New Phytologist

Research

Arbuscular mycorrhizal hyphopodia and germinated spore exudates trigger Ca^{2+} spiking in the legume and nonlegume root epidermis

Mireille Chabaud[1]*, Andrea Genre[2]*, Björn J. Sieberer[1], Antonella Faccio[2], Joëlle Fournier[1], Mara Novero[2], David G. Barker[1] and Paola Bonfante[2]

[1]Laboratory of Plant–Microbe Interactions, UMR CNRS-INRA 2594/441, F-31320 Castanet-Tolosan, France; [2]Department of Plant Biology, University of Turin and IPP-CNR, Viale Mattioli 25, 10125 Turin, Italy

Author for correspondence:
Paola Bonfante
Tel: +39 011 6705965
Email: p.bonfante@ipp.cnr.it

Received: *18 June 2010*
Accepted: *11 August 2010*

New Phytologist (2011) **189**: 347–355
doi: 10.1111/j.1469-8137.2010.03464.x

Key words: arbuscular mycorrhiza, calcium spiking, common SYM pathway, fungal–plant signalling, *Gigaspora* spp., *Medicago truncatula*, nuclear-localized cameleon, spore exudates.

Summary

- The aim of this study was to investigate Ca^{2+} responses to endosymbiotic arbuscular mycorrhizal (AM) fungi in the host root epidermis following pre-infection hyphopodium formation in both legumes and nonlegumes, and to determine to what extent these responses could be mimicked by germinated fungal spore exudate.
- Root organ cultures of both *Medicago truncatula* and *Daucus carota*, expressing the nuclear-localized cameleon reporter NupYC2.1, were used to monitor AM-elicited Ca^{2+} responses in host root tissues.
- Ca^{2+} spiking was observed in cells contacted by AM hyphopodia for both hosts, with highest frequencies correlating with the epidermal nucleus positioned facing the fungal contact site. Treatment with AM spore exudate also elicited Ca^{2+} spiking within the AM-responsive zone of the root and, in both cases, spiking was dependent on the *M. truncatula* common SYM genes *DMI1/2,* but not on the rhizobial Nod factor perception gene *NFP*.
- These findings support the conclusion that AM fungal root penetration is preceded by a SYM pathway-dependent oscillatory Ca^{2+} response, whose evolutionary origin predates the divergence between asterid and rosid clades. Our results further show that fungal symbiotic signals are already generated during spore germination, and that cameleon-expressing root organ cultures represent a novel AM-specific bio-assay for such signals.

Introduction

Arbuscular mycorrhizal (AM) fungi are ecologically important biotrophic plant symbionts, providing host roots with vital mineral nutrients in exchange for photosynthates and a privileged ecological niche essential for fungal development and propagation (Smith & Read, 2008). Following spore germination, AM fungi respond to host-secreted molecules, such as sesquiterpene lactones, by intense hyphal ramification within the vicinity of the root (Buée *et al.*, 2000; Akiyama *et al.*, 2005). This leads to root contact and the differentiation of fungal adhesion structures, known as hyphopodia, on nonroot hair epidermal cells referred to as atrichoblasts (Harrison, 2005; Parniske, 2008). In return, the plant responds to so far unidentified fungal signals with modifications in gene expression (Kosuta *et al.*, 2003; Kuhn *et al.*, 2010) and the organization of a polarized cytoplasmic assembly, termed the prepenetration apparatus (PPA), in hyphopodia-contacted cells (Genre *et al.*, 2005). PPA-dependent intracellular infection is a conserved mechanism across plant species (Genre *et al.*, 2008), and is associated with the construction of the specialized interface compartment surrounding the infection hypha, a hallmark of the AM association (Parniske, 2008).

Certain of these plant responses depend on a signal transduction pathway which is partly shared with the well-characterized pathway required for the initiation of the nitrogen-fixing root nodule symbiosis formed between

*These authors contributed equally to this work.

legumes and soil rhizobia (Parniske, 2008). In the model legume *Medicago truncatula*, this so-called 'common SYM' pathway (Kistner & Parniske, 2002) includes a putative cation channel (MtDMI1) located in the nuclear membrane (Ané *et al.*, 2004; Riely *et al.*, 2007) and a leucine-rich repeat (LRR) receptor-like kinase (MtDMI2) of unknown function (Endre *et al.*, 2002). The third shared component (MtDMI3) is a nuclear-localized calcium and calmodulin-dependent kinase (CCaMK) (Mitra *et al.*, 2004; Smit *et al.*, 2005). Specific rhizobial lipochito-oligosaccharide signals, known as nodulation factors (NFs), trigger oscillations (or spiking) of the intracellular Ca^{2+} concentration (Oldroyd & Downie, 2006). This response is dependent on DMI1 and DMI2, whereas the downstream DMI3 CCaMK is believed to decipher the oscillating calcium signal, leading to the activation of a regulatory cascade and specific gene transcription (Gleason *et al.*, 2006).

The fact that hyphopodia are formed on roots of *dmi* mutants, but that these mutants are defective in both PPA formation and infection (Genre *et al.*, 2005), indicates that activation of the common SYM pathway is necessary for the epidermal cell remodelling which precedes fungal colonization. This leads to the hypothesis that Ca^{2+} signalling is required for the initiation of PPA formation (Genre *et al.*, 2005; Parniske, 2008). Evidence for a role for Ca^{2+} signalling during the early stages of the AM association has come from a study in *M. truncatula* showing that *DMI1/2*-dependent cytoplasmic Ca^{2+} spiking can be observed in a percentage of root hairs in the vicinity of ramifying AM fungal hyphae (Kosuta *et al.*, 2008). Since the responding root hairs were not contacted by the fungus, this implies that the putative fungal signal triggering this host response is probably diffusible, in agreement with findings from other studies using a variety of biological assays to detect symbiotic AM signals (Kosuta *et al.*, 2003; Olah *et al.*, 2005; Navazio *et al.*, 2007; Gutjahr *et al.*, 2009; Kuhn *et al.*, 2010).

As stated earlier, AM infection in the majority of legume and nonlegume hosts is initiated from hyphopodia formed on nonroot hair atrichoblasts. One of the principal objectives of this study was therefore to examine whether Ca^{2+} signalling responses could be observed in hyphopodia-contacted atrichoblasts before infection. For this purpose, we made use of a nuclear-targeted 'cameleon' calcium reporter (35S:NupYC2.1) expressed in root organ cultures (ROCs) of both *M. truncatula* and the nonlegume *Daucus carota*. ROCs are particularly interesting material for such studies since they can be successfully infected and colonized by AM fungi (Boisson-Dernier *et al.*, 2001), but are unable to associate with rhizobia (Akashi *et al.*, 2003). The NupYC2.1 reporter is a fusion between the nuclear protein nucleoplasmin and the cameleon YC2.1, and has recently been used to demonstrate rhizobial NF-elicited Ca^{2+} spiking within root hair nuclei of *M. truncatula* roots (Sieberer *et al.*, 2009). This nuclear-localized cameleon is a convenient tool for monitoring Ca^{2+} signalling in atrichoblasts, since these epidermal cells lack the cytoplasm-rich apical region characteristic of growing root hairs.

We report, in this article, that sustained nuclear Ca^{2+} spiking can be detected in hyphopodium-contacted epidermal cells of both *M. truncatula* and carrot roots, thus demonstrating that fungal-activated Ca^{2+} spiking occurs in the cell type targeted by AM hyphopodia, and is most probably part of an ancient plant signalling pathway predating the divergence between the rosid and asteroid clades. In addition, we have been able to show that exudates of germinated AM spores (but not purified rhizobial NFs) are able to trigger nuclear Ca^{2+} spiking in the outer root tissues of ROCs, and that this response is limited to the AM-responsive root zone. Ca^{2+} spiking responses to both hyphopodia formation and AM exudate application are dependent on genes of the common SYM pathway in *M. truncatula*, but independent of the *NFP* gene encoding the lysine motif-receptor-like kinase (LysM RLK) which mediates NF perception (Arrighi *et al.*, 2006). Together, these findings provide additional evidence that Ca^{2+} spiking is a key component of a highly conserved AM-activated signalling pathway required for intracellular fungal infection, and furthermore suggest that cameleon-expressing ROCs can provide valuable AM-specific bio-tests for the future characterization of fungal symbiotic signals.

Materials and Methods

Plant and fungal materials

Medicago truncatula genotype Jemalong A17 and the mutants *nfp-2* (Arrighi *et al.*, 2006), *dmi1-1*, *dmi2-2* and *dmi3-1* (Catoira *et al.*, 2000; Wais *et al.*, 2000) were used in this study, as well as the horticultural *Daucus carota* var. sativus. *Agrobacterium rhizogenes*-transformed ROCs expressing the 35S:NupYC2.1 construct (Sieberer *et al.*, 2009) were obtained according to Boisson-Dernier *et al.* (2001) for *M. truncatula* and to Bécard & Fortin (1988) for carrot. Transformed roots with a high level of nuclear fluorescence were selected 21 days after inoculation, decontaminated and subcultured on M medium at 25°C in the dark for subsequent use as ROCs. The AM fungi used in this study were *Gigaspora margarita* isolate BEG 34 (International Bank for the Glomeromycota, University of Kent, UK) and *G. gigantea* isolate HC/F E30 (Herbarium Cryptogamicum Fungi, University of Torino, Italy). The latter is characterized by a strong cytoplasmic autofluorescence (Séjalon-Delmas *et al.*, 1998).

For AM-targeting experiments, spores were pregerminated and placed in Petri dishes with fresh root cultures covered with Biofolie 25™ (Dutscher SAS, Brumath, France), as described in Genre *et al.* (2005). *Medicago truncatula*

ROCs were grown in vertically oriented Petri dishes to favour the development of a regular fishbone-shaped root system (Chabaud *et al.*, 2002). The nongravitropic carrot ROCs were initially grown horizontally and then switched to vertical growth following fungal inoculation in order to facilitate hyphal targeting of young lateral roots. The dishes were visually screened to detect highly ramifying hyphae and epidermal cells contacted by newly formed hyphopodia.

Production of the fungal exudate and root treatment

Batches of 100 surface-sterilized *G. margarita* spores were placed in 1 ml of sterile distilled water and incubated for 7 d at 30°C in the dark to induce germination (germination rate > 90%). The fungal germination medium was recovered by pipetting, concentrated 10-fold using a Lio5P lyophilizer (Cinquepascal, Milan, Italy) and then stored at −20°C.

Segments of primary roots carrying one or two young laterals (2–4 cm in length) were placed in a 2-mm-thick microchamber on a microscope slide containing sterile distilled water. After placing the root segments in the microchamber, they remained responsive to exudate for several hours. The water in the microchamber was rapidly (< 30 s) substituted by 100 μl of exudate before initiating confocal image acquisition. To prevent a cold-shock response, all solutions were warmed to 25°C before use.

For NF treatment, aqueous solutions at a final concentration of 10^{-8} M were prepared from a concentrated stock (10^{-3} M) of purified *Sinorhizobium meliloti* NF (kindly provided by Allan Downie, JIC, Norwich, UK), and root explants were treated as above. The activity of the NF stock was confirmed by eliciting epidermal-specific p*MtENOD11-GUS* reporter gene activity in seedlings of the transgenic *M. truncatula* line L416 (Charron *et al.*, 2004).

Confocal microscopy and measurement of changes in nuclear Ca^{2+} levels

Confocal microscopy was used for all the fluorescence resonance energy transfer (FRET)-based cameleon experiments described in this work, thereby avoiding the background root fluorescence which would have been encountered using epifluorescence microscopy and which could have masked potential Ca^{2+} responses in atrichoblasts. FRET-based ratio imaging for the detection and plotting of relative changes in nuclear Ca^{2+} levels corresponding to yellow fluorescent protein (YFP) to cyan fluorescent protein (CFP) fluorescence intensity changes over time (Miyawaki *et al.*, 1997, 1999) was performed according to Sieberer *et al.* (2009). The pinhole was set to 4–5 Airy Units in order to increase the thickness of the optical sectioning and thereby limit the likelihood of the nuclei moving out of focus during confocal scanning. A scanning resolution of 512 × 512 pixels was chosen to allow rapid imaging, and frames were collected every 5 s. Bright-field images were acquired simultaneously using the transmission detector of the microscope. For studies of hyphopodia-associated Ca^{2+} spiking, a minimum of 20 contacted epidermal cells from at least three independent roots (between 50% and 75% responding) was examined for each plant species and each mutant line, with the exception of *M. truncatula nfp-2* roots for which only six cells were observed. For exudate treatments, a minimum of 10 reactive root epidermal cells (approximately 75% responding) was observed for each treatment and, in all cases, the ratio imaging presented in Figs 4 and 5 was initiated immediately following exudate addition.

Statistical analysis

The width of 50 peaks for either hyphopodium-contacted (seven nuclei from six independent roots) or exudate-treated (12 nuclei from four independent roots) epidermal cells of *M. truncatula* roots was measured manually. As the data for the exudate-elicited responses do not fit a normal distribution (Kolmogorov–Smirnov test, $P < 0.05$), the respective median values were compared using the Kruskal–Wallis test.

Results

Hyphopodium development triggers nuclear Ca^{2+} spiking in *M. truncatula* and *D. carota* root epidermal cells

By using the *in vitro*-targeted inoculation system developed by Chabaud *et al.* (2002), we were able to visually monitor the progressive growth of *G. gigantea* hyphae until contact with the host root and the subsequent development of hyphopodia on the epidermal surface of *M. truncatula* ROCs expressing the 35S:NupYC2.1 construct (Sieberer *et al.*, 2009). Fungal contact sites were selected for FRET-based confocal microscopy (Fig. 1a,b), and a study of over 100 atrichoblast nuclei revealed significant nuclear Ca^{2+} spiking in almost one-half of these nonroot hair epidermal cells contacted by hyphopodia. The irregularity of the oscillatory signal is well illustrated in the 10-min-long recordings taken from three adjacent contacted epidermal cells shown in Fig. 1c–e. Fig. 2 shows that *c.* 45% of hyphopodium-contacted cells spiked between 3 and 10 times over the 10-min observation period. By comparison, the large majority of atrichoblasts from control noninoculated roots showed no spiking (e.g. Fig. 1k), and the remaining 30% a maximum of only one or two isolated spikes (Fig. 2). It is striking that the highest oscillation frequencies of 7–10 peaks per 10 min were only observed in cells for which the nucleus had moved to a position facing the site of hyphopodium

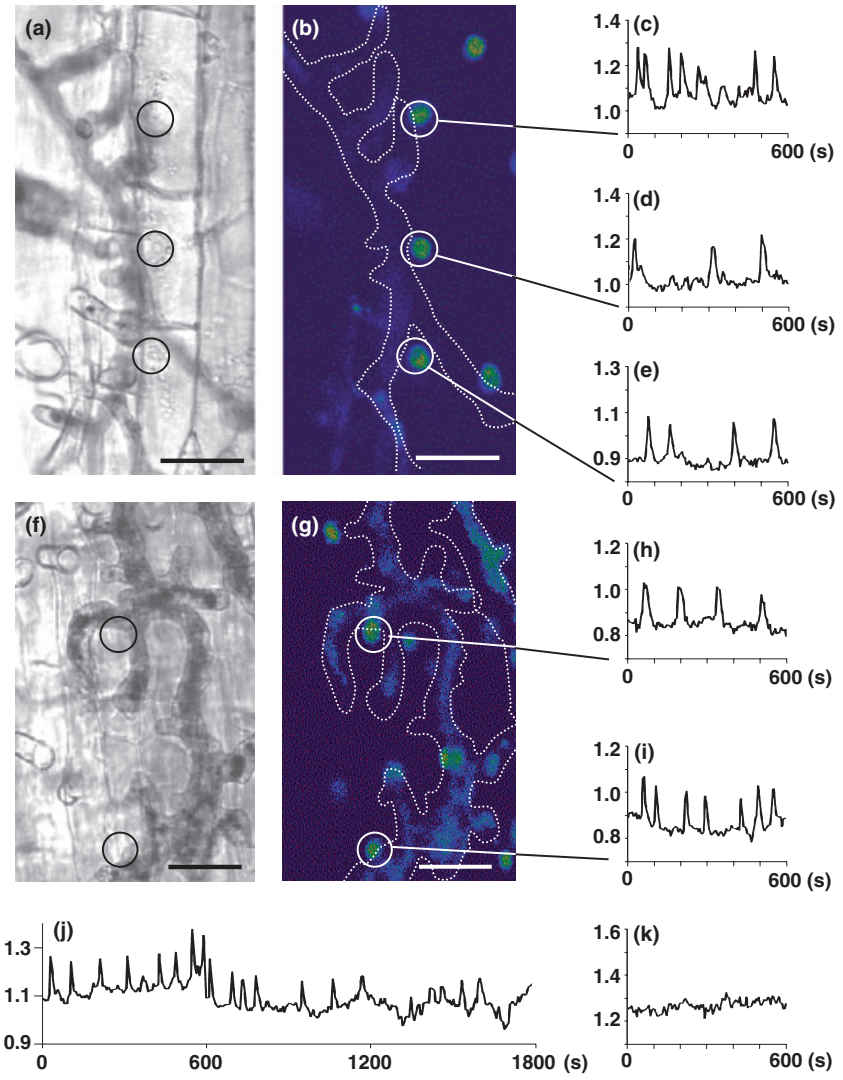

Fig. 1 Nuclear Ca^{2+} spiking in root epidermal cells contacted by arbuscular mycorrhizal (AM) hyphopodia. Top view of the epidermis of *Medicago truncatula* (a, b) and *Daucus carota* (f, g) roots contacted by *Gigaspora* hyphae. The bright-field images (a, f) show hyphopodia adhering to the root surface. The fungal outline is indicated by a dotted line in the corresponding pseudo-coloured fluorescence images (b, g) that show the NupYC2.1-labelled nuclei of the underlying epidermal cells. Circles identify the nuclei of hyphopodium-contacted cells (atrichoblasts) whose corresponding fluorescence resonance energy transfer (FRET) plots [yellow fluorescent protein (YFP) to cyan fluorescent protein (CFP) ratio] are presented in (c–e) and (h, i), and show repeated Ca^{2+} spiking over the 10-min acquisition period. (j) Nuclear spiking of a contacted epidermal cell of *M. truncatula* recorded continuously over 30 min. (k) Absence of Ca^{2+} spiking in an epidermal cell of a noninoculated *M. truncatula* root. Bars, 20 μm.

contact (Fig. 2). This transcellular migration corresponds to the first visible response of the epidermal cell nucleus to the formation of the hyphopodium (Genre *et al.*, 2005). Although only 15% of these cells showed high-frequency spiking during the 10-min observation period, statistical analysis confirms that this difference is significant with respect to cells for which the nucleus had not migrated.

In addition to those atrichoblasts directly contacted by AM hyphopodia, we also observed that a small percentage (~20%) of the surrounding epidermal cells (one to two cells distant from ramifying hyphae and hyphopodia) showed significant Ca^{2+} spiking with between three and six spikes over the 10-min period (Fig. 2). This suggests that diffusible fungal factors could be responsible for short-distance Ca^{2+} spiking responses in the epidermis, in line with the results of Kosuta *et al.* (2008). Finally, sustained nuclear Ca^{2+} spiking could be followed in certain hyphopodium-contacted epidermal cells for up to several hours (30 min continuous spiking is illustrated in Fig. 1j). In contrast with the variability in the spike periodicity, the width of the spikes in response to pre-infection AM contact was relatively homogeneous (27 ± 1.6 s). This spike duration lies within the 15–40 s range previously observed for NF-elicited nuclear spiking in root hairs (Sieberer *et al.*, 2009).

Since AM fungi can associate with *c.* 80% of angiosperms, and since endosymbiosis-associated Ca^{2+} spiking has never been studied outside of the legume family, we decided to extend this study to the nonlegume host *D. carota*. We therefore introduced the NupYC2.1 cameleon into ROCs of *D. carota* and performed similar *Gigaspora*-targeting experiments as for *Medicago*. Fig. 1f–i shows that nuclear Ca^{2+} spiking is also triggered in carrot atrichoblasts in contact with AM hyphopodia, with a similar spiking pattern to that observed in *M. truncatula*. We therefore conclude that nuclear Ca^{2+} spiking is a prepenetration response to AM hyphopodium formation on the host root epidermis and is shared by both legume and nonlegume hosts.

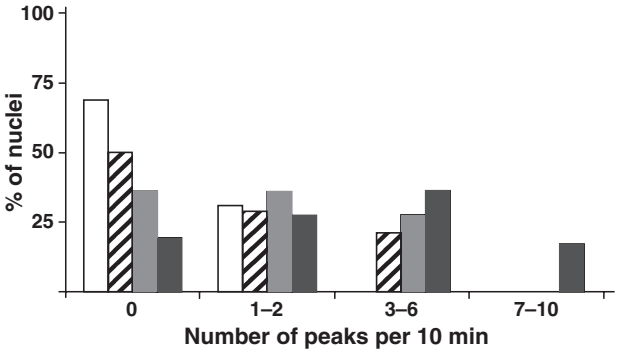

Fig. 2 Distribution of Ca^{2+} spiking responses in nuclei of hyphopodium-contacted and neighbouring epidermal cells. The levels of Ca^{2+} spiking responses, represented by the total number of peaks recorded over a 10-min observation period, are presented for the following four categories of epidermal cells: atrichoblasts from control noninoculated roots (white bars, n = 42); atrichoblasts not in direct contact with the arbuscular mycorrhizal (AM) fungus, but in the immediate vicinity (one to two cells distant) of hyphopodia (hatched bars, n = 52); atrichoblasts directly contacted by hyphopodia but without nuclear positioning facing the site of fungal attachment (grey bars, n = 47); and hyphopodia-contacted atrichoblasts with the nucleus facing the contact site (black bars, n = 88). High-frequency spiking nuclei (7–10 spikes per 10 min) were only observed in cells for which the nucleus had migrated towards the site of hyphopodium contact. Statistical analysis shows that the two populations of cells represented by grey and black bars are significantly different (χ^2 = 10.26, P < 0.01) with regard to the high-frequency spiking category of nuclei.

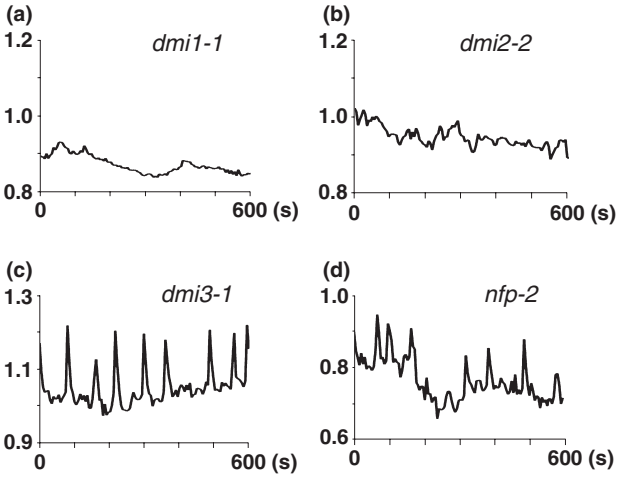

Fig. 3 *Medicago truncatula dmi1/2* mutants are defective in arbuscular mycorrhizal (AM) fungal-induced nuclear spiking. The Ca^{2+} spiking response induced by hyphopodium contact is absent in both *dmi1-1* (a) and *dmi2-2* (b) mutant lines. By contrast, hyphopodium-triggered Ca^{2+} spiking is normal for *dmi3-1* (c) and *nfp-2* (d) mutants.

The common SYM genes *DMI1* and *DMI2* are required for hyphopodium-elicited Ca^{2+} spiking in *M. truncatula*

In order to determine whether hyphopodium-induced Ca^{2+} spiking is dependent on the common SYM signal transduction pathway that is also implicated in rhizobial NF signal transduction (Parniske, 2008), we performed parallel experiments using NupYC2.1-expressing *M. truncatula* root cultures carrying mutations in the *DMI1*, *DMI2* and *DMI3* genes. Fig. 3 shows that nuclear Ca^{2+} spiking is absent in *dmi1-1* and *dmi2-2* mutant roots, whereas the *dmi3-1* mutant displays pronounced spiking in hyphopodium-contacted cells. This indicates that the hyphopodium-dependent signals which trigger nuclear Ca^{2+} oscillations are transduced via the common SYM pathway, and is consistent with the downstream role of the nuclear-localized CCaMK as the decoder of Ca^{2+} spiking (Oldroyd & Downie, 2006). Finally, experiments performed with a mutant line (*nfp-2*) defective for NF perception in *M. truncatula* showed that the activation of hyphopodium-induced epidermal Ca^{2+} spiking is not dependent on the LysM RLK which mediates NF recognition (Arrighi *et al.*, 2006) (Fig. 3d). This result is consistent with the fact that *nfp* mutants are not defective in AM colonization (Ben Amor *et al.*, 2003).

AM fungal exudate elicits *DMI1/2*-dependent nuclear Ca^{2+} spiking in the root epidermis

Based on the results of Kosuta *et al.* (2008) and our observation that Ca^{2+} responses are not limited to hyphopodium-contacted atrichoblasts, we then asked whether exudates of germinated AM spores (*G. margarita*), known to contain bioactive molecules (Navazio *et al.*, 2007; Gutjahr *et al.*, 2009), could also induce Ca^{2+} spiking in the *M. truncatula* root epidermis. We found that the application of a 10× concentrated fungal exudate induced pronounced nuclear Ca^{2+} spiking in the majority (*c.* 75%, n = 100) of the atrichoblasts located between 10 and 20 mm from the root tip (Figs 4b, 5a). This region corresponds to the developing and mature root hair zone, which is the primary target for AM root infection (Chabaud *et al.*, 2002; Genre *et al.*, 2005, 2008). Root hairs within this zone also responded to the spore exudate (Fig. 4c), although with poorly-defined and more irregular peaks. Water controls failed to elicit any Ca^{2+} spiking (Fig. 4d), and nonconcentrated (1×) fungal exudate only triggered low-frequency spiking (Fig. 4e). Finally, only very limited spiking was observed in the distal zone of the root (Fig. 4a), and no Ca^{2+} spiking was detected in the root elongation zone (Fig. 4f).

Similar results were obtained for the NupYC2.1-expressing carrot ROCs (Fig. 5b,c), further showing that the capacity of the host plant to respond to AM fungi via intracellular Ca^{2+} signalling is probably widespread among host plants. For both *Medicago* and carrot roots, Ca^{2+} spiking was generally initiated within the first 10 min following fungal exudate application and lasted for at least 30–40 min. As with the Ca^{2+} spiking responses to fungal

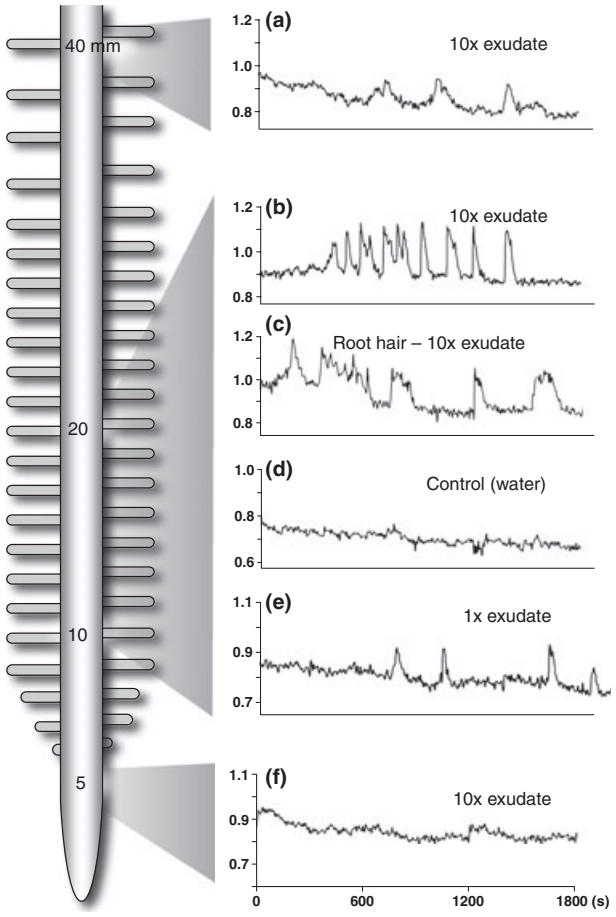

Fig. 4 Epidermal Ca^{2+} spiking responses to fungal exudate vary according to position and cell type along the *Medicago truncatula* root. (a) Treatment with 10× concentrated fungal exudate elicited only limited nuclear Ca^{2+} oscillations within atrichoblasts located 40 mm from the root tip. (b) Atrichoblasts located 10–20 mm from the tip showed the strongest Ca^{2+} spiking responses to 10× fungal exudate. (c) Root hairs within the 10–20-mm zone responded to 10× fungal exudate with broader and more irregular peaks. (d) No spiking was observed following treatment with distilled H_2O. (e) Spiking was less pronounced in atrichoblasts following 1× exudate application. (f) No response was observed to the fungal exudate within the root elongation zone.

hyphopodia, both plants showed a wide variability in the overall spiking pattern, with periodicities for *M. truncatula* ranging from two to five spikes per 10 min. Interestingly, the spike width median of 50 s (Fig. 6) was almost double that of the width median of the oscillations induced in response to hyphopodium contact. Finally, it should be underlined that treatment with purified *S. meliloti* NFs (10^{-8} M) failed to activate spiking in *M. truncatula* ROCs (20 cells analysed from four independent roots; Fig. 5d), in line with the fact that these roots cannot be nodulated by rhizobia (Akashi *et al.*, 2003).

It is also possible to visualize the nuclear Ca^{2+} spiking variability elicited by germinated AM spore exudates by concomitant time-lapse imaging of the YFP to CFP ratio for a number of adjacent outer root cells (Supporting Information Video S1). Interestingly, both epidermal and outer cortical cell nuclei were included within the selected focal plane, thus revealing that low-frequency spiking is also elicited in the outer cortex in response to the fungal exudate. This time-lapse illustrates the cell autonomy of the Ca^{2+} spiking responses in adjacent cells, a feature previously described for NF-induced cytoplasmic and nuclear spiking in *M. truncatula* root hairs (Miwa *et al.*, 2006; Sieberer *et al.*, 2009).

Finally, we confirmed that Ca^{2+} spiking elicited by the fungal exudate is blocked in the AM-responsive zones of *dmi1-1* (Fig. 5e) and *dmi2-2* (Fig. 5f) roots, and unaltered in epidermal cells of *dmi3-1* and *nfp-2* mutants (Fig. 5g,h, respectively), as shown earlier for hyphopodium-induced Ca^{2+} spiking. In conclusion, bioactive molecules present in AM fungal exudates trigger robust Ca^{2+} spiking in root epidermal cells of both legume and nonlegume ROCs. In *M. truncatula*, this response is dependent on the activation of the common SYM pathway, and is most pronounced in atrichoblasts located in the root zone susceptible to AM infection.

Discussion

Ca^{2+} spiking is part of an ancient signalling pathway required for AM infection

In vivo imaging of ROCs expressing the nuclear-specific NupYC2.1 cameleon construct has revealed that nuclear-localized Ca^{2+} spiking is induced in nonroot hair epidermal cells (atrichoblasts) in response to contact with AM fungal hyphopodia in both *M. truncatula* and *D. carota* roots. For *M. truncatula*, hyphopodium-elicited Ca^{2+} spiking is dependent on the *DMI1* and *DMI2* genes, but not on *DMI3*, consistent with the positioning of Ca^{2+} spiking downstream of DMI1 and DMI2 and the potential decoding of this secondary messenger by the nuclear CCaMK (Lévy *et al.*, 2004). Since the activation of the common SYM pathway is required for the organization of the PPA (Genre *et al.*, 2005), we hypothesize that the nuclear Ca^{2+} oscillatory response is a prerequisite for the initiation of this specific cellular remodelling which is essential for subsequent intracellular colonization by the AM fungus. In this context, it should be underlined that high-frequency nuclear spiking (7–10 peaks per 10 min) was only observed in atrichoblasts in which the nucleus had moved towards the hyphopodium contact site. This initial nuclear movement is the first observable cellular response to fungal contact and is independent of the *DMI1/2* genes (Genre *et al.*, 2005). Therefore, the physical proximity of the host nucleus to the adhered fungus may be a requisite for the establishment of the high-frequency Ca^{2+} spiking signature necessary for PPA formation. Since only 15% of cells with the nucleus positioned facing the hyphopodium show

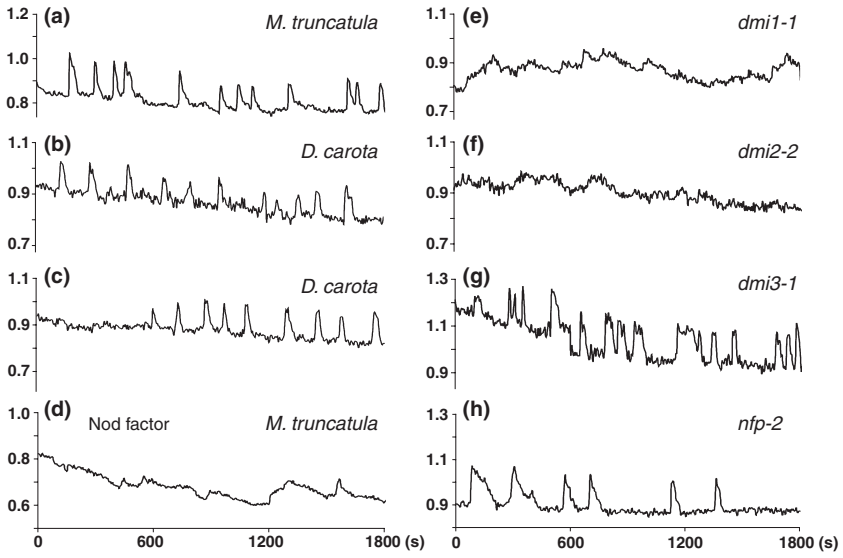

Fig. 5 Nuclear Ca^{2+} spiking in the *Medicago truncatula* and *Daucus carota* root epidermis treated with arbuscular mycorrhizal (AM) fungal exudate. (a–c) 10× fungal exudate induced nuclear Ca^{2+} spiking in atrichoblasts located in the responsive zone (10–20 mm from the tip) of *M. truncatula* (a) and carrot (b, c) root organ cultures. (d) No Ca^{2+} spiking was elicited in response to 10^{-8} M *Sinorhizobium meliloti* Nod factor treatment of *M. truncatula* roots. (e–h) Although fungal exudate failed to induce nuclear Ca^{2+} spiking in *dmi1-1* (e) and *dmi2-2* (f) mutants, Ca^{2+} responses were unaltered in *dmi3-1* (g) and *nfp-2* (h) mutants.

high-frequency spiking, we deduce that this particular signature is likely to be present during only a limited period following nuclear migration. Finally, the fact that Kosuta *et al.* (2008) were unable to observe Ca^{2+} spiking in root hairs several days after hyphae had contacted the epidermis presumably indicates that the fungal signal responsible for eliciting spiking is no longer being produced locally once the fungus has initiated infection. Indeed, our time lapse observations reveal that infection can be very rapid (within 24 h) following hyphopodium contact.

Notwithstanding the fact that common SYM genes (and in particular the gene encoding CCaMK) are highly conserved amongst all mycorrhizal plant species including bryophytes (Zhu *et al.*, 2006; Markmann *et al.*, 2008; Chen *et al.*, 2009; Wang *et al.*, 2010) and have been shown to be essential for the establishment of AM symbioses in monocots such as rice (Chen *et al.*, 2007; Banba *et al.*, 2008), this is the first time that a Ca^{2+} spiking response to symbiotic stimuli has been observed in a nonlegume. The fact that very similar nuclear Ca^{2+} spiking profiles can be detected in the carrot root epidermis in contact with AM hyphopodia argues that this response is a conserved element of AM fungal signal transduction predating the divergence between asterids and rosids, both belonging to the angiosperm eudicot clade (Wang *et al.*, 2009). Since Ca^{2+} spiking is a key feature of rhizobial NF signal transduction in both temperate and tropical legumes (Capoen *et al.*, 2010), it is therefore likely that the oscillatory intracellular Ca^{2+} signal is a hallmark of plant root endosymbioses across the plant kingdom.

Ca^{2+} spiking responses can be elicited by AM fungal exudates

Our results show that sustained nuclear Ca^{2+} spiking is not restricted to hyphopodium-contacted cells, but can also be induced in a small percentage of neighbouring cells which are not in direct contact with the fungus, albeit at lower spiking frequencies. This implies that Ca^{2+} spiking can be activated either by diffusible fungal factors and/or host cell-to-cell communication. The role of diffusible fungal factors would certainly be consistent with the observations of Kosuta *et al.* (2008) that cytosolic Ca^{2+} spiking responses can be elicited in noncontacted *M. truncatula* root hairs in the vicinity of ramifying AM hyphae. Furthermore, we have been able to show that nuclear Ca^{2+} spiking can also be activated by germinated spore exudates in both *Medicago* and carrot epidermal cells from the root zone susceptible to AM infection, thus indicating that AM signal molecules capable of activating the common SYM pathway are already secreted during AM spore germination in the absence of the host plant. Navazio *et al.* (2007) exploited similar fungal exudates to those used in this study to

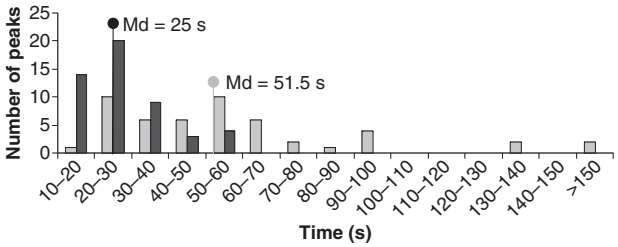

Fig. 6 Variability of spike width for exudate-induced Ca^{2+} spiking compared with that associated with hyphopodium-activated Ca^{2+} spiking. Spike widths were measured manually for 50 peaks in each case (black bars for hyphopodium-elicited spiking, and grey bars for exudate-induced spiking). In the case of the exudate-induced spikes, the data could not be modelled by a normal distribution (Kolmogorvo–Smirnov test, $P < 0.05$). The spike width medians (25 s for hyphopodium-elicited spikes vs 52 s for exudate-induced spikes) are statistically significantly different according to the Kruskal–Wallis test ($P < 0.05$).

elicit a rapid Ca^{2+} transient in aequorin-expressing soybean cells. However it is difficult to compare these results with those obtained using cameleon-transformed roots since differentiated root epidermal cells are likely to respond differently to the cotyledon-derived soybean cell suspensions.

Previous studies in *M. truncatula* have provided evidence for the existence of diffusible AM signals based on a variety of host responses including the activation of gene expression (Kosuta *et al.*, 2003; Kuhn *et al.*, 2010), the stimulation of lateral root branching (Olah *et al.*, 2005) and the activation of starch-related metabolic pathways (Gutjahr *et al.*, 2009). Certain of these responses appear to be SYM pathway-dependent (Olah *et al.*, 2005; Gutjahr *et al.*, 2009; Kuhn *et al.*, 2010), whereas others are not (Kosuta *et al.*, 2003; Kuhn *et al.*, 2010), thus suggesting that AM fungi generate a number of distinct biologically active molecules with different cellular targets and corresponding signalling pathways. We have found that Ca^{2+} spiking elicited by the fungal exudate in the responsive zone of the root is generally broader and more variable in width when compared with the relatively homogeneous spikes elicited by hyphopodium contact. This difference indicates that the bio-active molecules released by the presymbiotic mycelium may differ from the fungal signals generated following hyphopodium differentiation on the outer root surface.

During their co-evolution with symbiotic microbes, legumes may have needed to modulate Ca^{2+} spiking responses in order to discriminate between rhizobial and AM interactions. It is therefore intriguing that both cytoplasmic (Kosuta *et al.*, 2008) and nuclear (this paper) pre-infection Ca^{2+} spiking responses elicited in *M. truncatula* epidermal tissues in response to AM fungi appear to be significantly less regular than those elicited by rhizobial NFs in root hairs (Kosuta *et al.*, 2008; Sieberer *et al.*, 2009). However, it remains to be shown whether such differences in the pattern of Ca^{2+} spiking represent key determinants in transducing the respective endosymbiotic signals into appropriate cellular responses. Finally, the observation that NFs are unable to induce Ca^{2+} spiking in ROCs is of particular interest, since this implies that it is now possible to distinguish between the activation of the two endosymbiotic SYM-dependent signal transduction pathways. We therefore anticipate that cameleon-expressing ROCs will provide useful AM-specific bio-assays for the future identification and characterization of fungal signals perceived through the common SYM pathway.

Acknowledgements

We thank David Douds for kindly providing an inoculum of *G. gigantea* and Allan Downie for supplying the *S. meliloti* NF. This research was funded by an Italian Converging Technologies project funded by Regione Piemonte (ref. CIPE-BIOBIT), an Italian project (ref. PRIN 2008), a French National Research Agency project (ref. ANR-08-BLAN-0029-01) and a joint project financed by the French National Centre for Scientific Research (ref. PICS 4267). Confocal microscopy carried out at the Laboratory of Plant–Microbe Interactions made use of the Microscopy-Imagery Platform facilities belonging to the Federated Research Institute (IFR40), Pole of Plant Biotechnology, Toulouse, France. Confocal microscopy in Torino was performed at the Laboratory of Advanced Microscopy, Department of Plant Biology, Torino, Italy.

References

Akashi R, Kawano T, Hashiguchi M, Kutsuna Y, Hoffmann-Tsay SS, Hoffmann F. 2003. Super roots in *Lotus corniculatus*: a unique tissue culture and regeneration system in a legume species. *Plant and Soil* **255**: 27–33.

Akiyama K, Matsuzaki K, Hayashi H. 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* **435**: 824–827.

Ané JM, Kiss GB, Riely BK, Penmetsa RV, Oldroyd GED, Ayax C, Levy J, Debellé F, Baek JM, Kalo P *et al.* 2004. *Medicago truncatula DMI1* required for bacterial and fungal symbioses in legumes. *Science* **303**: 1364–1367.

Arrighi JF, Barre A, Ben Amor B, Bersoult A, Soriano LC, Mirabella R, de Carvalho-Niebel F, Journet EP, Ghérardi M, Huguet T *et al.* 2006. The *Medicago truncatula* lysine motif-receptor-like kinase gene family includes *NFP* and new nodule-expressed genes. *Plant Physiology* **142**: 265–279.

Banba M, Gutjahr C, Miyao A, Hirochika H, Paszkowski U, Kouchi H, Imaizumi-Anraku H. 2008. Divergence of evolutionary ways among common Sym genes: CASTOR and CCaMK show functional conservation between two symbiosis systems and constitute the root of a common signaling pathway. *Plant and Cell Physiology* **49**: 1659–1671.

Bécard G, Fortin JA. 1988. Early events of vesicular–arbuscular mycorrhiza formation on Ri T-DNA transformed roots. *New Phytologist* **108**: 211–218.

Ben Amor B, Shaw SL, Oldroyd GED, Maillet F, Penmetsa RV, Cook D, Long SR, Dénarié J, Gough C. 2003. The *NFP* locus of *Medicago truncatula* controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation. *Plant Journal* **34**: 495–506.

Boisson-Dernier A, Chabaud M, Garcia F, Becard G, Rosenberg C, Barker DG. 2001. *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. *Molecular Plant–Microbe Interactions* **14**: 695–700.

Buée M, Rossignol M, Jauneau A, Ranjeva R, Bécard G. 2000. The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. *Molecular Plant–Microbe Interactions* **13**: 693–698.

Capoen W, Oldroyd G, Goormachtig S, Holsters M. 2010. *Sesbania rostrata*: a case study of natural variation in legume nodulation. *New Phytologist* **186**: 340–345.

Catoira R, Galéra C, de Billy F, Penmetsa RV, Journet EP, Maillet F, Rosenberg C, Cook D, Gough C, Dénarié J. 2000. Four genes of *Medicago truncatula* controlling components of a Nod factor transduction pathway. *Plant Cell* **12**: 1647–1665.

Chabaud M, Venard C, Defaux-Petras A, Bécard G, Barker DG. 2002. Targeted inoculation of *Medicago truncatula in vitro* root cultures reveals *MtENOD11* expression during early stages of infection by arbuscular mycorrhizal fungi. *New Phytologist* **156**: 265–273.

Charron D, Pingret JL, Chabaud M., Journet EP, Barker DG. 2004. Pharmacological evidence that multiple phospholipid signaling pathways link Rhizobium nodulation factor perception in *Medicago truncatula* root hairs to intracellular responses, including Ca^{2+} spiking and specific ENOD gene expression. *Plant Physiology* **136**: 3582–3593.

Chen CY, Fan C, Gao MQ, Zhu HY. 2009. Antiquity and function of CASTOR and POLLUX, the twin ion channel-encoding genes key to the evolution of root symbioses in plants. *Plant Physiology* **149**: 306–317.

Chen CY, Gao MQ, Liu JY, Zhu HY. 2007. Fungal symbiosis in rice requires an ortholog of a legume common symbiosis gene encoding a Ca^{2+}/calmodulin-dependent protein kinase. *Plant Physiology* **145**: 1619–1628.

Endre G, Kereszt A, Kevei Z, Mihacea S, Kalo P, Kiss GB. 2002. A receptor kinase gene regulating symbiotic nodule development. *Nature* **417**: 962–966.

Genre A, Chabaud M, Faccio A, Barker DG, Bonfante P. 2008. Prepenetration apparatus assembly precedes and predicts the colonization patterns of arbuscular mycorrhizal fungi within the root cortex of both *Medicago truncatula* and *Daucus carota*. *Plant Cell* **20**: 1407–1420.

Genre A, Chabaud M, Timmers T, Bonfante P, Barker DG. 2005. Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in *Medicago truncatula* root epidermal cells before infection. *Plant Cell* **17**: 3489–3499.

Gleason C, Chaudhuri S, Yang TB, Munoz A, Poovaiah BW, Oldroyd GED. 2006. Nodulation independent of rhizobia induced by a calcium-activated kinase lacking autoinhibition. *Nature* **441**: 1149–1152.

Gutjahr C, Novero M, Guether M, Montanari O, Udvardi M, Bonfante P. 2009. Presymbiotic factors released by the arbuscular mycorrhizal fungus *Gigaspora margarita* induce starch accumulation in *Lotus japonicus* roots. *New Phytologist* **183**: 53–61.

Harrison MJ. 2005. Signaling in the arbuscular mycorrhizal symbiosis. *Annual Review of Microbiology* **59**: 19–42.

Kistner C, Parniske M. 2002. Evolution of signal transduction in intracellular symbiosis. *Trends in Plant Science* **7**: 511–518.

Kosuta S, Chabaud M, Lougnon G, Gough C, Dénarié J, Barker DG, Bécard G. 2003. A diffusible factor from arbuscular mycorrhizal fungi induces symbiosis-specific *MtENOD11* expression in roots of *Medicago truncatula*. *Plant Physiology* **131**: 952–962.

Kosuta S, Hazledine S, Sun J, Miwa H, Morris RJ, Downie JA, Oldroyd GED. 2008. Differential and chaotic calcium signatures in the symbiosis signaling pathway of legumes. *Proceedings of the National Academy of Sciences, USA* **105**: 9823–9828.

Kuhn H, Küster H, Requena N. 2010. Membrane steroid-binding protein 1 induced by a diffusible fungal signal is critical for mycorrhization in *Medicago truncatula*. *New Phytologist* **185**: 716–733.

Lévy J, Bres C, Geurts R, Chalhoub B, Kulikova O, Duc G, Journet EP, Ané JM, Lauber E, Bisseling T *et al.* 2004. A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. *Science* **303**: 1361–1364.

Markmann K, Giczey G, Parniske M. 2008. Functional adaptation of a plant receptor-kinase paved the way for the evolution of intracellular root symbioses with bacteria. *PLoS Biology* **6**: e68.

Mitra RM, Gleason CA, Edwards A, Hadfield J, Downie JA, Oldroyd GED, Long SR. 2004. A Ca^{2+}/calmodulin-dependent protein kinase required for symbiotic nodule development: gene identification by transcript-based cloning. *Proceedings of the National Academy of Sciences, USA* **101**: 4701–4705.

Miwa H, Sun J, Oldroyd GED, Downie JA. 2006. Analysis of calcium spiking using a cameleon calcium sensor reveals that nodulation gene expression is regulated by calcium spike number and the developmental status of the cell. *Plant Journal* **48**: 883–894.

Miyawaki A, Griesbeck O, Heim R, Tsien RY. 1999. Dynamic and quantitative Ca^{2+} measurements using improved cameleons. *Proceedings of the National Academy of Sciences, USA* **96**: 2135–2140.

Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, Ikura M, Tsien RY. 1997. Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* **388**: 882–887.

Navazio L, Moscatiello R, Genre A, Novero M, Baldan B, Bonfante P, Mariani P. 2007. A diffusible signal from arbuscular mycorrhizal fungi elicits a transient cytosolic calcium elevation in host plant cells. *Plant Physiology* **144**: 673–681.

Olah B, Brière C, Bécard G, Dénarié J, Gough C. 2005. Nod factors and a diffusible factor from arbuscular mycorrhizal fungi stimulate lateral root formation in *Medicago truncatula* via the *DMI1/ DMI2* signalling pathway. *Plant Journal* **44**: 195–207.

Oldroyd GED, Downie JA. 2006. Nuclear calcium changes at the core of symbiosis signalling. *Current Opinion in Plant Biology* **9**: 351–357.

Parniske M. 2008. Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature Reviews. Microbiology* **6**: 763–775.

Riely BK, Lougnon G, Ané JM, Cook DR. 2007. The symbiotic ion channel homolog DMI1 is localized in the nuclear membrane of *Medicago truncatula* roots. *Plant Journal* **49**: 208–216.

Séjalon-Delmas N, Magnier A, Douds DD, Bécard G. 1998. Cytoplasmic autofluorescence of an arbuscular mycorrhizal fungus *Gigaspora gigantea* and nondestructive fungal observations *in planta*. *Mycologia* **90**: 921–926.

Sieberer BJ, Chabaud M, Timmers AC, Monin A, Fournier J, Barker DG. 2009. A nuclear-targeted cameleon demonstrates intranuclear Ca^{2+} spiking in *Medicago truncatula* root hairs in response to rhizobial nodulation factors. *Plant Physiology* **151**: 1197–1206.

Smit P, Raedts J, Portyanko V, Debellé F, Gough C, Bisseling T, Geurts R. 2005. NSP1 of the GRAS protein family is essential for rhizobial Nod factor-induced transcription. *Science* **308**: 1789–1791.

Smith SE, Read DJ. 2008. *Mycorrhizal symbiosis*. London, UK: Elsevier.

Wais RJ, Galera C, Oldroyd G, Catoira R, Penmetsa RV, Cook D, Gough C, Dénarié J, Long SR. 2000. Genetic analysis of calcium spiking responses in nodulation mutants of *Medicago truncatula*. *Proceedings of the National Academy of Sciences, USA* **97**: 13407–13412.

Wang B, Yeun L, Xue JY, Liu Y, Ané JM, Qiu YL. 2010. Presence of three mycorrhizal genes in the common ancestor of land plants suggests a key role of mycorrhizas in the colonisation of land by plants. *New Phytologist* **186**: 514–525.

Wang H, Moore MJ, Soltis PS, Bell CD, Brockington SF, Alexandre R, Davis CC, Latvis M, Manchester SR, Soltis DE. 2009. Rosid radiation and the rapid rise of angiosperm-dominated forests. *Proceedings of the National Academy of Sciences, USA* **106**: 3853–3858.

Zhu HY, Riely BK, Burns NJ, Ane JM. 2006. Tracing nonlegume orthologs of legume genes required for nodulation and arbuscular mycorrhizal symbioses. *Genetics* **172**: 2491–2499.

Supporting Information

Additional supporting information may be found in the online version of this article.

Video S1 AM fungal exudate elicits cell autonomous nuclear Ca^{2+} spiking in *Medicago truncatula* wild-type roots expressing NupYC2.1.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.