

Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca^{2+} spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone

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Summary

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- The primary objective of this study was to identify the molecular signals present in arbuscular mycorrhizal (AM) germinated spore exudates (GSEs) responsible for activating nuclear Ca^{2+} spiking in the *Medicago truncatula* root epidermis.
- *Medicago truncatula* root organ cultures (ROCs) expressing a nuclear-localized cameleon reporter were used as a bioassay to detect AM-associated Ca^{2+} spiking responses and LC-MS to characterize targeted molecules in GSEs.
- This approach has revealed that short-chain chitin oligomers (COs) can mimic AM GSE-elicited Ca^{2+} spiking, with maximum activity observed for CO4 and CO5. This spiking response is dependent on genes of the common SYM signalling pathway (*DMI1/DMI2*) but not on *NFP*, the putative *Sinorhizobium meliloti* Nod factor receptor. A major increase in the CO4/5 concentration in fungal exudates is observed when *Rhizophagus irregularis* spores are germinated in the presence of the synthetic strigolactone analogue GR24. By comparison with COs, both sulphated and nonsulphated Myc lipochito-oligosaccharides (LCOs) are less efficient elicitors of Ca^{2+} spiking in *M. truncatula* ROCs.
- We propose that short-chain COs secreted by AM fungi are part of a molecular exchange with the host plant and that their perception in the epidermis leads to the activation of a SYM-dependent signalling pathway involved in the initial stages of fungal root colonization.

Introduction

Arbuscular mycorrhizal (AM) associations are ancient plant symbioses playing a central role in most land ecosystems, both by improving soil nutrient uptake and by contributing to protection against fungal pathogens. It has been proposed that the establishment of the AM endosymbiosis requires a molecular dialogue between the plant and fungal partners. Strong evidence suggests that a key component of this dialogue is the perception of host-secreted strigolactones, which activate fungal development and, in particular, the intense hyphal ramification in the vicinity of the root which precedes colonization of the root tissue (Akiyama *et al.*, 2005; Besserer *et al.*, 2006). However, the obligate biotrophy of AM fungi and the inability to perform genetics on these microbes have greatly hampered the characterization of the reciprocal fungal signalling molecules. Most of our current knowledge on symbiotic signalling derives from parallel studies on the *Rhizobium*–legume symbiosis, where it has been shown that these nitrogen-fixing bacteria synthesize specific decorated

lipochito-oligosaccharides (LCOs) termed Nod factors (Dénarié & Cullimore, 1993). In addition, plant genetics has led to the characterization of several genes essential for initiating both root endosymbioses (*MtDMI1*, *MtDMI2* and *MtDMI3* in the case of the model legume *Medicago truncatula*), thus defining a shared ‘common SYM’ signalling pathway (Parniske, 2008). This pathway also relies on calcium-mediated signalling, and in particular the triggering of nuclear-associated Ca^{2+} spiking (Oldroyd & Downie, 2006). Upstream of the common SYM pathway, perception of Nod factors requires the *MtNFP* gene, encoding a LysM Receptor-Like Kinase (Arrighi *et al.*, 2006). However, although *NFP* plays some role in host responses to Myc LCOs (Maillet *et al.*, 2011; Czaja *et al.*, 2012), it is not essential for AM colonization, since *nfp* mutants have a wildtype (WT) AM phenotype (Ben Amor *et al.*, 2003).

Since the bacterial symbiosis evolved more recently than the AM association (60 vs 450 million yr ago), it is currently thought that rhizobia have exploited the ancient mycorrhizal signalling pathway by mimicking symbiotic fungal signals. Therefore, by analogy with bacterial Nod factors, it has been proposed that AM fungi release signal molecules (Myc factors) essential for

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recognition of the fungal partner (Albrecht *et al.*, 1999; Catoira *et al.*, 2000). This has led to the hypothesis that modern legumes possess two separate branches that feed into the common SYM pathway, one for each type of root symbiont. Thus, hypothetical AM Myc factors should be able to elicit SYM pathway-dependent early plant responses that are specific to mycorrhizal colonization and their activity is likely to be independent on the NFP Nod factor receptor. In addition, and in contrast to Nod factors, Myc factors should be active on a wide variety of plant hosts (including, of course, nonlegumes), as there is no evidence for strict host specificity in the AM association.

A number of studies have indicated the production of diffusible fungal signals by either AM hyphae, germinating spores or mycorrhizal roots. The responses to these fungal signals include changes in intracellular calcium concentration (Navazio *et al.*, 2007; Chabaud *et al.*, 2011) and gene transcriptional activation (Kosuta *et al.*, 2003; Weidmann *et al.*, 2004; Drissner *et al.*, 2007; Kuhn *et al.*, 2010; Mukherjee & Ané, 2011; Ortu *et al.*, 2012). Not all of these responses are dependent on the common SYM pathway, suggesting the existence of alternative signalling pathways and possibly also a variety of fungal factors. Recently, LCOs have been identified at low concentrations in mycorrhizal root exudates and in germinating AM spore exudates using bioassays originally established for Nod factor characterization (Maillet *et al.*, 2011). These so-called Myc LCOs are active in stimulating lateral root development and overall levels of mycorrhization. Furthermore, genetic studies have shown that, as for Nod factors, enhanced lateral root development in response to Myc LCOs is SYM pathway-dependent. Finally, this biological response is totally or partially dependent upon the *NFP* gene depending on the Myc LCO concentration.

Since Ca^{2+} signalling in the form of sustained intracellular oscillations is a central feature of the common SYM pathway, *in vivo* fluorescent Ca^{2+} reporters known as cameleons have proved to be particularly useful tools for evaluating cellular responses to microbial symbiotic signals in the host epidermis (Miwa *et al.*, 2006; Kosuta *et al.*, 2008). Purified rhizobial Nod factors elicit characteristically regular Ca^{2+} spiking in both the nucleus and cytoplasm of *M. truncatula* root hairs (Ehrhardt *et al.*, 1996; Sieberer *et al.*, 2009), whereas germinated spore exudates (GSEs) of the AM fungus *Gigaspora margarita* trigger nonregular spiking in root epidermal atrichoblasts of the same legume as well as in the nonlegume *Daucus carota* (Chabaud *et al.*, 2011). Importantly, AM GSEs are active on *M. truncatula* root organ cultures (ROCs), consistent with the fact that ROCs can be successfully colonized by AM fungi. Furthermore, this AM-elicited calcium signalling is dependent on the common SYM genes *DMI1* and *DMI2* and independent of *NFP*. By contrast, rhizobia are unable to nodulate ROCs, and *Sinorhizobium meliloti* Nod factors fail to induce Ca^{2+} spiking in the ROC epidermis (Chabaud *et al.*, 2011).

In this article, we have made use of ROCs expressing the nuclear-localized Nup-YC2.1 cameleon as a Nod-independent bioassay to identify and characterize potential symbiotic signal molecules of AM fungal origin. We have discovered that the nonregular spiking observed in response to *G. margarita* GSEs

(Chabaud *et al.*, 2011; this study) is also observed in response to exudates of *G. rosea* and the distantly related glomeromycete *Rhizophagus irregularis*. By contrast, GSEs of the biotrophic pathogen *Colletotrichum trifolii* are unable to trigger spiking in the *M. truncatula* ROC epidermis. The ROC/cameleon bioassay, coupled to mass spectrometric analysis, have together revealed that short-chain chitin oligomers (COs) elicit an analogous nuclear spiking signature and furthermore are present in the AM GSEs. Importantly, this CO-activated Ca^{2+} signalling is SYM pathway-dependent and NFP-independent. The addition of the synthetic strigolactone GR24 results in a striking increase both in the CO concentrations in *R. irregularis* GSEs and in the Ca^{2+} spiking activity of these exudates. These findings lead to the proposal that short-chain chitin oligomers generated by AM fungi are novel signals perceived in epidermal tissues of host plants and capable of activating an AM-related signalling pathway.

Materials and Methods

Plant materials

Medicago truncatula Gaertn. genotype Jemalong A17 and the derived mutants *nfp-2* (Arrighi *et al.*, 2006), *dmi1-1* and *dmi2-2* (Catoira *et al.*, 2000; Wais *et al.*, 2000) were used in this study. *M. truncatula* ROCs expressing the 35S:NupYC2.1 construct (Sieberer *et al.*, 2009) were obtained previously (Chabaud *et al.*, 2011) and propagated in vertical dishes to favour a regular fish-bone-shaped root system (Chabaud *et al.*, 2002). Wildtype A17 and mutant *nfp-2* composite plants were obtained following transformation with *Agrobacterium rhizogenes* according to Boisson-Dernier *et al.* (2001). Two weeks after transformation, composite plantlets were transferred to vertical square dishes ($12 \times 12 \text{ cm}^2$) containing M medium (Bécard & Fortin, 1988) without sucrose, and covered with Biofolie 25TM (Lumox, Sarstedt, France) as described in Genre *et al.* (2005), and with the lower part of the dish wrapped in black plastic to protect the root from light. Roots from composite plants were used following 11–15 d of culture on M medium without sucrose.

Nongravitropic ROCs from the horticultural *Daucus carota* var. *sativus* expressing the 35S:NupYC2.1 were obtained previously (Chabaud *et al.*, 2011) according to Bécard & Fortin (1988) and were grown horizontally. The *Arabidopsis thaliana* ecotype Columbia was transformed by flower dipping with *A. tumefaciens* strain GV3101 carrying the 35S:NupYC2.1 construct according to Clough & Bent (1998). Transformed T1 and T2 progenies were selected on 50 mg l^{-1} kanamycin. Seeds from a single T2 line with high-level fluorescence were surface-sterilized and grown on M medium without sucrose in vertical dishes, with the lower part wrapped in black plastic. Lateral roots from 9-d-old plantlets were used for the test with chitin oligomers.

Fungal materials and preparation of germinated spore exudates

The AM fungi used in this study were *Gigaspora margarita* (BEG 34, International Bank for the Glomeromycota, University of

Kent, UK), *Gigaspora rosea* (DAOM 194757, National Mycological Herbarium, Ottawa, Canada) and *Rhizophagus irregularis* (previously called *Glomus intraradices* (DAOM 197198); Krüger *et al.*, 2012). Germinated spore exudates (GSEs) from *G. margarita* and *G. rosea* were prepared as described in Chabaud *et al.* (2011). Batches of 100 surface-sterilized *G. margarita* or *G. rosea* spores were placed in 1 ml of sterile H₂O and incubated in the dark for 7 d at 30°C (2% CO₂) to induce germination (germination rate > 90%). The GSE was recovered by pipetting, concentrated 10-fold using a lyophilizer and then stored at -20°C. One hundred microlitre aliquots (equivalent to 100 spores) were used in the ROC Ca²⁺ spiking bioassay. Chitinase treatment of the *G. margarita* GSE was performed using 1 mg ml⁻¹ chitinase from *Streptomyces griseus* (ref C6137; Sigma-Aldrich) in sterile H₂O for 16 h at room temperature. This enzyme complex degrades chitin to its monomer subunits.

To prepare *R. irregularis* GSEs, 125 × 10³ sterile spores (Agronutrition, Labège, France) were placed in 40 ml sterile H₂O and incubated for 7 d in the dark at 30°C (2% CO₂). When GSEs were prepared in the presence of strigolactone, GR24 was added to a final concentration of either 10⁻⁶ or 10⁻⁸ M. After incubation, the spores were removed by filtration (0.45 µm nitrocellulose filters; Millipore) and a butanol extraction performed on the GSE. The aqueous phase was lyophilized and resuspended in 100 µl H₂O. Mass spectrometric analysis was performed on a 10 µl aliquot (400-fold concentrated compared with the GSE – equivalent to 12.5 × 10³ spores), and the same quantity used in the ROC Ca²⁺ spiking bioassay after diluting 10-fold in H₂O (40-fold concentrated compared with the GSE).

Colletotrichum trifolii spores were prepared as described in Torregrosa *et al.* (2004). A total of 10⁷ spores were diluted in 100 ml of sterile H₂O. After 24 h incubation at 24°C, the germinated spores were pelleted by centrifugation at 5000 g for 15 min and the GSE was recovered for analysis. *C. trifolii* GSEs were lyophilized and resuspended in 400 µl H₂O. Mass spectrometry analysis was performed directly using 10 µl of the GSE (250-fold concentrated – equivalent to 2.5 × 10⁵ spores) and the ROC/Ca²⁺ spiking bioassay using 100 µl of the GSE concentrate.

Bioassays for Ca²⁺ spiking using roots expressing the NupYC2.1 reporter

Several cm-long young lateral root segments (second-order) from either ROCs or composite plants, or young third-order lateral roots grown under Biofolie membrane (to limit root hair development), were excised and placed in a microchamber and treated with 100 µl of the solution to be tested as described in Chabaud *et al.* (2011). In the case of *Arabidopsis*, the entire plantlet was placed on the slide, with the whole root system in the microchamber. Confocal FRET-based ratio imaging for detecting and plotting relative changes of nuclear Ca²⁺ concentrations corresponding to yellow fluorescent protein (YFP) to cyan fluorescent protein (CFP) fluorescence intensity changes over time (Miyawaki *et al.*, 1997) was performed according to Sieberer

et al. (2009) using a Leica TCS SP2 AOBS confocal laser-scanning microscope equipped with a long-distance HCX Apo L NA 40X 0.80 water-immersion objective or a HCX PL APO 40X 0.85 dry objective (Leica Microsystems GmbH, Wetzlar, Germany). Fluorescence intensity for both the CFP and YFP moieties of NupYC2.1 were collected, setting the pinhole diameter at 4–5 Airy units, exciting the probe at 458 nm (80% Ar laser) and recording the emitted fluorescence at 470–500 and 530–570 nm, respectively. Transmitted-light images were acquired simultaneously to confirm cell identity. Images were scanned at a resolution of 512 × 512 pixels and collected every 5 s over a period of 30 min after treatment. Average YFP and CFP fluorescence intensities were calculated for each nucleus using Leica LCS or Image J software (NIH, Bethesda, MD, USA). Values were then exported to a Microsoft Excel spreadsheet in order to calculate the YFP/CFP ratio for each time frame. Ratio values were then plotted over time to obtain a graphical representation of FRET intensity, corresponding to relative Ca²⁺ concentration variations in the nucleoplasm. As previously reported (Chabaud *et al.*, 2011), nuclear spiking in arachidoblasts was never observed in control untreated roots or after the addition of GSEs or chitin-based elicitors. Ca²⁺ peaks were then counted manually and frequency distribution histograms of peak numbers were prepared as shown in Fig. 3 and Supporting Information Fig. S2. The total numbers of epidermal cell nuclei and independent roots analysed for each test are presented in Table S1.

Biochemicals used for bioassays

Short-chain chitin oligomers were purchased from either Yaizu Suisankagaku Industry Corporation (Tokyo, Japan) or Seikagaku Biobusiness Corporation (Tokyo, Japan), and in addition CO3 and CO4 were kindly provided by Klaus Tietjen (Bayer Crop Science, Monheim, Germany). CO8 was kindly provided by Dr Naoto Shibuya from Meiji University, Kawasaki, Japan. The structure and purity of the commercial chitin oligomers were confirmed by MRM analysis, using two MRM transitions for each CO (see the following section). These analyses revealed that each CO was uncontaminated by either longer- or shorter-length COs and that there were no traces of any LCOs. In addition, the COs were fully *N*-acetylated. Stock solutions of chitin oligomers were prepared in H₂O at 10⁻³ M and stored at -20°C. *S. meliloti* Nod factor solutions were freshly prepared from a concentrated 10⁻³ M stock (ethanol : water (1 : 1 (v/v))). Both sulphated and nonsulphated Myc LCOs were kindly provided by Eric Samain, Hugues Dri-guez, Sébastien Fort (CNRS, Grenoble, France) and Fabienne Maillat, Jean Dénarié (LIPM, Castanet-Tolosan, France). Myc LCOs were freshly prepared as C16:1 : C18:0 (1 : 1) mixtures from concentrated 10⁻⁴ M stocks (acetonitrile : water (1 : 1 (v/v))). In the case of nonsulphated Myc LCOs, dilutions were performed in such a way as to ensure that the final acetonitrile concentration was at least 0.5%. Finally, biochemical analysis was used to confirm the concentrations of the less water-soluble molecules (CO8 and nonsulphated LCOs) used in the bioassays.

Biochemical analyses of GSEs

Chito-oligosaccharides were detected using a 4000 Q Trap mass spectrometer with a Turbo V ESI source in the positive mode, coupled to an Agilent 1100 series HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA). High-performance liquid chromatography (HPLC) separation was performed using a hypercarb column (5 μm , 2 \times 100 mm; Hypercarb, Thermo; Suginta *et al.*, 2009). For HPLC analysis, 10 μl of GSE solutions was directly injected. Solutions of acetic acid : water (1 : 10³ (v/v); A) and acetic acid : acetonitrile (1 : 10³ (v/v); B) were pumped at 0.3 ml min⁻¹. The gradient used was from 100% A to 100% B in 30 min.

The COs were identified in the MRM mode by monitoring the transitions from parent ion > common daughter ions (*m/z*: 204, 407, 610), and for quantification using the MRM transitions 628 > 204 *m/z* (CO3), 831 > 204 *m/z* (CO4), 1034 > 204 *m/z* (CO5), 1237 > 204 *m/z* (CO6), 1643 > 204 *m/z* (CO8). As an example, Fig. S1 illustrates a comparison of the MS/MS spectra for the CO4 standard and CO4 present in the AM fungal GSE. In addition, Table S2 compares the observed retention times and corresponding parent ions for short-chain CO standards and the GSE COs. Note that since retention times can vary slightly between experimental sessions, CO standards were always injected before and after the GSE samples. Blank controls confirmed the absence of residual signal carryover.

In order to determine the concentration of COs in the GSEs, a CO4 standard was added to the GSE before analysis. Since the CO4 signal obtained was threefold lower than expected due to the complexity of the matrix, this additional factor was integrated for determining the true concentrations of COs present within the GSEs.

Results

Short-chain chitin oligomers mimic AM exudate-elicited Ca²⁺ spiking activity in the host root epidermis

In order to determine to what extent the intracellular Ca²⁺ signaling previously observed in the host root epidermis in response to *G. margarita* spore exudates (Chabaud *et al.*, 2011) could be generalized to other AM fungal species, we isolated GSEs both from a second *Gigaspora* species (*G. rosea*) and from the distantly related *R. irregularis*. Fig. 1 shows that sustained Ca²⁺ spiking could be observed in *M. truncatula* ROC atrichoblasts expressing the nuclear-localized cameleon NupYC2.1 when treated with either the *Gigaspora* or *Rhizophagus* GSEs. In all cases, the spiking profiles were highly irregular in terms of both the periodicity and the total number of spikes recorded over the 30 min period following the addition of the exudates. No correlation could be established between the AM species and the Ca²⁺ spiking profile, which exhibited considerable cell-to-cell variation as previously reported for *G. margarita* (Chabaud *et al.*, 2011).

In order to characterize the bioactive molecules released by germinating AM fungal spores and responsible for triggering nonregular Ca²⁺ spiking in the host epidermis, we initially

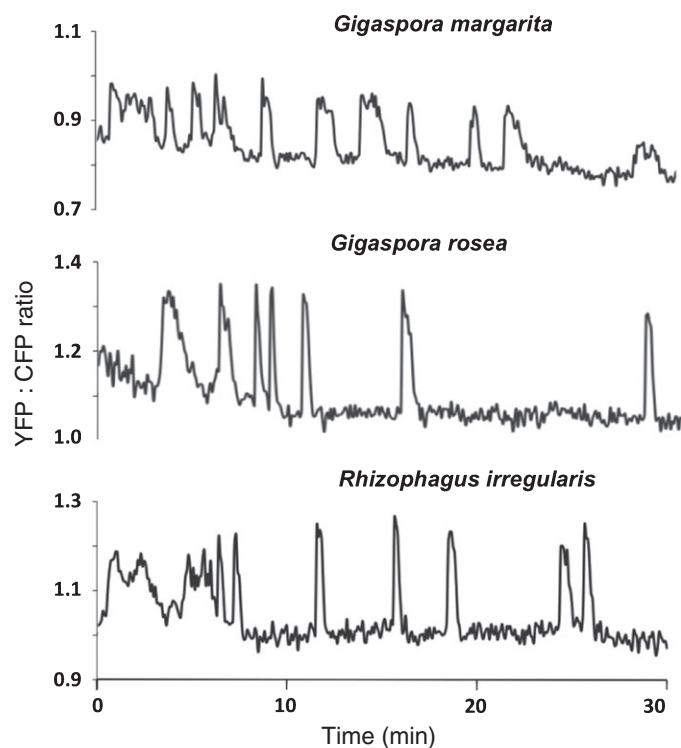
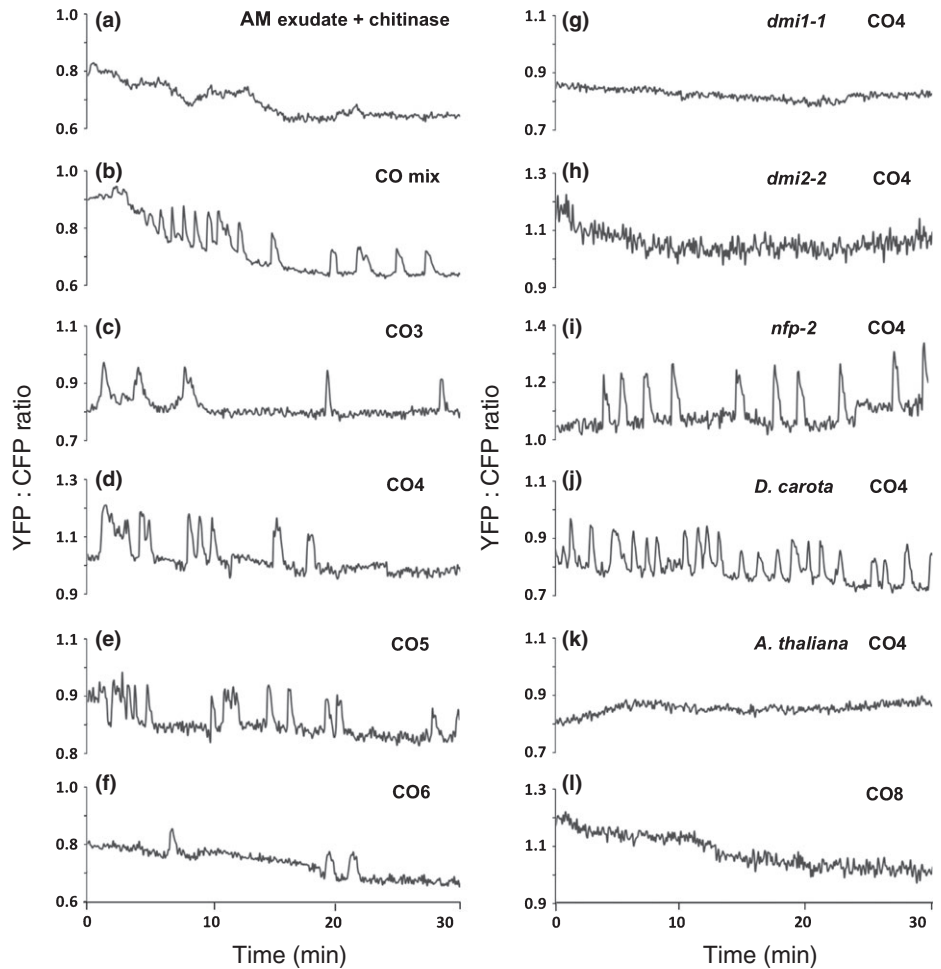


Fig. 1 Nuclear Ca²⁺ spiking in the *Medicago truncatula* epidermis in response to germinated spore exudates of various arbuscular mycorrhizal (AM) species. Nonregular Ca²⁺ spiking responses were observed in the nuclei of *M. truncatula* root organ culture (ROC) atrichoblasts expressing the nuclear cameleon NupYC2.1 after the addition of germinated spore exudates (GSEs) from both *Rhizophagus* and *Gigaspora* AM species (see the Materials and Methods section). Data are presented as yellow fluorescent protein : cyan fluorescent protein (YFP : CFP) ratios (arbitrary units) and roots were imaged every 5 s for 30 min following GSE treatment.

evaluated the chitinase-sensitivity of the *G. margarita* exudate. Fig. 2(a) shows that chitinase treatment completely abolished the spiking response, thus indicating a chitin-based structure for the putative symbiotic fungal factors. As part of a screen for potential candidate signals, initial tests performed on a mixture of chitin oligomers (COs) comprising 1–6 *N*-acetyl glucosamine residues revealed that the CO mix (10⁻⁸ M) was sufficient to activate sustained nonregular nuclear Ca²⁺ spiking in ROC epidermal cells (Fig. 2b). Subsequent experiments performed with the identical concentration of individual chitin oligomers (CO3–6; Fig. 2c–f) showed that maximal Ca²⁺ spiking was obtained with chitotetraose and chitopentaose (see Fig. S2a for a semiquantitative analysis). In all cases, the Ca²⁺ spiking profiles closely resembled the nonregular fungal GSE-elicited spiking. Finally, although all the Ca²⁺ responses presented in Fig. 2(a–f) were measured in ROC atrichoblast cells, short-chain COs also triggered nonregular spiking in root hair nuclei, as previously shown for *G. margarita* GSEs (Chabaud *et al.*, 2011).

Having established that short-chain COs were able to elicit nonregular Ca²⁺ spiking in *M. truncatula* ROCs, we then examined whether this response requires activation of the common SYM pathway. The fact that spiking failed to be elicited in either *dmi1* or *dmi2* mutant lines after addition of 10⁻⁸ M CO4 (Fig. 2g,h) implies that the common SYM pathway is indeed

Fig. 2 Short-chain chitin oligomers mimic the Ca^{2+} spiking activity of arbuscular mycorrhizal (AM) germinated spore exudates. *Medicago truncatula* root organ cultures (ROCs) expressing the NupYC2.1 cameleon were used as a bioassay to identify symbiotic AM signals. (a) Chitinase treatment of the *Gigaspora margarita* germinated spore exudate (GSE) suppresses Ca^{2+} spiking. (b) Nonregular Ca^{2+} spiking elicited in response to a 10^{-8} M mixture of chitin oligomers (CO1–6). (c–f) Representative Ca^{2+} spiking in response to individual short-chain COs (CO3–6) added at 10^{-8} M. (g–i) The CO-induced Ca^{2+} spiking response is dependent on the common SYM pathway genes *DMI1* and *DMI2*, but independent of the putative Nod factor receptor *NFP*. (j, k) 10^{-8} M CO4 elicits spiking in the root epidermis of the nonlegume AM host *Daucus carota* but not the nonmycorrhizal plant *Arabidopsis thaliana*. (l) As shown in Supporting Information Fig. S1(b), >50% of atrichoblasts fail to respond to the defense response elicitor CO8 (10^{-8} M). Data are presented as yellow fluorescent protein : cyan fluorescent protein (YFP : CFP) ratios (arbitrary units).



required for signal transduction upstream of nuclear Ca^{2+} signaling. Furthermore, our experiments also revealed that CO-elicited spiking was unaltered in an *nfp* mutant background (Fig. 2i). Thus, as for the *G. margarita* GSE, CO perception in ROCs is not dependent on the putative Nod factor receptor *NFP*.

In order to evaluate Ca^{2+} spiking responses to short-chain chitin oligomers in nonlegumes, we made use of available *D. carota* ROC lines expressing Nup-YC2.1 (Chabaud *et al.*, 2011). We had previously observed that *G. margarita* GSEs were able to activate spiking in carrot roots (Chabaud *et al.*, 2011), and Fig. 2(j) shows that 10^{-8} M CO4 is also an efficient trigger of Ca^{2+} spiking in this AM host. We then introduced the same nuclear-localized cameleon into the nonAM host *A. thaliana* (see the Materials and Methods section). Significantly, chitotetraose failed to elicit spiking in the root epidermis of *A. thaliana* plants (Fig. 2k).

Finally, in order to compare Ca^{2+} spiking responses between short-chain COs and longer COs known to elicit plant defence responses (Shibuya & Minami, 2001; Shimizu *et al.*, 2010), we treated *M. truncatula* ROCs with 10^{-8} M chitooctaose. Figs 2(l) and S2 show that CO8 is a very poor elicitor of spiking in *Medicago* roots, with responses even lower than those observed with CO6. In conclusion, short-chain chitin oligomers (CO4/5

are the most active) are able to trigger nonregular Ca^{2+} spiking in ROCs of AM host plants via the common SYM pathway and independently of *NFP*.

Strigolactone boosts short-chain CO concentration in AM germinated spore exudates

Having shown that short-chain COs elicit similar Ca^{2+} spiking responses in epidermal tissues of AM hosts as compared with AM fungal GSEs, we then investigated the chitin oligomer composition of these exudates (see the Materials and Methods section). Typical profiles obtained by multiple reaction monitoring (MRM) for CO3, CO4, CO5 and CO6 species are shown in Fig. S3. Quantitative data corresponding to the peak areas for each chitin oligomer are presented in Table 1, and reveal broadly similar concentrations for the four chitin oligomers. When these values are converted to concentrations using CO standards (see the Materials and Methods section), our data reveal that CO3–6 are each present in the *R. irregularis* GSE in the concentration range $0.3\text{--}1.2 \times 10^{-9}$ M. Since the Ca^{2+} spiking experiments were performed with $40\times$ concentrated GSEs, this means that the most active short-chain COs are present at a total concentration of *c.* 5×10^{-8} M. Similar results were obtained for GSEs of *G. rosea* (e.g. 0.9×10^{-9} M for CO4),

Table 1 Concentrations of short-chain chitin oligomers (COs) present in *Rhizopagus irregularis* germinated spore exudates (GSEs) are significantly increased in the presence of the synthetic strigolactone GR24

	GSE – GR24 (counts $\times 10^{-3}$)	GSE + GR24 (counts $\times 10^{-3}$)	GR24 stimulation
CO3	33.5 \pm 1.5	154 \pm 7.0	$\times 4.6$
CO4	44.7 \pm 2.3	1690 \pm 95	$\times 38$
CO5	28.7 \pm 1.7	1970 \pm 120	$\times 69$
CO6	17.0 \pm 5.6	263 \pm 18	$\times 15$

The addition of GR24 (10^{-6} M) to the germination medium of *R. irregularis* spores leads to major increases in CO concentrations and, in particular, in the concentrations of CO4/5. The figures (total counts $\times 10^{-3}$) refer to the sum of the peak areas corresponding to each CO species (both α and β forms). Note that the CO quantification presented in this table was performed on a different *R. irregularis* GSE from that illustrated in Supporting Information Fig. S3.

although detailed analyses were more difficult to perform for *Gigaspora* species because of the limited availability of spores. Thus, the amounts of short-chain COs present in the various AM GSEs used in the Ca^{2+} spiking bioassay are consistent with the spiking profiles obtained with purified 10^{-8} M COs (Fig. 2).

In the case of the endosymbiotic rhizobial/legume association, the perception of secreted plant flavonoids by the soil bacterium is essential for optimizing Nod factor synthesis before root infection. To date, strigolactones are the only signalling components of host origin found to be essential for the AM symbiosis (Gomez-Roldan *et al.*, 2008; Kretzschmar *et al.*, 2012). We therefore examined whether the addition of the synthetic strigolactone GR24 modified the concentrations of secreted COs present in the AM GSEs. The inclusion of 10^{-6} M GR24 in the germination medium of *R. irregularis* spores (see the Materials and Methods section) led to a major increase (*c.* 50-fold) in the concentrations of both chitotetraose and chitopentaose (Table 1). On the other hand, the increase was less important (five- to 15-fold) for the CO3 and CO6 species. In terms of nuclear Ca^{2+} spiking activity, the positive effect of strigolactone can be seen by comparing the responses observed in atrichoblasts treated with GSEs prepared in either the absence or presence of 10^{-6} M GR24 (Fig. S4). The major stimulation in short-chain chitin oligomer levels correlates with significantly enhanced spiking over the 30 min observation period. Indeed, when we add increasing concentrations of CO4 (up to 10^{-6} M) to *M. truncatula* ROCs, we observe an increase in the percentage of spiking atrichoblasts and also an overall increase in spiking intensity (not shown). In addition, it should be underlined that CO3–6 concentrations in the *R. irregularis* GSE were still enhanced even when the GR24 concentration was lowered to 10^{-8} M, but in this case increases were limited to between three and eightfold (not shown). By contrast, when 10^{-6} M GR24 was included in the germination medium of *G. rosea* spores, we observed only modest increases (three- to fourfold) in short-chain CO concentrations. Finally, we were unfortunately

unable to detect any LCOs in the butanol fractions of the *R. irregularis* GSEs tested. We presume that this is due to the low concentrations of Myc LCOs present in these GSEs (Maillet *et al.*, 2011) and the fact that the MS sensitivity for the detection of LCOs is *c.* 100-fold lower than that for COs.

Germinated spore exudates from a pathogenic fungus do not elicit Ca^{2+} spiking in ROCs

If, as these data suggest, the short-chain COs present in AM GSEs are primarily responsible for epidermal Ca^{2+} spiking responses in *M. truncatula* roots, exudates from other chitin-rich biotrophic fungi would also be expected to elicit spiking in the ROC bioassay. To evaluate this, we prepared exudates from germinated spores of the *M. truncatula* hemibiotrophic pathogenic fungus *Colletotrichum trifolii* (see the Materials and Methods section). Mass spectrometric analysis revealed that CO4 was indeed present at similar concentrations in these exudates compared with AM GSEs (not shown). However, to our surprise, *C. trifolii* exudates failed to elicit any Ca^{2+} spiking in the *M. truncatula* root epidermis and furthermore a negative response was obtained even when the exudate was supplemented with 10^{-8} M purified CO4 (Fig. S5). We therefore conclude that components of the pathogenic fungal exudate must be inhibiting the CO-elicited epidermal spiking.

Short-chain COs are more active than LCOs in eliciting NFP-independent Ca^{2+} spiking

Having shown that short-chain COs are efficient elicitors of NFP-independent Ca^{2+} spiking in *M. truncatula* ROCs, it was important to examine the activities of other known chitin-based symbiotic signals. We had previously demonstrated that 10^{-8} M *S. meliloti* Nod factors were unable to trigger spiking in ROCs (Chabaud *et al.*, 2011), and this result is presented in histogram format in Fig. 3. Indeed, even at the much higher concentration of 10^{-5} M, these sulphated LCOs elicited only a limited spiking response (Fig. 3). Strikingly, this Nod factor-elicited spiking in the ROC epidermis resembles the nonregular Ca^{2+} spiking triggered by AM GSEs and COs, and furthermore this response is NFP-independent (Fig. S6a,b). Together, this suggests that the identical signalling pathway is being activated by both 10^{-8} M COs and the 10^3 -fold higher concentrations of sulphated LCO.

Recently, Maillet *et al.* (2011) have described several LCO species present in low concentrations in exudates of AM-colonized carrot roots and in GSEs of *R. irregularis*. These LCOs are *N*-acylated with either C16:0 or C18:1 lipid chains and either sulphated or nonsulphated on the reducing end of the chitin backbone, but, unlike Nod factors, they are not *O*-acetylated (for a comparison of structures, see Fig. S7). Experiments using *M. truncatula* ROCs expressing the Nup-YC2.1 cameleon showed that 10^{-8} M sulphated Myc LCOs (a mix of C16:0 and C18:1) were totally unable to activate Ca^{2+} spiking (Fig. 3). However, as for the *S. meliloti* Nod factors, a low-level spiking response could be detected when the concentration of sulphated

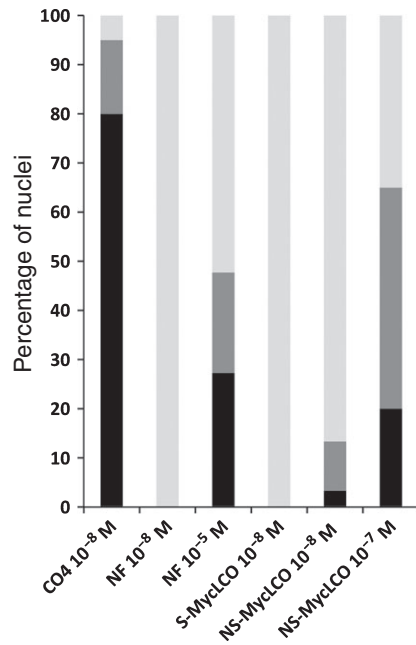


Fig. 3 Ca²⁺ spiking activities of various lipochito-oligosaccharides in the *Medicago truncatula* root organ culture epidermis. *Medicago truncatula* roots expressing NupYC2.1 were treated with different lipochito-oligosaccharides (LCOs) including *Sinorhizobium meliloti* Nod factors (NF) and both sulphated (S) and nonsulphated (NS) Myc LCOs at the indicated concentrations. For each treatment, between 15 and 45 atrichoblasts from at least two independent roots were imaged over 30 min (see also Supporting Information Table S1). The histograms show the percentage of nuclei with zero peaks (light grey), one to two peaks (dark grey) or \geq three peaks (black) throughout the entire 30 min imaging period.

Myc LCOs was increased to 10⁻⁵ M (not shown). By contrast, nonsulphated Myc LCOs elicited moderate spiking at 10⁻⁷ M (responses in *c.* 50% of cells) and a low-level spiking could be detected at 10⁻⁸ M (Fig. 3). Again, this nonregular spiking is maintained in an *nfp* mutant background (Fig. S6c,d), suggesting that Myc-LCOs are perceived in ROCs with a lower affinity by

the same NFP-independent pathway as short-chain COs. In conclusion, these data indicate that the addition of the sulphate residue and the fatty acid chain to the chitin backbone leads to a significant reduction in NFP-independent Ca²⁺ spiking activity in ROCs.

Althoughameleon-expressing ROCs have proved to be excellent tools to identify and characterize AM-specific signalling molecules, because of the lack of interference with host responses to rhizobial LCOs, it was essential to examine Ca²⁺ spiking responses elicited in whole plants by short-chain chitin oligomers. Since preliminary experiments had shown no differences in responses to either Nod factor or CO4 when applied to roots from either regenerated transgenic plants or WT composite plants (not shown), we present here a comparison of responses in both WT and *nfp* backgrounds exclusively using composite plants. A typical spiking profile elicited by 10⁻⁹ M *S. meliloti* Nod factors in root hairs of composite *M. truncatula* plants expressing 35S:Nup-YC2.1 is shown in Fig. 4(a). As previously demonstrated, this characteristic spiking is totally absent in an *nfp* mutant background (Ben Amor *et al.*, 2003), and we have confirmed that this is the case even when the Nod factor concentration is increased to 10⁻⁵ M (Fig. 4b). On the other hand, in line with the results obtained using ROCs, 10⁻⁸ M CO4 elicits a less frequent and less regular Ca²⁺ spiking profile in root hairs of the whole WT plant (Fig. 4c). Furthermore, the fact that the CO4-induced spiking is maintained in an *nfp* background (Fig. 4d) confirms that the putative Nod factor receptor is not required for AM-related signal perception in whole plants. In conclusion, the characteristics of the Ca²⁺ spiking response elicited by short-chain chitin oligomers appear to be identical whether in *M. truncatula* plants or root organ cultures, whereas spiking responses to LCOs such as *S. meliloti* Nod factors differ radically in terms of spiking signature, required LCO concentration and NFP dependency. These results argue that COs and sulphated LCOs activate distinct Ca²⁺-mediated signalling pathways in intact plants.

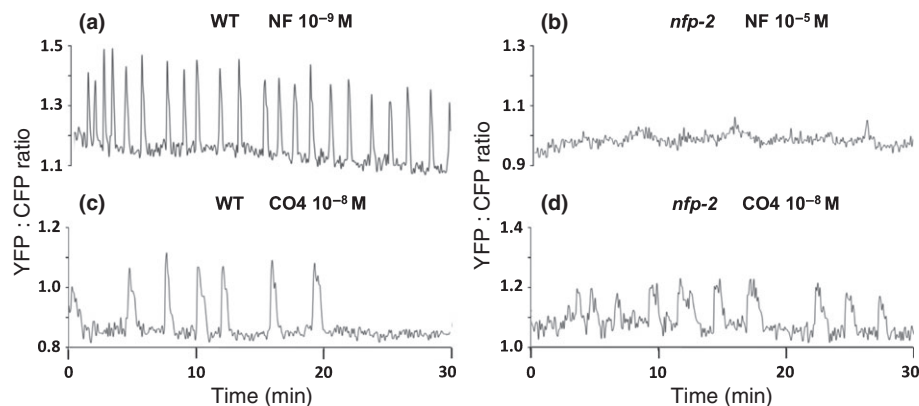


Fig. 4 Comparison of Ca²⁺ spiking responses elicited by either Nod factors or chitotetraose on roots of both wildtype (WT) and *nfp* mutant plants. Composite *Medicago truncatula* plants expressing NupYC2.1 were used to compare the activities of Nod factors (NF) and chitin oligomers (COs) on whole plants. (a) Characteristic high-frequency regular spiking elicited in a root hair of a composite WT plant following treatment with 10⁻⁹ M *Sinorhizobium meliloti* NF. (b) Absence of spiking in root hairs of a composite *nfp-2* mutant treated with 10⁻⁵ M NF. (c) As is the case for root organ cultures (ROCs), nonregular and lower frequency spiking is induced in root hairs of WT composite plants treated with 10⁻⁸ M CO4. (d) Similarly, and in contrast with NF, 10⁻⁸ M CO4 elicits nonregular spiking in roots of *nfp-2* composite plants. Data are presented as yellow fluorescent protein : cyan fluorescent protein (YFP : CFP) ratios (arbitrary units) and roots were imaged every 5 s for 30 min following NF/CO4 treatment.

Discussion

Short-chain chitin oligomers activate an AM-related signalling pathway

Essential steps of host-microbe recognition for both the rhizobial and AM endosymbioses take place before root colonization when the appropriate microbial signals are perceived by root epidermal tissues. This leads to the activation of respective host signal transduction pathways, of which a conserved 'common SYM' segment includes both the triggering of intracellular Ca^{2+} spiking (Oldroyd & Downie, 2006) and its decoding via the essential nuclear-localized calcium and calmodulin-dependent kinase (CCaMK = DMI3). By making use of *M. truncatula* root organ cultures expressing a nuclear-localizedameleon as a Nod-independent bioassay for AM signals, we provide evidence in this article that short-chain chitin oligosaccharides are able to trigger an AM-related signalling pathway and that the two most active chitin oligomers (CO4 and CO5) are present in AM GSEs at concentrations compatible with the Ca^{2+} spiking activities of the exudates. Furthermore, major increases in both the CO4/5 concentrations and the associated Ca^{2+} spiking activities of *R. irregularis* exudates were observed when the synthetic strigolactone GR24 was included in the germination medium. Besserer *et al.* (2006, 2008) have shown that the mitochondrial and energy metabolism of AM fungi is rapidly triggered in response to GR24. Therefore, the increase of COs observed here could simply be a consequence of a general metabolic boost. However, the fact that GR24-dependent increases in CO concentrations were significantly higher for the most active CO4 and CO5 compared with the other short-chain COs suggests that this qualitative modification in the exudates corresponds to a change in the physiological status of the AM fungus. By comparison, preliminary experiments failed to reveal any significant modification to short-chain CO concentrations in *C. trifolii* exudates in response to GR24 treatment (not shown). Genetic experiments have confirmed that the CO-elicited spiking in *M. truncatula* ROCs is dependent on the common SYM pathway genes *MtDMI1* and *MtDMI2*, and furthermore that inactivation of the *MtNFP* gene, encoding the LysM RLK essential for Nod factor perception (Arrighi *et al.*, 2006), did not modify the Ca^{2+} spiking activity of short-chain chitin oligomers. This is consistent with the recent demonstration that the putative Nod factor receptors from *Lotus japonicus*, NFR1 and NFR5, can directly bind *Mesorhizobium loti* Nod factors *in vitro* with a significantly higher affinity compared to the chitin oligomer CO5 (Broghammer *et al.*, 2012). Finally, the discovery that 10^{-8} M CO4 is equally active in eliciting nuclear Ca^{2+} spiking on roots of nonlegume hosts such as *D. carota* (and not on roots of the nonhost *A. thaliana*) provides additional evidence in favour of the hypothesis that chito-oligosaccharides are novel factors capable of activating an AM-related transduction pathway.

In this context, it is important to underline the fact that Walker *et al.* (2000) and Oldroyd *et al.* (2001) were the first to show that short-chain COs could elicit SYM pathway-dependent intracellular Ca^{2+} spiking in root hairs of pea and *M. truncatula*

seedlings respectively. At the time it was not envisaged that these molecules could be symbiotic fungal signals and the fact that experiments were performed on whole plants made it difficult to discriminate between 'Nod' and 'Myc' signalling. We show in this article that 10^{-8} M CO4 can elicit nonregular and *NFP*-independent spiking in the root epidermis of whole *M. truncatula* plants as well as in ROCs. The fact that much higher CO4 concentrations (10^{-4} M) were required to induce Ca^{2+} spiking in the experiments performed by Oldroyd *et al.* (2001) on root hairs of 1-d-old seedlings suggests that the sensitivity to CO signals probably depends on various factors, including the stage of root development (Chabaud *et al.*, 2011) and the epidermal cell type (atrachoblasts vs root hairs).

How do plants distinguish between chitin-based pathogenic and symbiotic signals?

Chitinaceous molecules are widespread microbial signals and their perception is essential for plants to discriminate between a variety of biotic stimuli. It is now known that receptors with extracellular LysM motifs are required for the recognition of both rhizobial LCOs in legumes (Limpens *et al.*, 2003; Radutoiu *et al.*, 2003) and chitin-based defence elicitors in model plants such as rice and *Arabidopsis* (Kaku *et al.*, 2006; Miya *et al.*, 2007). In the latter case, the length of the chitin oligomer appears to be crucial, since receptor affinity drops dramatically if the long-chain octamer is replaced by the short-chain tetramer or pentamer (Shibuya *et al.*, 1996). By contrast, our experiments based on the *M. truncatula* ROC bioassay have revealed the reverse correlation, with maximal Ca^{2+} spiking elicited by the chitin tetra/pentamers compared with the longer hexamer and octamer. Interestingly, Liu *et al.* (2012) have recently shown that activation of the AtCERK1 immune receptor requires long-chain chitin oligomer-induced dimerization, and that this activation is attenuated by the presence of short-chain oligomers such as CO5. Further work will, of course, be necessary to attribute a role for short-chain CO signals in the AM endosymbiosis, unrelated to plant defence. Evidence for the role of LysM-type receptors during root mycorrhization has recently come from RNAi studies using the nonlegume *Parasponia* (Op den Camp *et al.*, 2011), although it should be underlined that these findings relate to arbuscule formation in the root cortex rather than to the initial stages of root infection. Since the *M. truncatula nfp* mutant has a WT AM phenotype (Ben Amor *et al.*, 2003), it is conceivable that alternative LysM-type receptor(s) are involved in perceiving short-chain COs in *M. truncatula*. Detailed phylogenetic analysis of this extensive gene family within *M. truncatula* (Arrighi *et al.*, 2006) and across plant species (Zhang *et al.*, 2007), coupled with inactivation approaches for candidate genes and the AM-specific ROC bioassay, should now provide the means of identifying potential Myc factor receptors in both legume and nonlegume mycorrhizal plants.

It is also of interest in this context that, despite the presence of short-chain COs in the GSE of the pathogenic hemibiotrophic fungus *C. trifolii*, Ca^{2+} spiking was not detected in *M. truncatula* roots following treatment with the *Colletotrichum* exudate. Since

the addition of exogenous CO₄ (10⁻⁸ M) was unable to compensate for this lack of response, we deduce that *C. trifolii* GSEs contain either CO₄-inhibitors/competitors, or other components that prevent CO₄ activity. Finally, in the case of the hemibiotrophic association between the AM host *Oryza sativa* and *Magnaporthe oryzae*, Marcel *et al.* (2010) have shown that the rice CcCaMK homologue to *M. truncatula* DMI3 is not essential for this pathogenic interaction. This therefore suggests that a SYM pathway-related oscillatory Ca²⁺ signal is not required to establish this pathogenic fungal–plant association. Together, these findings argue that both the perception of short chitin oligomers and the activation in the root epidermis of nuclear Ca²⁺ spiking via the SYM pathway are specifically required to set in motion the AM fungal symbiosis. Additional experiments to show the link between CO-induced Ca²⁺-spiking and effective root colonization are required to confirm this hypothesis.

Roles for different chitin-based signals in the AM symbiosis

After many years of obscurity, the molecular language used by AM fungi to communicate to their plant hosts is finally emerging. Maillet *et al.* (2011) have identified several species of Myc LCOs present at low concentrations in exudates of AM-colonized carrot roots and *R. irregularis* GSEs. These Myc LCOs, as is the case for their rhizobial counterparts, are active in stimulating lateral root development (Maillet *et al.*, 2011), and, as a consequence, capable of increasing the root surface available for mycorrhizal colonization. Depending upon the concentration of Myc LCOs, this biological activity is partially or totally dependent on the putative Nod factor receptor NFP (Maillet *et al.*, 2011).

We now show that additional chitinous signals of AM origin in the form of nondecorated short-length chitin oligomers are able to activate a Ca²⁺-mediated signalling pathway in root epidermal tissues, most probably via a distinct LysM-type receptor. Our experiments using *M. truncatula* ROCs demonstrate that short-chain COs are more active than either sulphated or nonsulphated Myc LCOs in triggering AM-dependent Ca²⁺ spiking. Conversely, it has been shown that 10⁻⁸ M chitin tetraose is unable to stimulate root branching in *M. truncatula* plants (Oláh *et al.*, 2005) and that sulphated LCOs are more active than nonsulphated LCOs in stimulating lateral root development (Maillet *et al.*, 2011). In this context, a recent article describes a transcriptomic analysis of host gene expression in *M. truncatula* roots in response to either Nod factors or Myc LCOs (Czaja *et al.*, 2012). Despite the overall low levels of gene induction, the authors demonstrate that Myc LCO-induced gene expression is dependent on the *NFP* gene. This finding further underlines differences in the mechanism of host perception for AM-generated COs and LCOs. Together, this suggests that AM fungi may use two different chitin-based, signals as compared with a single multifunctional LCO for rhizobial species. Finally, it is striking that irregular Ca²⁺ spiking in the *Medicago* epidermis is elicited by AM fungal contact at sites of hyphopodia formation (Chabaud *et al.*, 2011), by all the *Gigaspora* and *Rhizophagus* GSEs tested so far (Fig. 1) and also by purified short-chain COs (Fig. 2). It remains to be determined what this irregular spiking signifies, and whether it

contributes to the specificity of downstream host cellular responses.

After spore germination in the soil, AM hyphae undergo intensive ramification in the immediate vicinity of host roots, probably due to the perception of secreted strigolactones. This ramification amplifies direct hyphal contact with the root outer surface and the differentiation of the preinfection adhesion structures known as hyphopodia. Mutations in the common SYM signalling pathway of *M. truncatula* result in failure to elaborate the intracellular prepenetration apparatus (PPA) following hyphopodium formation (Genre *et al.*, 2005) and this presumably leads to infection arrest at the root surface. It is therefore likely that critical fungal–plant signalling takes place at this particular stage of the interaction, and this is supported by the observation of intense Ca²⁺ spiking within nuclei of atrichoblasts directly contacted by hyphopodia (Chabaud *et al.*, 2011). The findings reported in this paper suggest that simple chitin oligomers (or closely related molecules) may be generated at the fungal–plant interface as part of the two-way preinfection molecular exchange involving strigolactones. From an evolutionary perspective, it is conceivable that the establishment of the dialogue between AM fungi and primitive host plants may have exploited simple products of their chitin metabolism as unsophisticated signals. This is also consistent with the fact that the AM association is both widespread and with no apparent host–fungal specificity. In addition to the triggering of common SYM pathway-dependent nuclear Ca²⁺ spiking, it will, of course, be important in the future to identify downstream mycorrhization-specific genes activated by short-chain COs. It is also an open question as to whether other important signalling molecules are generated at the fungal–host interface created after hyphopodium contact and adhesion. For example, the presence of surface signals, the secretion of additional effectors, and localized increase in exudate concentration could all contribute to the successful recognition of AM fungi as endosymbionts and their subsequent host-regulated entry into the plant root.

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Supporting Information

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

Fig. S1 Comparison of MS/MS spectra for the CO4 standard and the AM GSE.

Fig. S2 Semi-quantitative analysis of Ca²⁺ spiking activities of various chitin oligomers.

Fig. S3 Identification of short-chain COs in *Rhizophagus irregularis* GSEs.

Fig. S4 Effect of GR24 addition on the Ca²⁺ spiking activity of *R. irregularis* GSEs.

Fig. S5 *Colletotrichum trifolii* GSEs do not activate Ca²⁺ spiking in the *M. truncatula* root epidermis.

Fig. S6 Nonregular Ca²⁺ spiking elicited in *M. truncatula* ROCs by both rhizobial Nod factors and nonsulphated Myc LCOs.

Fig. S7 Structures of COs and LCOs tested in this article.

Table S1 Total number of roots/epidermal cells analysed for each root (ROC) treatment, corresponding to the data presented in Figs 1–3 and S2

Table S2 LC MS/MS retention times for parent ions corresponding to fully acetylated short-chain chitin oligomers (CO3–6) present in both commercial standards and AM GSEs

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