fused with the N-terminus of GFP, was engineered. The use of GFP allows easy verification of the intracellular delivery of the protein cargo by TAT. Germinated spores (7 d) of the AM fungus G. margarita were incubated for 10 min with 3 µM TAT-GFP and then extensively washed to get rid of any residual protein aspecifically bound to the fungal surface. In addition to the expected autofluorescence of the spores in the green channel (after excitation with blue light at 450/490 nm), a GFP-specific fluorescent signal could be observed inside the fungal hyphae (Fig. 1a,a′). The fluorescence was still present after 48 h, with some residual fluorescence detectable even after 72 h (data not shown), suggesting relative stability of the TAT-imported fusion protein. In control samples, incubated with H2O only, it was possible to observe only the autofluorescence of the spores, whereas young hyphae did not exhibit any fluorescent signal (Fig. 1b,b′). When G. margarita germinated spores were incubated with GFP (3 µM for 10 min) alone, not fused with the TAT peptide, no detectable fluorescence signal was observed in the hyphal network (Fig. 1c,c′), indicating the specificity of the TAT-mediated protein cargo import.

In order to ascertain that the fluorescence observed in the samples incubated with TAT-GFP (Fig. 1a,a′) does not derive from residual fusion protein not specifically attached to the surface of the hyphal network, a mild proteolytic treatment with trypsin (0.05% for 5 min), commonly used during in vitro subculturing of mammalian cells growing in a monolayer (Frangini et al., 2013), was carried out after incubation

with TAT-GFP. Although the gentle trypsinization effectively removed some residual protein attached to the surface of some spores, it did not eliminate hyphal fluorescence (Supporting Information Fig. S1). The efficiency of TAT-GFP digestion by trypsin was checked by SDS-PAGE analysis (Fig. S1). This result confirmed that the TAT-GFP chimera was indeed internalized inside the cytoplasm of G. margarita hyphae. Taken together, these results indicate the potential of the TAT peptide to be used as a vehicle for protein internalization into AM fungal mycelia.

The TAT peptide can import the Ca2+-sensitive bioluminescent protein aequorin into AM fungal hyphae

Next, the possibility of using a TAT-aequorin fusion protein as a novel tool to monitor Ca2+ dynamics in AM fungi was evaluated. We have recently demonstrated that the basic domain of the TAT protein efficiently targeted the attached bioluminescent Ca2+ reporter aequorin to the cytoplasm of plant cells (Zonin et al., 2011). To verify whether the fusion protein TAT-aequorin could be internalized by AM fungi, G. margarita spores were germinated for different time intervals and subsequently incubated with TAT-aequorin (30 μM) for 10 min. After exhaustive washing with H2O and reconstitution of apoaequorin with its prosthetic group coelenterazine, luminescence measurements were carried out by injecting an equal volume of discharge solution (1 M CaCl2 and 30% ethanol). Although almost no luminescence was detected in samples from either ungerminated spores (2 d) or spores that had just started their germination (4 d), a high level of luminescence was recorded in fully germinated spore (7 d) samples. The light signal was found to significantly decrease again in 14-d germinated spores ($P < 0.05$; Student's t-test) (Fig. 2). No luminescence was detected in fungal samples incubated with aequorin, not fused to the TAT peptide (data not shown).

Germinated spores showing the optimal level of mycelium development (7 d) were incubated with TAT-aequorin (30 μM) for increasing time intervals. Immunoblot analysis of total protein extracts demonstrated that the fusion protein, absent in control samples, was rapidly and progressively internalized into treated samples. The uptake occurred as fast as 5 min after cell incubation with TAT-aequorin and the level of internalized protein slightly increased in the next 24 h (Fig. 3a). No immunoreactive bands were detected in protein extracts from G. margarita samples incubated with free aequorin (not fused to TAT), used at the same concentration (30 μM) and for the same time intervals (5 min to 24 h) as TAT-aequorin (Fig. 3b).

Nevertheless, luminescence levels emitted by fungal mycelia that had been incubated with TAT-aequorin and with coelenterazine to reconstitute the functional holoprotein were found to be insufficient to enable Ca2+ measurement assays to be carried out (Fig. 2). It was only possible to record high levels of luminescence (c. 107 counts) by using an in vitro reconstitution assay, that is, reconstitution of the TAT-delivered aequorin with coelenterazine after the lysis of the fungal mycelium (data not shown). The discrepancy between the luminescence levels obtained after in vivo and in vitro reconstitution of the TAT-delivered photoprotein suggests that a potentially limiting factor for the use of TAT-delivered aequorin as an effective tool for Ca2+ measurements in AM fungi may be poor penetration of coelenterazine through the fungal cell wall. An alternative explanation may be inadequate lysis of the fungal mycelium at the end of the experiment at the luminometer, when the remaining active aequorin pool has to be discharged.

Ca2+-mediated perception of environmental signals by AM fungi

In order to achieve a slight loosening of the AM fungal cell wall, thus facilitating the internalization of coelenterazine and/or TAT-aequorin to levels suitable for Ca2+ monitoring, germinated spores of G. margarita were pretreated for 4 h with 5 mg ml−1 of a mixture of fungal cell wall-degrading enzymes (Australys Zimolysine). The viability of the fungal mycelium, assessed by staining with SYTO 9 and propidium iodide, was found not to be affected by this mild treatment (Fig. S2). Additional changes in the experimental protocol were found to be necessary in order to obtain luminescence levels suitable for conversion to intracellular Ca2+ values (3.09 × 105 ± 0.32 × 105 total counts; n = 21): (1) the use in the Ca2+ measurement assays of samples composed of a large number of germinated spores of G. margarita (c. 200); (2) overnight reconstitution with coelenterazine; (3) the addition of the detergent Igepal CA-630 (4%, v/v) to the discharge solution used at the end of each experiment at the luminometer (Mithöfer & Mazars, 2002). Once successfully set up, TAT-aequorin-based Ca2+ measurements demonstrated the occurrence of a tight fungal homeostatic control of the intracellular free Ca2+ concentration ([Ca2+]i) under resting conditions. In particular, the basal [Ca2+]i was found to reach values comparable to those usually found in all eukaryotic cells (c. 100 nM) (Fig. 4). Upon stimulation with different environmental stimuli, G. margarita was found to respond with rapid and transient intracellular Ca2+ changes, characterized by different kinetics. In particular, a mechanical perturbation, simulated by the injection of one volume of H2O, induced a modest perturbation of the [Ca2+]i, which peaked at c. 0.44 μM and rapidly dissipated within 30 s (Fig. 4a). A cold shock (injection of ice-cold H2O; Fig. 4b) and salt stress (0.3 M NaCl; Fig. 5c) were found to trigger in germinated spores of G. margarita transient intracellular Ca2+ changes characterized by higher peak amplitudes (c. 0.79 μM and c. 0.57 μM, respectively) and longer durations (c. 2 min and 5 min, respectively). In order to test the effect of strigolactones, the plant signalling molecules that are known to play a crucial role in the early steps of the AM symbiosis (Akiyama et al., 2005; Besserer et al., 2006), germinated spores of G. margarita were challenged with the synthetic analogue GR24 (10−6 M). As shown in Fig. 4(d), GR24 was found to evoke a rapid and remarkable [Ca2+]i elevation (c. 0.90 μM after 10 s) which dissipated within 3–4 min. When higher doses (2 × 10−6 and 5 × 10−6 M) were used, the amplitude of the GR24-induced Ca2+ transient was found to be dose-dependent (Fig. 4d). In response to 10−7 M GR24, a Ca2+ trace superimposable on that obtained with 10−6 M was recorded, whereas a lower dose (10−8 M) did not seem to trigger any Ca2+ response above the touch response displayed in Fig. 4(a) (data not shown), suggesting intrinsic limitations in the sensitivity of the TAT-aequorin-based Ca2+ assays.

To confirm the Ca2+-mediated perception of strigolactones by G. margarita, fungal mycelia were challenged with EGO5, another recently designed synthetic strigolactone analogue (Prandi et al., 2011). EGO5 induced a Ca2+ transient characterized by a similar kinetics, although the dose (10−5 M) requested to trigger a comparable Ca2+ response was ten times higher than for GR24 (Fig. 5). Taken together, these results demonstrate a strict control on [Ca2+]i in the AM fungus G. margarita and the occurrence of Ca2+-based sensing mechanisms for detecting and responding to several relevant stimuli, in particular strigolactones, symbiosis-related signals.

Discussion

In this work we have demonstrated that the cell-penetrating peptide TAT, an amino acid sequence derived from the protein transduction domain of the HIV-1 TAT protein, could efficiently translocate two covalently linked protein cargoes (GFP and aequorin) into the AM fungus G. margarita.

Whereas the level of some internalized CPP-fused proteins was found to rapidly decrease over 24 h of cellular uptake in plant cells (Zonin et al., 2011), the TAT-GFP and TAT-aequorin fusion proteins used in this study were still fully detectable in the cytoplasm of G. margarita after 24 h. The presence of some residual intracellular fluorescence in the fungal hyphae even after 72 h of TAT-GFP incubation suggests a longer and more favourable time frame of TAT-delivered protein bioavailability in AM fungi.

Previous studies carried out in the Basidiomycota fungus Antrodia cinnamomea treated with a mixture of GFP and the nona-arginine CPP (R9) demonstrated that noncovalent protein transduction, that is, the cellular internalization of proteins noncovalently bound to arginine-rich intracellular delivery peptides, was ineffective in this fungus (Liu et al., 2008). Based on our results, it seems that the mechanism of cellular uptake in covalent or noncovalent protein transduction may vary, and/or that there can be differences in the CPP penetrating capabilities in different fungi. As the use of CPPs to mediate protein internalization without a covalent link with their cargoes has been demonstrated to work in different animal and plant systems (Wang et al., 2006), further investigations are needed to assess if protein transduction by CPPs can be effected even in a noncovalent fashion in AM fungi.

The intracellular delivery of the bioluminescent Ca2+ reporter aequorin by means of the TAT peptide used as a cell-penetrating tool offered the possibility of monitoring the concentration of intracellular free Ca2+ and its dynamic variations in AM fungi. The calcium ion is known to act as an intracellular messenger in a wide range of signalling processes in bacteria, plants, fungi and animals (Dominguez, 2004; Clapham, 2007; Dodd et al., 2010; Cai & Clapham, 2012). Although many fungal species utilize a relatively simple Ca2+ homeostasis and signalling machinery, the complex Ca2+ signalling machinery present in basal fungi supports the notion that some components of the Ca2+ signalling toolkit might have been lost after the animal–fungi divergence (Cai & Clapham, 2012). Up-regulation of AM fungal genes related to Ca2+ homeostasis and signalling has been reported during both early (hyphopodium) and late (arbuscule) stages of symbiotic interactions with host roots, strongly suggesting the involvement of calcium as a signalling system in fungal processes leading to root colonization (Breuninger & Requena, 2004; Requena et al., 2007; Liu et al., 2013). Nevertheless, direct measurements of Ca2+ dynamics have so far been hampered by technical difficulties in delivering either fluorescent Ca2+ dyes or genetically encoded Ca2+ probes into AM fungi, because of the unique biological traits of Glomeromycota.

Recombinant aequorin expression has previously been shown to be a valuable method for the monitoring of Ca2+ signalling in filamentous fungi. Changes in [Ca2+]cyt were monitored in different Ascomycota fungi, such as the phytopathogenic fungus Phyllosticta ampelicida (Shaw et al., 2001), Neurospora crassa and Aspergillus niger (Nelson et al., 2004; Benčina et al., 2005), after challenge with several physico-chemical stimuli. Those studies demonstrated Ca2+-mediated sensing by fungal hyphae of various physiologically perturbing treatments, such as mechanical

perturbation, hypo-osmotic shock, and exposure to high concentrations of external Ca2+.

In this work we have set up a novel strategy based on the TAT-aided translocation of aequorin into AM fungi. Although the thick and tough cell wall of G. margarita spores (Sward, 1981) seemed to represent an insurmountable barrier for the TAT-cargo protein complexes, TAT-aequorin was found to be efficiently internalized by fully developed (7 d) fungal mycelia. The finding that, after 1 h of incubation with TAT-aequorin, 14-d germinated spores emitted a light signal significantly weaker than that of 7-d germinated spores is likely to be attributable to the obligate biotrophic nature of AM fungi, which need a plant partner in order to complete their life cycle (Bonfante & Requena, 2011): if they do not encounter a host plant, fungal hyphae stop their growth, retract their cytoplasm, and form septa separating viable from empty hyphal segments (Logi et al., 1998). The establishment of efficient aequorin-based Ca2+ measurement assays required controlled pretreatment of AM fungal mycelia with a mixture of fungal cell wall-degrading enzymes before the incubation with TAT-aequorin and coelenterazine. This initial step, which did not affect the viability of the fungal mycelium, allowed for a slight loosening of the fungal cell wall, thus improving the reconstitution of the active holoprotein inside AM fungal mycelia. The results obtained indicate that the AM fungus G. margarita is able to maintain the free [Ca2+]i at concentrations as low as those recorded in animal and plant cells (c. 100 nM). Moreover, these data demonstrate the use of transient changes in [Ca2+]cyt for the transduction of environmental signals of both an abiotic and a biotic nature in the AM fungus G. margarita. AM fungal germinated spores were found to respond to environmental cues, such as mechanical perturbation, cold and salt stress, with stimulus-specific Ca2+ signatures. Of particular interest is the Ca2+ response of G. margarita to strigolactones, the plant signalling molecules known to play a crucial role in the early steps of AM symbiosis (Xie et al., 2010; Ruyter-Spira et al., 2013; Zwanenburg & Pospišil, 2013). The induction of a rapid and transient elevation in [Ca2+]i in response to both the synthetic strigolactones GR24 and EGO5 indicates the Ca2+-mediated perception of plant signals related to symbiosis by AM fungi. This result, together with the recent demonstration of an intracellular Ca2+ change as an early essential step in the symbiotic signalling pathway activated by plant flavonoids in the nitrogen-fixing bacterium Rhizobium leguminosarum (Moscatiello et al., 2010; Arrigoni et al., 2013), suggests that both microbial symbionts – AM fungi and rhizobia – possess Ca2+-based sensing systems for detecting and transducing symbiosis-specific plant host signals.

Future work will be focused on the design and evaluation of TAT-fused fluorescent Ca2+ reporter proteins, such as Cameleon. This would allow Ca2+ imaging experiments to be performed in single AM fungal hyphae, thus circumventing the need for large quantities of AM germinated spores as in aequorin-based Ca2+ assays. The possibility of performing Ca2+ imaging experiments at the single cell level would be particularly important for the detection of potential Ca2+ spiking events. Another attractive possibility is the use of a mutagenized TAT peptide (Cardarelli et al., 2008) to selectively target genetically encoded Ca2+ reporters to the nucleus, thus enabling analysis of Ca2+ oscillations within this compartment. This would be particularly relevant in view of the observed up-regulation of a nuclear Ca2+/calmodulin-dependent protein kinase in Glomus intraradices during mycorrhizal development (Liu et al., 2013). Pulsatile cytoplasmic Ca2+ signatures have recently been revealed by means of the recombinant expression of a Cameleon Ca2+ sensor in some plant pathogenic filamentous fungi (Kim et al., 2012).

Moreover, the potential of CPPs as a genetic engineering tool in AM fungi deserves to be considered in the future. Indeed, the effective use of CPPs for gene transfer and gene silencing has been reported in both animal and plant cells (see Chugh et al., 2010 and Bechara & Sagan, 2013 for reviews).

In conclusion, the TAT-mediated delivery of a Ca2+ reporter protein is a welcome technical advance which has permitted the measurement of stimulus-specific Ca2+ signals in an AM fungus. The detection of strigolactone-induced intracellular Ca2+ changes in G. margarita helps to improve our current understanding of chemical communication during AM symbiosis establishment, and confirms the versatility of calcium as a common signalling system underlying many beneficial plant–microbe interactions (Oldroyd, 2013; Venkateshwaran et al., 2013). The TAT-based strategy for protein internalization in AM fungi lends itself as an interesting alternative to previously reported methods of transient transformation of AM fungi (Harrier & Millam, 2001; Helber & Requena, 2008), and may represent a powerful tool for the deciphering of some of the still elusive features of AM fungi, which have just started to be unravelled by the recent sequencing of the Rhizophagus irregularis genome (Tisserant et al., 2013).

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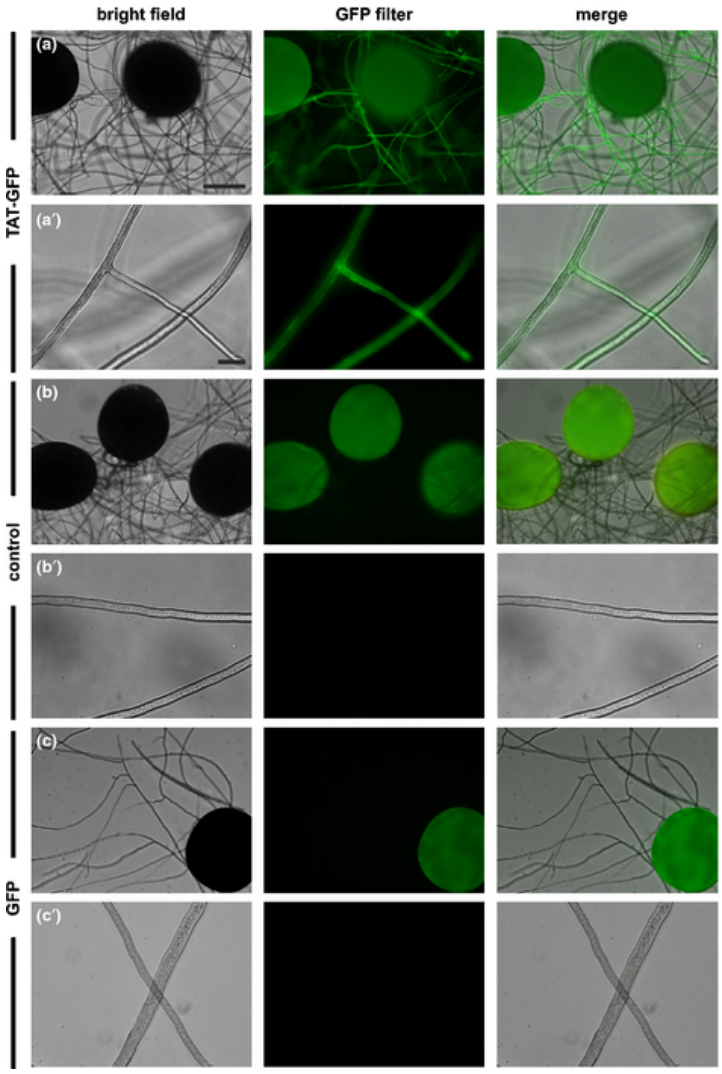

Figure 1.
Internalization of the TAT-GFP fusion protein into the arbuscular mycorrhizal (AM) fungus Gigaspora margarita. Germinated spores of G. margarita (7 d) were incubated for 10 min with: (a, a′) 3 μM TAT-GFP, (b, b′) H2O only, or (c, c′) 3 μM GFP. After the differential treatments, fungal samples were extensively washed with H2O. Bright field and fluorescence microscopy images are shown. Bars: (a–c) 250 μm; (a′–c′) 25 μm.

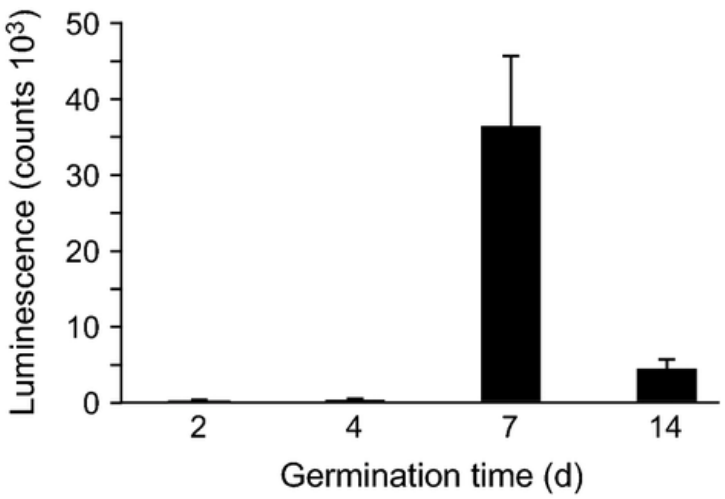

Figure 2.
Luminescence measurements in Gigaspora margarita at different times of spore germination, after 1 h of incubation with the TAT-aequorin fusion protein. Luminescence emitted by samples of 100 fungal germinating spores was measured, after injection of an equal volume of 1 M CaCl2 and 30% ethanol. Data are the means ± SE of three independent experiments.

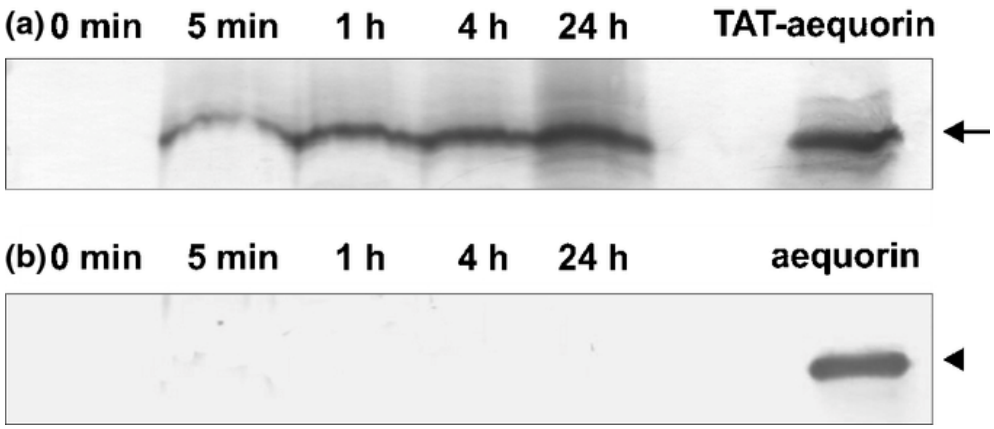

Figure 3.
Immunoblot analysis of TAT-mediated aequorin delivery into Gigaspora margarita mycelia. Germinating spores (7 d) of G. margarita were incubated with 30 μM TAT-aequorin (a) or 30 μM aequorin (b) for the reported time intervals. Total protein extracts (30 μg) were separated by 12% SDS-PAGE, transferred onto PVDF membranes and incubated with an anti-polyHis antibody. Purified 26-kDa TAT-aequorin (a; 0.4 μg; arrow), and 22-kDa aequorin (b; 0.4 μg; arrowhead) were used as positive controls.

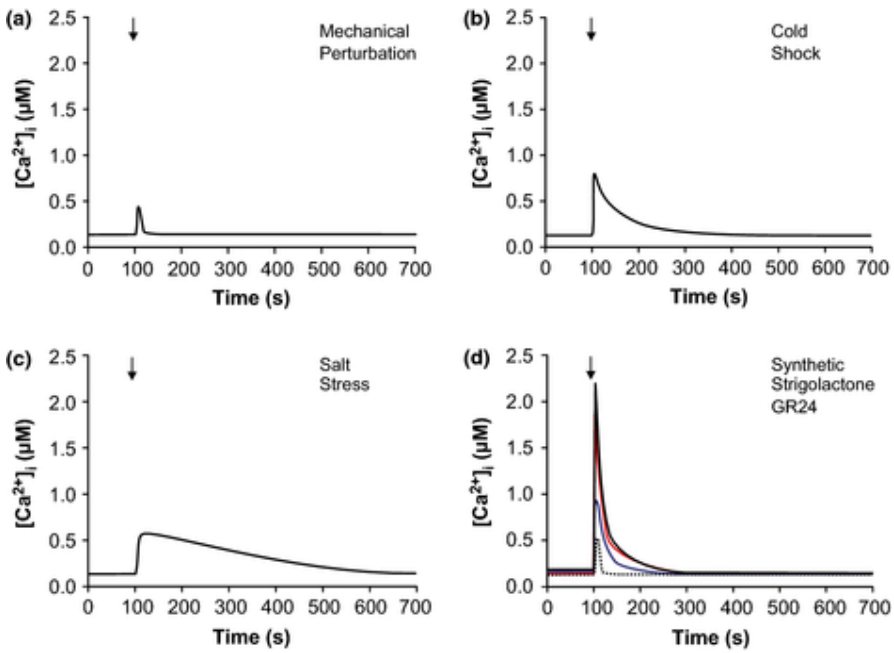

Figure 4.
Monitoring of intracellular free Ca2+ concentration ([Ca2+]i) in Gigaspora margarita. Germinated fungal spores (7 d) were incubated with TAT-aequorin (30 µM for 1–2 h) before Ca2+ measurement assays. The following stimuli were applied (arrow) after 100 s: (a) mechanical perturbation (one volume of H2O); (b) cold shock (ice-cold H2O); (c) salt stress (0.3 M NaCl), (d) the synthetic strigolactone GR24 (blue line, 10−6 M; red line, 2 × 10−6 M; black line, 5 × 10−6 M) or the solvent control (dotted line). The traces are representative of three independent experiments which gave very similar results.

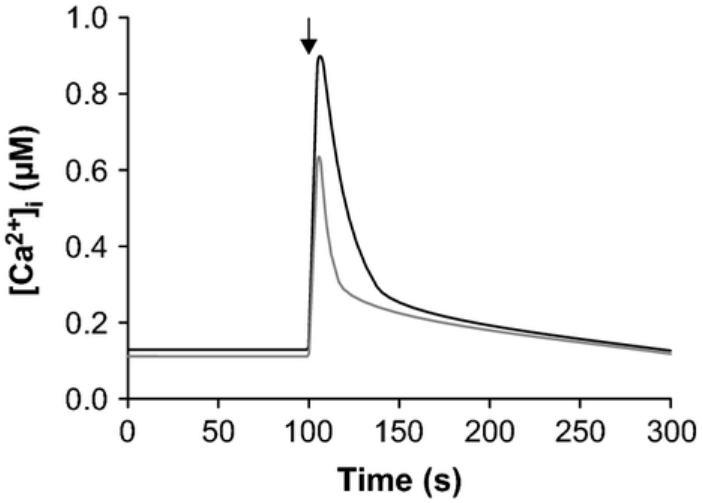

Figure 5.
Comparison of the effects of the synthetic strigolactone analogues GR24 and MEB10 on [Ca2+]i in Gigaspora margarita. Ca2+ assays were performed in G. margarita germinated spores (7 d) previously incubated with TAT-aequorin (30 µM for 1–2 h). Black trace, GR24 (10−6 M); grey trace, EGO5 (10−5 M).