Mycorrhizal lipochitinoligosaccharides (LCOs) depolarize root hairs of \textit{Medicago truncatula}

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Abstract

Arbuscular Mycorrhiza and Root Nodule Symbiosis are symbiotic interactions with a high benefit for plant growth and crop production. Thus, it is of great interest to understand the developmental process of these symbioses in detail. We analysed very early symbiotic responses of \textit{Medicago truncatula} root hair cells, by stimulation with lipochitinoligosaccharides specific for the induction of nodules (Nod-LCOs), or the interaction with mycorrhiza (Myc-LCOs). Intracellular micro electrodes were used, in combination with Ca\textsuperscript{2+} sensitive reporter dyes, to study the relations between cytosolic Ca\textsuperscript{2+} signals and membrane potential changes. We found that sulfated Myc- as well as Nod-LCOs initiate a membrane depolarization, which depends on the chemical composition of these signaling molecules, as well as the genotype of the plants that were studied. A successive application of sulfated Myc-LCOs and Nod-LCOs resulted only in a single transient depolarization, indicating that Myc-LCOs can repress plasma membrane responses to Nod-LCOs. In contrast to current models, the Nod-LCO-induced depolarization precedes changes in the cytosolic Ca\textsuperscript{2+} level of root hair cells. The Nod-LCO induced membrane depolarization thus is most likely independent of cytosolic Ca\textsuperscript{2+} signals and nuclear Ca\textsuperscript{2+} spiking.

Introduction

Roots establish two symbiotic associations with microbes that both play an important role in plant nutrition. An ancient symbiosis with Arbuscular Mycorrhizal fungi (AM) is found in approximately 80\% of land plants, which evolved roughly 450 million years ago [1, 2]. The Root Nodule Symbiosis (RNS) emerged only within the last 60 million years and is confined to the family of legume plants (\textit{Fabaceae}) with the exception of Parasponia (\textit{Cannabaceae}) [3, 4]. Both, AM and RNS are of great importance for plant nutrition, as AM fungi supply plants with various nutrients from the soil [5], whereas the rhizobium bacteria in the nodules provide fixed nitrogen sources [6, 7].

Early steps in the development of AM and RNS are very similar, despite of the large differences in their morphology and the nature of the symbiotic microbes. Both, AM fungi and nitrogen-fixing bacteria exudate diffusible symbiotic signals, consisting of a chitinoligosaccharide
backbone (CO) that is composed of four, or five, N-acetylg glucosamine residues (GlcNAc). An N-acyl group is attached to the non-reducing terminal sugar and gives rise to molecules defined as lipochitinoligosaccharides (LCOs) [8, 9]. LCOs involved in Nodulation (Nod) can have further substitutions on the glucosamine subunits, such as methylation, acetylation, glycosylation and sulfation, moreover the length and degree of saturation of the N-acyl residue can vary [10–12]. Less is known about the LCOs extruded by AM fungi, but *Glomus intraradices* exudates a mixture of sulfated, and non-sulfated, simple LCOs, whereas Palmitic acid (C16:0) and Oleic acid (C18:1) are the major N-acyl substitutions. Structural differences between Nod-and Myc-LCOs are limited. Nod-LCOs carry an O-acetyl group at position 6 of the non-reducing end, which is missing in Myc-LCOs [9].

During the development of AM and RNS symbiotic relations, LCOs are recognized by pairs of lysin motif domain receptor like kinases (LysM-RLKs), located in the plasma membrane of the host plant cell. In *Medicago truncatula* Nod-LCOs are perceived by the LysM receptor kinase 3 (*MtLYK3*) and Nod factor perception (*MtNFP*) [13, 14], whereas the Nod factor receptors 1 (*LjNFR1*) and *LjNFR5* were found in *Lotus japonicus* [15, 16]. *MtLYK3* forms a receptor complex with the leucin rich repeat receptor like kinase (LRR-RLK) *MtDMI2* (*LjSYMRRK*), which is also essential for Nod-LCO perception [17, 18]. These Nod-LCO receptors are homologous to the receptors for chitin fragments, which trigger Microbe Associated Molecular Patterns (MAMPs)-dependent pathogen defense responses [19, 20].

Because of the strong similarity between Nod- and Myc-LCOs, both stimuli may affect the plasma membrane potential in a similar manner. We therefore used intracellular electrodes to measure membrane potential changes of *Medicago truncatula* root hairs to Nod- and Myc-LCOs. This approach revealed that root hairs depolarize upon stimulation with sulfated Myc-LCOs, but not in response to non-sulfated Myc-LCOs. Sulfated Myc-LCOs repress responses to Nod-LCOs, which suggests that both stimuli share components of early steps in the signaling pathway. Interestingly, no correlation was observed between the depolarization and intracellular Ca$^{2+}$ signals, which suggests that LCOs evoke a plasma membrane depolarization in a Ca$^{2+}$-independent manner.

**Materials and methods**

**Plant material and experimental conditions**

Seeds of *Medicago truncatula* Gaertn cv. Jemalong A17 and cv. R108-1 were surface-sterilized with concentrated sulphuric acid for approximately 10 min, rinsed six times with sterile distilled water and then kept in 2% (v/v) sodium hypochloride for 2 min. Thereafter, seeds were washed with sterile distilled water six times and seed moisture expansion was allowed for 4 h before seeds were spread on 1% (w/v) agar in water (Agar Agar Kobe I, Roth, www.carlroth.com). Plates were sealed with parafilm (Parafilm, www.parafilm.com), inverted, wrapped with aluminum foil and vernalized in 4˚C for 96 h [21, 22]. After vernalization, *M. truncatula* seeds were incubated for 24 h in the dark at room temperature. Germinated seeds were moistened with sterile distilled water (pH 7.0) to allow the removal of seed coats. For microelectrode impalement of root hair cells, the *M. truncatula* seedlings were grown for 2 h in a climate chamber with a light intensity 84 μmol m$^{-2}$ s$^{-1}$ on agar plates with the following solution 0.1 mM CaCl$_2$, 0.1 mM NaCl, 0.1 mM KCl and 5 mM MES/Tris, pH 7.0, supplemented with 1.5% (w/v) Plant Agar (Duchefa, www.duchefa-biochemie.com). Electrophysiological measurements were performed in a bath solution with 0.1 mM CaCl$_2$, 0.1 mM NaCl, 0.1 mM CsCl and 5 mM MES/Tris, pH 7.0. Seedlings incubated with this bath solution for 1–2 h were fixed in an experimental chamber with double-sided adhesive tape. Roots were immobilized with small stripes of parafilm.
Three different Myc-LCOs, synthesized by a two steps chemo-biotechnological process [9, 23] were used. Myc-LCO-IV-C18:1 and Myc-LCO-IV-C18:1- S are both N-acylated by oleic acid (C18:1) and differ only in the presence or absence of a sulfate group (S) on one glucosamine subunit. Myc-LCO-IV-C16:0-S is also sulfated, but it is N-acylated by palmitic acid (C16:0). The three Myc LCOs, as well as the *Sinorhizobium meliloti* Nod LCOs (NodSm-IV-C16:2-S), were dissolved in 50% (v/v) ethanol to obtain a 1 mM stock solution. For all measurements the Nod- and Myc-LCOs were diluted in bath solution to a concentration of 100 nM. LCOs were applied via a perfusion system based on gravitation.

**Experimental setup for impalement with microelectrodes and electrical configuration**

The experimental chamber with a *M. truncatula* seedling was mounted in the focal plane of an upright microscope (Axioskop 2FS, Zeiss, [http://www.zeiss.com](http://www.zeiss.com)). A water immersion objective (W Plan-Achroplan, 40x/0.8 W, Zeiss) was used to visualize the root hairs and to monitor the impalement of single root hairs with microelectrodes using a piezo-driven micromanipulator (MM3A, Kleindiek Nanotechnik, [www.nanotechnik.com](http://www.nanotechnik.com)). The microelectrodes were pulled from borosilicate glass capillaries (inner diameter 0.58 mm, outer diameter 1.0 mm, with filament; Hilgenberg, [www.hilgenberg-gmbh.com](http://www.hilgenberg-gmbh.com)) using a horizontal laser puller (P2000; Sutter Instruments, [www.sutter.com](http://www.sutter.com)). The reference electrode consisted of a 300 mM KCl, 2% agarose salt bridge, connected to an Ag/AgCl half-cell and it was placed in the bath solution.

The microelectrodes were filled with 300 mM KCl for membrane potential recordings and connected via Ag/AgCl half-cells either to the head stage of an IPA-2 microelectrode amplifier (Applicable Electronics, [www.applicableelectronics.com](http://www.applicableelectronics.com)), or an HS180 headstage (HS180, BioLogic, [http://www.bio-logic.info](http://www.bio-logic.info)). The input resistance of the headstages was either $>10^{11}$ (IPA-2) or $10^{11}$ Ω (HS180). Free running membrane potentials were recorded at 50 Hz, either using the WinEDR software [24, 25] (John Dempster, University of Strathclyde, [spider.science.strath.ac.uk](http://spider.science.strath.ac.uk)) with an NI USB 6259 interface (National Instruments, [www.ni.com](http://www.ni.com)) or with the PULSE software (v. 8.74; Heka [http://www.heka.com](http://www.heka.com)) using an LIH-1600 interface (HEKA). FURA2-dextran was current injected by applying a negative current of 4–10 nA for 1–2 min., through the electrode of which the tip was filled with 0.5 μl of 20 μM FURA2-dextran (Mw 10000, Thermo Fisher, [www.thermofisher.com](http://www.thermofisher.com)).

**Fluorescence microscopy**

The fluorescent calcium indicator dye FURA2-dextran was excited by 200 ms UV flashlight from a VisiChrome high-speed polychromator system (Visitron Systems, Germany; [www.visitron.de](http://www.visitron.de)), at 345- and 395-nm wavelengths and a time interval of 5 s [26, 27]. The FURA2-Dextran emission signal passed a dichroic mirror (FT 395; Zeiss, Germany) and was filtered by a 510-nm bandpass filter (D510/40M; Chroma Technology). All fluorescence signals were recorded by a Quantum 512SC camera (Photometrics, USA; [www.photometrics.com](http://www.photometrics.com)) using Visiview software (Visitron Systems). The Image J software package was used (FIJI version, National Institute of Health, [https://imagej.net/Welcome](https://imagej.net/Welcome)), to correct images for background fluorescence, calculate FURA2 ratio values and obtain pseudocolored images. The FURA2-dextran signals are shown as the ratio of the emission signals obtained with excitation light of 345 nm and 390 nm.

**Data analysis**

The membrane potential data recorded with the WinEDR, or the PULSE, software were imported and analyzed in Microsoft Excel. Average data and standard error of the mean (SE)
Fig 1. Nod-LCO induced plasma membrane potential depolarizations in root hairs of two *M. truncatula* genotypes. (A) Transmitted light image of a *M. truncatula* root hair impaled with a microelectrode. Scale bar represents 10 μm. (B) Representative membrane potential changes evoked by application of 100 nM Nod-LCO (NodSM-IV,
Results

Nod-LCO- and Myc-LCO-induced membrane potential depolarizations

The membrane potential depolarization of root hair cells represents one of the first steps evoked by Nod-LCOs in the host plants [28–30], but it is unknown if this response is provoked by Myc-LCOs. We tested the impact of a Sinorhizobium meliloti Nod-LCO (NodSM-IV, C16:2, S), on the membrane potential of M. truncatula roots using the micro electrode impalement technique (Fig 1A). Root hair cells were kept in bath solutions with Cs+ to block K+ uptake channels and hyperpolarize the plasma membrane potential. Under these conditions, membrane potentials were recorded that range from -138 mV to -187 mV (n = 37) in M. truncatula genotype R108-1, whereas slightly more hyperpolarized values from -156 mV to -224 mV were found in genotype A17 (n = 11). Stimulation with 100 nM of the Nod-LCO (NodSM-IV, C16:2, S) induced a transient depolarization in both genotypes, after a lag phase of 45 s (SE = 3.74, n = 32) in R108-1 and 34 s (SE = 2.6, n = 9) in A17 (Fig 1B and 1C). Despite of the less hyperpolarized membrane potential of R108-1, Nod-LCOs triggered a two-fold larger membrane depolarization in this genotype, as compared to A17 (Fig 1B and 1C). On average, the maximal depolarization was 21 mV (SE = 2.8, n = 37) in R108-1, whereas it was only 10 mV (SE = 2.7, n = 11) in A17. The time span to reach the depolarization maximum also varied, it was 211 s (SE = 10.7, n = 32) in R108-1, but only 109 s (SE = 7.9, n = 9) in A17.

Myc-LCOs have a similar chemical nature as Nod-LCOs and to some extend both stimuli provoke the same responses in host plants [31]. Indeed, root hair cells of M. truncatula R108-1 depolarized in response to the sulfated Myc-LCO (Myc-LCO-IV, C16:0, S) with the same magnitude, as to the Nod-LCO (NodSM-IV, C16:2, S; 19 mV, SE = 6.1, n = 9) (Fig 2A). However, the response to another Myc-LCO (Myc-LCO-IV, C18:1, S) was smaller (9 mV, SE = 3.6, n = 8) (Fig 2B). In contrast to both sulfated Myc-LCOs, the non-sulfated Myc-LCO (Myc-LCO-IV, C18:1) did not affect the membrane potential of M. truncatula root hair cells (Fig 2C).

Because of their similarities in chemical nature, Nod- and Myc-LCOs may compete for a single perception mechanism in root hairs. This possibility was tested by application of a sulfated Myc-LCO, prior to stimulation with Nod-LCOs. In line with a common perception module, the presence of the sulfated Myc-LCO repressed plasma membrane responses to Nod-LCOs (Fig 3A and 3B). However, non-sulfated Myc-LCOs were not effective, as root hairs remained responsive to the Nod-LCOs, despite of the presence of the non-sulfated Myc-LCOs (Fig 3C).

Nod-LCO-induced membrane depolarization precedes cytosolic calcium-spiking

Stimulus-induced depolarizations of the plant plasma membrane are often linked to a transient elevation of the cytosolic Ca2+ concentration [32, 33]. The ratiometric Ca2+ indicator FURA2 is rapidly transported into vacuoles of root hairs of M. truncatula and we therefore current injected FURA2-dextran with micro electrodes, which results in a stable cytoplasmic...
localization of the dye (Fig 4A). Stimulation with 100 nM Nod-LCO evoked regular oscillations of the cytosolic free Ca\(^{2+}\) concentration after a lag phase of approximately 8 min (Fig 4B and 4C). Pseudocolour images, indicating the FURA2 345 nm/390 nm excitation ratio, show that the repetitive increases in cytosolic Ca\(^{2+}\) concentration occurred predominantly in a...
Mycorhizal lipochitinoligosaccharides-induced depolarization of root hairs

Figure captions:

(a) 30
  20
  10
  0
  -10
  -5
  ΔV (mV)

Myc-LCO-IV
C16:0,S

Nod SM-IV
C16:2,S

(b) 20
  10
  0
  -10
  -5
  ΔV (mV)

Myc-LCO-IV
C16:0,S

Nod SM-IV
C16:2,S

(c) 25
  20
  15
  10
  5
  0
  -5
  -10
  ΔV (mV)

Myc-LCO-IV
C18:1

Nod SM-IV
C16:2,S
A subcellular compartment that includes the nucleus (Fig 4A). Repetitive spikes of the intracellular Ca$^{2+}$ concentration were observed in 16 out of 23 cells. The frequency and the shape of the Ca$^{2+}$ peaks varied between cells, but no elevation of the cytosolic Ca$^{2+}$ concentration was observed within the first minutes, during which the plasma membrane depolarizes (compare Figs 1 and 4). This indicates that the Nod-LCO-induced depolarization of the plasma membrane precedes the repetitive transient Ca$^{2+}$ elevations in the cytosol of root hairs.

We tested the hypothesis that the Nod-LCO-evoked depolarization occurs before the Ca$^{2+}$ signals are provoked, by re-impaling root hairs that were loaded with FURA2-dextran. In these cells, a KCl-filled electrode measures the membrane potential, while the cytosolic Ca$^{2+}$ level was monitored with FURA2 (Fig 4D). This approach revealed that Nod-LCOs first caused a depolarization of the plasma membrane, which is followed by the occurrence of repetitive Ca$^{2+}$ spikes that are associated with the nucleus.

**Discussion**

**Membrane depolarization**

A transient depolarization of the plasma membrane is an early response evoked by several biotic stimuli, in a variety of cell types. Previously, these responses have been described for Nod-LCOs, as well as the structurally related chitin fragments and several other MAMPs [28–30, 33–37]. Our study revealed that sulfated Myc-LCOs trigger a transient depolarization of *M. truncatula* root hairs, but non-sulfated Myc-LCOs did not. At a concentration of 100 nM, non-sulfated Myc-LCOs can stimulate root branching (RBS) [9], as well as the expression of symbiosis specific genes [22] and provoke Ca$^{2+}$ signals in cells of lateral roots [38], but they do not evoke a plasma membrane depolarization (Fig 2C). The depolarization of root cells may thus depend on another perception mechanism as the one that provokes Ca$^{2+}$ signals, root branching and gene expression.

In contrast to the recognition of non-sulfated Myc-LCOs, the perception mechanism of sulfated Myc-LCOs is likely to be shared with that of Nod-LCOs, as the successive application of two stimuli resulted only in a single transient depolarization (Fig 3A and 3B). This suggests that both types of stimuli are acting through the same signaling pathway. A similar conclusion was drawn by Sun et al. (2015), who measured similar cytosolic Ca$^{2+}$ signals evoked by Myc- or Nod-LCOs [38].

**Cytosolic Ca$^{2+}$-signals**

Changes in the cytosolic Ca$^{2+}$ concentration that occur simultaneously with the plasma membrane depolarization have been recorded earlier with intracellular ion-selective electrodes [39]. Depending on the side of impalement, these electrodes either recorded an increase, or decrease, of the cytosolic Ca$^{2+}$ level in growing root hair cells. These early Ca$^{2+}$ signals were not detected by other researchers that used FURA2-dextran in *M. truncatula*, suggesting that they are confined to the plasma membrane [40–42]. Instead, FURA2 or genetically encoded Ca$^{2+}$-sensors detected repetitive elevations of the Ca$^{2+}$ level after stimulation with Nod-LCOs, which occur at a later stage as the plasma membrane depolarization [27, 40–44].

Here we show that in root hairs of *M. truncatula*, the first transient increase of the cytosolic Ca$^{2+}$ concentration was not observed within 4 min. after application of Nod-LCOs (Fig 4B).
Mycorrhizal lipochitooligosaccharides-induced depolarization of root hairs

(a) Image showing root hair depolarization over time.

(b) Graph showing depolarization ratio over time.

(c) Graph showing depolarization ratio over time at a different scale.

(d) Graph showing a different type of depolarization response.

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Nod-LCO-induced depolarizations occurred earlier and normally peaked 3 min after application of the stimulus (Fig 1B). We thus conclude that the depolarization precedes the cytosolic Ca\(^{2+}\) signals associated with the nucleus, which was confirmed by simultaneous measurement of both responses (Fig 4D). The repetitive Ca\(^{2+}\) peaks are thus unlikely to provoke membrane potential changes, but instead activate Ca\(^{2+}\)/calmodulin-dependent protein kinases (CCaMKs) that are required for nodule formation [45–48]. Myc-LCOs trigger similar Ca\(^{2+}\) signals in root hair cells [38], which are also decoded by CCaMK leading to arbuscular branching [49].

**Outlook**

A plasma membrane depolarization is the earliest response that can be observed upon stimulation of *M. truncatula* root cells with Nod- or Myc-LCOs. This response even precedes the cytosolic Ca\(^{2+}\) signals in root hairs, which are a central element in the signaling chain that leads to nodule formation [31]. Even though the Nod-LCO-induced depolarization already was recognized in 1992 [28], we still do not know the nature of the ion channels that are addressed by the signaling pathway. Felle et al. (1998) showed that during the depolarization anions are released from roots and thus suggested that anion channels are activated by Nod-LCOs [50]. In *Arabidopsis* the SLAC/SLAH- and ALMT-gene families encode anion channels, of which all SLAC/SLAH- and several ALMT-members are targetted to the plasma membrane. Target repression of homologous genes in *M. truncatula* may reveal if genes of these families indeed encode anion channels that are activated by Nod- and Myc-LCOs.

The magnitude of the Nod-LCO-induced depolarization is strongly dependent on the genotype of the *M. truncatula* line that is studied (Fig 1B). Genotypic variation thus may be exploited to search for genes that are involved in the Nod- and Myc-LCO-dependent membrane potential change. This experimental approach may take advantage of mutant collections that have been generated for *M. truncatula* with use of the Tnt1 retrotransponson [51]. Future studies that adress the variation in plasma membrane responses, thus are likely to give insights in the sequence of events that occurs within the first minutes after stimulation with LCOs extruded by symbiotic bacteria and fungi.

**Supporting information**

S1 Table. Data of Nod-LCO induced plasma membrane potential depolarizations in root hairs of two *M. truncatula* genotypes, as shown in Fig 1.

(XLSX)

S2 Table. Data that reveal the impact of selected Myc-LCOs on the membrane potential of *M. truncatula*, genotype R108-1 root hair cells, as shown in Fig 2.

(XLSX)
S3 Table. Data on the interference of Myc-LCOs, with Nod-LCO-induced depolarization of root hairs, as shown in Fig 3.
(XLSX)

S4 Table. Data that reveal that the Nod-LCO-induced depolarization is not linked to cytosolic Ca$^{2+}$ signals, as shown in Fig 4.
(XLSX)

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