Evidence for calcium-mediated perception of plant symbiotic signals in aequorin-expressing Mesorhizobium loti

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Abstract

Background: During the interaction between rhizobia and leguminous plants the two partners engage in a molecular conversation that leads to reciprocal recognition and ensures the beginning of a successful symbiotic integration. In host plants, intracellular \( \text{Ca}^{2+} \) changes are an integral part of the signalling mechanism. In rhizobia it is not yet known whether \( \text{Ca}^{2+} \) can act as a transducer of symbiotic signals.

Results: A plasmid encoding the bioluminescent \( \text{Ca}^{2+} \) probe aequorin was introduced into Mesorhizobium loti USDA 3147T strain to investigate whether a \( \text{Ca}^{2+} \) response is activated in rhizobia upon perception of plant root exudates. We find that \( M. \text{loti} \) cells respond to environmental and symbiotic cues through transient elevations in intracellular free \( \text{Ca}^{2+} \) concentration. Only root exudates from the homologous host Lotus japonicus induce \( \text{Ca}^{2+} \) signalling and downstream activation of nodulation genes. The extracellular \( \text{Ca}^{2+} \) chelator EGTA inhibits both transient intracellular \( \text{Ca}^{2+} \) increase and inducible \( \text{nod} \) gene expression, while not affecting the expression of other genes, either constitutively expressed or inducible.

Conclusion: These findings indicate a newly described early event in the molecular dialogue between plants and rhizobia and highlight the use of aequorin-expressing bacterial strains as a promising novel approach for research in legume symbiosis.
A successful symbiosis is the result of an elaborate developmental program, regulated by the exchange of molecular signals between the two partners [3]. During growth in the rhizosphere of the host plant, rhizobia sense compounds secreted by the host root and respond by inducing bacterial nodulation (nod) genes which are required for the synthesis of rhizobial signal molecules of lipo-chito-oligosaccharide nature, the Nod factors. In the host plant, the generation of intracellular Ca\textsuperscript{2+} oscillations triggered by Nod factors has been firmly established as one of the earliest crucial events in symbiosis signalling; these oscillations are transduced into downstream physiological and developmental responses [1]. It is not known whether there is a parallel key role for Ca\textsuperscript{2+} in rhizobia.

As in eukaryotic cells, Ca\textsuperscript{2+} is postulated to play essential functions in the regulation of a number of cellular processes in bacteria, including the cell cycle, differentiation, chemotaxis and pathogenicity [4,5]. Homeostatic machinery that is able to regulate intracellular free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) tightly is a prerequisite for a Ca\textsuperscript{2+}-based signalling system, and is known to be present in bacteria [6]. Ca\textsuperscript{2+} transport systems have been demonstrated in bacteria, with the identification of primary pumps and secondary exchangers, as well as putative Ca\textsuperscript{2+}-permeable channels [5,7]. Other Ca\textsuperscript{2+} regulatory components such as Ca\textsuperscript{2+}-binding proteins, including several EF-hand proteins, have been detected and have been putatively identified from genomic sequences [8,9].

In order to establish precisely when and how Ca\textsuperscript{2+} regulates processes in bacteria it is essential to measure [Ca\textsuperscript{2+}]\textsubscript{i} and its changes in live cells. This has proven difficult because of problems in loading fluorescent Ca\textsuperscript{2+} indicator dyes, such as fura-2, into bacterial cells. However, the recombinant expression of the Ca\textsuperscript{2+}-sensitive photoprotein aequorin, which has been demonstrated to be a suitable method to monitor [Ca\textsuperscript{2+}]\textsubscript{i}, changes accurately in eukaryotes [10-12], has been successfully applied also to bacteria. Challenge of *E. coli* [13-17] and the cyanobacterium *Anabaena* sp. PCC7120 [18-21] expressing aequorin with different stimuli resulted in the induction of transient variations of [Ca\textsuperscript{2+}]\textsubscript{i}, with specific Ca\textsuperscript{2+} signatures.

Here we report the introduction of a plasmid encoding aequorin in *Mesorhizobium loti*, the specific symbiont of the model legume *Lotus japonicus*, and the use of this reporter to examine the Ca\textsuperscript{2+} response of rhizobia to abiotic and biotic stimuli. The results obtained highlight the occurrence in *M. loti* of Ca\textsuperscript{2+}-based mechanisms for sensing and responding to cues originating in the rhizosphere.

**Results**

**Construction of an inducible reporter system for Ca\textsuperscript{2+} measurements in rhizobia**

The aequorin gene was cloned in the broad host-range expression vector pDB1 [22] under the control of the strong synthetic promoter P\textsubscript{syn}, regulated by the lacI\textsuperscript{q} repressor (see Additional file 1). The pAEQ80 plasmid was mobilized by conjugation into the type strain of *M. loti* (USDA 3147\textsuperscript{t}).

**Validation of the experimental system**

The functioning in *M. loti* of the pAEQ80 plasmid containing the aequorin gene was verified by evaluating the level of aequorin expression in an *in vitro* reconstitution assay. Light emitted by total soluble protein contained in the lysates from wild-type and aequorin-expressing *M. loti* cells was monitored after reconstitution of the apoprotein with coelenterazine. The strong luminescence signal detected in protein extracts from *M. loti* cells containing the aequorin construct and induced with IPTG confirmed the efficient level of aequorin expression (see Additional file 2).

We analysed whether the introduced pAEQ80 plasmid (10.5 kb) encoding aequorin or the expressed protein could affect bacterial cell growth and the symbiotic performance of *M. loti* cells. There is no significant effect on bacterial growth kinetics exerted either by the introduced plasmid or aequorin expression. Nodulation efficiency of *M. loti* pAEQ80 cells on the specific plant host *Lotus japonicus* was checked 4 weeks after bacterial inoculation on roots of seedlings grown on nitrogen-free medium. *L. japonicus* roots were found to be effectively nodulated by the transformed bacterial strain, with no differences in nodule number (5 \(\pm\) 1) and morphological parameters in comparison to seedlings inoculated with wild-type *M. loti*. The presence of bacteria inside nodules was verified by light microscopy (see Additional file 2). Green foliage was indicative of functional symbiosis.

The occurrence in *M. loti* cells of homeostatic control of the internal Ca\textsuperscript{2+} activity was then verified by preliminary Ca\textsuperscript{2+} measurement assays in a luminometer after *in vivo* reconstitution of apoequorin. Unperturbed exponentially growing rhizobial cells showed a steady-state intracellular free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) residing in the submicromolar range (around 500 nM) (see Additional file 2), demonstrating a tight regulation of [Ca\textsuperscript{2+}]\textsubscript{i}. No luminescence was detected either in cultures of the non-recombinant strain incubated with coelenterazine or in recombinant cells that had not been exposed to coelenterazine (data not shown), confirming that the recorded signal was due only to Ca\textsuperscript{2+}-dependent light emission from aequorin.
Environmental stimuli are sensed through transient $[Ca^{2+}]_i$ elevations by *M. loti*

To further validate the experimental system, abiotic stimuli which are known to trigger $[Ca^{2+}]_i$ changes in both plants [23] and cyanobacteria [18,19] were applied to apoaequorin-expressing *M. loti* cells. A mechanical perturbation, simulated by the injection of isoosmotic cell culture medium, resulted in a rapid $Ca^{2+}$ transient increase ($1.08 \pm 0.24 \mu M$) that decayed within 30 sec (Fig. 1A). This $Ca^{2+}$ trace, which is frequently referred to as a "touch response", is often observed after the hand-operated injection of any stimulus [24]. A similar $Ca^{2+}$ response characterized by an enhanced $Ca^{2+}$ peak of $2.14 \pm 0.46 \mu M$ was triggered by a simple injection of air into the cell suspension with a needle (Fig. 1A).

Cold and hypoosmotic shocks, caused by supplying three volumes of ice-cold medium and distilled water, respectively, induced $Ca^{2+}$ traces with distinct kinetics, e.g. different height of the $Ca^{2+}$ peak ($1.36 \pm 0.13 \mu M$ and $4.41 \pm$...
0.51 μM, respectively) and rate of dissipation of the Ca2+ signal (Fig. 1B and 1C). As a control, cells were stimulated with three volumes of growth medium at room temperature, (Fig. 1B) resulting in a Ca2+ trace superimposable on that of the touch response (Fig. 1A). These findings eliminate the possible effect of bacterial dilution on changes in Ca2+ homeostasis.

Challenge of M. loti with a salinity stress, which has recently been shown to affect symbiosis-related events in Rhizobium tropici [25], resulted in a [Ca2+] elevation of large amplitude (3.36 ± 0.24 μM) and a specific signature (Fig. 1C).

Variations in the extracellular Ca2+ concentration determined the induction of transient Ca2+ elevations whose magnitude was dependent on the level of external Ca2+. After a rapidly induced increase in [Ca2+]i, the basal Ca2+ level was gradually restored with all the applied external Ca2+ concentrations (Fig. 1D), confirming a tight internal homeostatic Ca2+ control, as previously shown for other bacteria [14,18].

All the above results indicate that aequorin-expressing M. loti cells comprise a functionally valid system with which to investigate the involvement of Ca2+ in intracellular transduction of environmental stimuli.

Host plant root exudates induce in M. loti a Ca2+ signal required for activation of nodulation genes

Root exudates from the symbiotically compatible legume L. japonicus were collected from 3-week-old seedlings axenically grown in water and applied to M. loti cells. The dose used for Ca2+ measurements was in the range that induced significant expression of nodA, nodB, nodC genes in M. loti (Fig. 2A). This concentration was found to trigger a transient [Ca2+]i change characterized by a very rapid increase (1.38 ± 0.23 μM Ca2+) followed by a second sustained major Ca2+ peak (2.01 ± 0.24 μM) at about 10 min (Fig. 2B), with a slow decay within the considered time

Figure 2
Effect of plant root exudates and tetronic acid on [Ca2+]i and nod gene expression in M. loti. A. Analysis of gene expression by semi-quantitative RT-PCR during control conditions (lane 1, white bars) and after 1 h treatment with L. japonicus root exudates (lane 2, black bars) or 1.5 mM tetronic acid (lane 2, striped bars). Relative transcript abundance was normalized against 16S rRNA. Data are the means ± SEM of three independent experiments. B, Monitoring of [Ca2+]i changes in M. loti cells challenged (arrow) with L. japonicus root exudates (black trace) or 1.5 mM tetronic acid (grey trace).
interval (30 min). The observed induction of transient \([\text{Ca}^{2+}]_i\) changes in \(M.\ loti\) cells suggests a \(\text{Ca}^{2+}\)-mediated perception of signalling molecules contained in host plant root exudates.

Flavonoids are components of root exudates that play a prominent role as inducers of structural \(nod\) genes in rhizobia. Although flavonoids have been detected in \(L.\ japonicus\) seeds [26], those that specifically activate the expression of \(nod\) genes in \(M.\ loti\) have not yet been identified [27, 28]. The most common flavonoids, known as \(nod\) gene inducers in other rhizobia (10 \(\mu\)M naringenin, luteolin, daidzein, kaempferol, quercetin dehydrate) were not able to trigger transient \(\text{Ca}^{2+}\) elevations in \(M.\ loti\) (data not shown). Tetronic acid, an aldonic acid previously reported to promote \(Nod\) factor biosynthesis in \(M.\ loti\) [29], was found to induce a detectable \(\text{Ca}^{2+}\) response (Fig. 2B). The kinetics of the \(\text{Ca}^{2+}\) trace was similar to that induced by crude root exudates, with a prompt \(\text{Ca}^{2+}\) spike (1.36 ± 0.16 \(\mu\)M \(\text{Ca}^{2+}\)) and a subsequent flattened dome (maximal \(\text{Ca}^{2+}\) value of 1.29 ± 0.08 \(\mu\)M reached around 15 min after the elicitor application). Notably, this second phase of the \(\text{Ca}^{2+}\) transient induced by tetronic acid only partially accounted for the larger \(\text{Ca}^{2+}\) increase recorded with the whole \(L.\ japonicus\) root exudates (Fig. 2B). Likewise, the level of \(nod\) gene expression induced by tetronic acid was found to be lower (though significantly different from the control, \(P < 0.05\)) than that generated by total root exudates (Fig. 2A).

Pretreatment of rhizobial cells with the extracellular \(\text{Ca}^{2+}\) chelator EGTA for 10 min effectively inhibited both the transient \(\text{Ca}^{2+}\) elevation (Fig. 3A) and \(nod\) gene activation (Fig. 3B) induced by \(L.\ japonicus\) root exudates. This indicates that the main source of the observed \(\text{Ca}^{2+}\) response is the extracellular medium, and that the elevation in \([\text{Ca}^{2+}]_i\) is required for \(nod\) gene induction. Cell viability, monitored by the BacLight Bacterial viability assay, was not altered by incubation with the \(\text{Ca}^{2+}\) chelator (Fig. 3C). The expression of both constitutive (glutamine synthetase II and 16S rRNA) and inducible (aequorin) genes was not significantly affected by EGTA treatment (Fig. 3D and 3E), ruling out possible general effects of extracellular \(\text{Ca}^{2+}\) chelation on gene induction.

To check host specificity of the \(\text{Ca}^{2+}\) signal, metabolite mixtures exuded by the non-host legumes soybean and \(Vicia\ sativa\) subsp. \(nigra\) were tested. After an initial rapid and steep \(\text{Ca}^{2+}\) rise (1.77 ± 0.34 \(\mu\)M), shared also by the response to \(L.\ japonicus\) root exudates, the \(\text{Ca}^{2+}\) transients triggered by non-host exudates show very different kinetics, such as a slow rate of decay of the \(\text{Ca}^{2+}\) level (Fig. 4A versus Fig. 2B). Pretreatment with EGTA also blocked these transient \(\text{Ca}^{2+}\) elevations (data not shown). The distinct \(\text{Ca}^{2+}\) signature activated by non-host legumes, together with the lack of activation of \(nod\) genes (Fig. 4B), suggests the possibility of \(\text{Ca}^{2+}\)-mediated perception by \(M.\ loti\) of molecules other than \(nod\) gene inducers, such as non-specific chemoattractants or other signalling molecules, e.g. proteins [30, 31] or plant cell wall fragments released during the detachment of border cells from the root tip [32], activating a different \(\text{Ca}^{2+}\) signalling pathway. Further confirmation of the specificity of the host plant-induced \(\text{Ca}^{2+}\) signalling comes from the complete absence of any detectable \(\text{Ca}^{2+}\) change and \(nod\) gene transcriptional activation by root exudates from a non-legume (tomato) (Fig. 4A and 4B).

**Discussion**

Even though \(\text{Ca}^{2+}\)-based signal transduction processes are well-established to underpin plant cell responses to rhizobial informational molecules, a possible involvement of \(\text{Ca}^{2+}\) as a messenger in rhizobia in response to plant symbiotic signals has not hitherto been considered. We approached this issue by constructing a \(M.\ loti\) strain expressing the bioluminescent \(\text{Ca}^{2+}\) indicator aequorin. The highly sensitive and reliable aequorin-based method is widely used to monitor the dynamic changes of \([\text{Ca}^{2+}]_i\), in both eukaryotic [33] and bacterial [18, 16] living cells and represents to date the tool of choice for monitoring \(\text{Ca}^{2+}\) changes in cell populations [11]. The effectiveness of this recombinant technique has been verified at more than one level, and the results obtained demonstrate the utility of aequorin as a probe to study the early recognition events in rhizobium-legume interactions from the bacterial perspective.

The generation of a well-defined and reproducible \(\text{Ca}^{2+}\) transient in \(M.\ loti\) cells in response to root exudates of the host plant \(L.\ japonicus\) containing \(nod\) gene inducers is indicative of \(\text{Ca}^{2+}\) participation in sensing and transducing diffusible host-specific signals. It cannot be ruled out that the biphasic pattern of the \(\text{Ca}^{2+}\) trace (Fig. 2B), monitored by the aequorin method, may be due to an instantaneous synchronized \(\text{Ca}^{2+}\) increase in cells immediately after stimulation, followed by a sustained \(\text{Ca}^{2+}\) response probably due to the sum of asynchronous oscillations occurring in single cells. \(\text{Ca}^{2+}\) oscillations, considered as a universal mode of signalling in eukaryotic cells [34-36] have been proposed to occur in bacteria as well [37].

The significant inhibition of \(nod\) gene expression obtained when the \(\text{Ca}^{2+}\) elevation is blocked indicates that an upstream \(\text{Ca}^{2+}\) signal is required for \(nod\) gene activation. The \(\text{Ca}^{2+}\) dependence of \(nod\) gene expression strongly suggests that the \([\text{Ca}^{2+}]_i\) change, evoked by \(L.\ japonicus\) exudates, represents an essential prerequisite to convey the plant symbiotic message into rhizobia. All the above results fulfil the criteria required to demonstrate that a \(\text{Ca}^{2+}\) transient is a crucial intermediate in a stimulus-
Figure 3 (see legend on next page)
response coupling [23] and confirm that Ca2+ signalling is operating in bacteria [5].

The inability of root exudates from non-host legumes and non legumes to duplicate the response induced by L. japonicus exudates (encoded in a distinct Ca2+ transient and downstream gene expression) further supports the symbiotic specificity of the host legume-induced Ca2+ signature. The possible relatedness to legume-rhizobium symbiosis of the signals contained in non-host legume exudates is supported by the absence of any Ca2+ response to non-legume exudates. In non-host legume root exudates M. loti cells may sense signalling molecules related to the symbiotic process but not strictly specific to the compatible host-microsymbiont pair, which may enable rhizobia to distinguish non-host from compatible plants.

Plant root exudates contain a pool of molecules, both stimulatory and inhibitory, of potential relevance to the molecular signal exchange between the two partners [3]. The use of entire natural mixtures secreted by plant roots represents the first step in the evaluation of rhizobium reactions to plant factors, providing information on the global Ca2+ responses occurring in the bacterial partner early in the symbiosis, even before a physical contact between the two interacting organisms. Further insights

Figure 3 (see previous page)

Effect of EGTA on the Ca2+ response and nod gene expression induced by L. japonicus exudates. A, M. loti cells were treated with L. japonicus root exudates (black trace) or pretreated with 5 mM EGTA 10 min before adding L. japonicus root exudates (grey trace). B, Top: RT-PCR analysis of control cells (lane 1), cells treated for 1 h with L. japonicus root exudates (lane 2) and cells pretreated with 5 mM EGTA 10 min before treatment with L. japonicus exudates (lane 3). Bottom: Relative percentage of nod gene induction in response to L. japonicus exudates in M. loti cells pretreated (striped bars) or not (black bars) with 5 mM EGTA. Normalization of transcript abundance was done against 16S rRNA. Data are the means ± SEM of three independent experiments. C, Viability, monitored with the BacLight Bacterial Viability kit, of M. loti cells in control conditions or incubated with 5 mM EGTA for 1 h 10 min. As positive control, cells were treated with 70% isopropanol. Live cells fluoresce green, dead cells fluoresce red. Bar = 10 μm. D, Top: RT-PCR analysis of the expression of the housekeeping gene glutamine synthetase II (GSII) in M. loti cells in the absence (-) or presence (+) of 5 mM EGTA. Bottom: Relative transcript abundance of GSII was normalized against 16S rRNA. Bars represent SEM. E, Top: RT-PCR analysis of the inducible aequorin (aeq) gene in M. loti cells in the absence (-) or presence (+) of 5 mM EGTA and 1 mM IPTG. Bottom: Relative transcript abundance of aeq was normalized against 16S rRNA. Bars represent SEM.

A

B

Figure 4

Monitoring [Ca2+] and nod gene expression in response to non-host legume and non-legume root exudates. Bacteria were challenged with root exudates from soybean (A, black trace; B, lane 2), V. sativa subsp. nigra (A, grey trace; B, lane 2) and tomato (A, light grey trace; B, lane 2). Control cells were treated with cell culture medium only (B, lane 1).
into the dynamics of the activated Ca$^{2+}$ change may come from the comparison with the Ca$^{2+}$ responses obtained by using fractionated root exudates or purified molecules. This would enable to assess the possible placement of the Ca$^{2+}$ signal within the NodD-flavonoid gene expression paradigm [38] in different species of rhizobia.

**Conclusion**

The above results demonstrate that *M. loti* cells sense host plant symbiotic cues through Ca$^{2+}$ and indicate that activation of nod genes requires an upstream Ca$^{2+}$ signal. Transgenic rhizobium strains expressing aequorin can be used as a novel approach to the dissection of early events in legume-rhizobium symbiosis, that may shed light on a previously uninvestigated facet - bacterial Ca$^{2+}$ signalling - of the two-way partner signal exchange and transduction.

**Methods**

**Chemicals**

Native coelenterazine was purchased from Molecular Probes (Leiden, The Netherlands). Molecular biology reagents were purchased from Promega Co. (Madison, WI, USA), Qiagen (Hilden, Germany) Clontech (Mountain View, CA, USA) and Invitrogen (Paisley, UK). Tetronic acid was obtained from Titolchimica (Rovigo, Italy). Flavonoids (naringenin, luteolin, daidzein, quercetin dehydrate) and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

**Bacterial strains and growth conditions**

*Mesorhizobium loti* strain USDA 3147$^T$ was kindly provided by Peter Van Berkum (USDA, Beltsville MD) and was grown in minimal BII medium [39] with or without 30 μg/ml kanamycin, as appropriate, at 28°C with shaking (170 rpm). *E. coli* was grown in LB medium at 37°C.

**Cloning of the apoaequorin gene and introduction into *M. loti***

The terms aequorin and apoaequorin refer to the bioluminescent protein with and without, respectively, the prokaryotic group coelenterazine. The apoaequorin cassette, given by the apoaequorin cDNA fused to the first 27 nucleotides encoding hemoagglutinin (HA1-AEQ) [40] was amplified by PCR with primers designed to obtain a 5’ *Xba*I site and to leave out the ATG start codon, already present into the P$_{syn}$ promoter of the expression vector pDB1 [22]. The correct translation frame was maintained by adding a nucleotide between the 5’ *Xba*I site and the apoaequorin gene. The primers used to obtain the apoaequorin cassette were: 5’-CCTACTCTAGATAAGCTT-TATGATGTTCCT-3’ and 5’TGATAGCATGCGAATTCT-CAGTGTATTAT-3’. PCR was run with the following parameters: 5 min at 94°C as start step; 30 s at 94°C, 30 s at 58°C, 1 s at 72°C for 30 cycle and 5 s at 72°C as a final step using PLATINUM® Taq DNA polymerase (Invitrogen). To obtain a 3’ Xbal site, the amplicon was then cloned into the pCR 2.1 plasmid by using TA Cloning® technology (Invitrogen), originating p2.1AEQ. Digestion with XbaI of this intermediate plasmid released the HA1-AEQ coding region, which was then ligated into the Xbal site of pDB1 under the control of the strong isopropyl-β-D-thiogalactoside (IPTG)-inducible synthetic promoter P$_{syn}$. The apoaequorin gene containing construct (pAEQ80, see Additional file 1) was mobilized to *M. loti* 3147$^T$ from *E. coli* by triparental conjugation using plasmid pRK2013 as helper [41]. Transconjugants were selected on BIII agar containing 50 μg/ml kanamycin.

**Growth kinetics of the recombinant strain**

To determine the effect of the plasmid presence and of apoaequorin expression on bacterial cell growth, *M. loti* wild-type or containing pAEQ80 (plus or minus IPTG) were grown in 30 ml of BIII medium (supplemented or not with 30 μg/ml kanamycin, as appropriate) as described above. Growth was determined by monitoring turbidity at 600 nm.

**In vitro *L. japonicus* nodulation tests**

*In vitro* nodulation studies were carried out as described by [42]. Briefly, seeds of *L. japonicus* B-129 Gifu were transferred after sterilization on 0.1% Jensen medium solidified with 1% agar. Inoculation with bacterial suspensions of *M. loti* wild-type or containing pAEQ80 (5·10$^7$ cells/root) was carried out 4 days after seed germination. *Lotus* seedlings, before and after infection, were grown at 24°C with 16 h light and 8 h dark. Growth and nodulation pattern were monitored for 4 weeks after inoculation. Microscopy observations were carried out with a Leica MZ16 stereo microscope equipped with a DFC 480 photocamera. To check the actual occurrence of bacteria inside the nodules, they were squeezed and the content stained with 5 μg/ml 4’,6-diamino-2-phenylindole (DAPI). Samples were observed with a Leica DMR fluorescence microscope. Images were acquired with a Leica IM500 digital camera.

**Expression of apoaequorin**

A loopful of *M. loti* USDA 3147$^T$ pAEQ80 grown on BIII plates was used to inoculate 30 ml of BIII medium supplemented with 30 μg/ml kanamycin and 1 mM IPTG and grown at 28°C overnight, until an absorbance at 600 nm of approximately 0.25 was reached (after about 18 h).

**In vitro reconstitution of apoaequorin to aequorin**

*M. loti* suspension cultures (300 ml) were grown to mid-exponential phase (A$_{600nm}$ = 0.25), pelletted by centrifugation at 3000 g for 10 min at 4°C, washed twice with fresh medium, and finally resuspended in 2 ml reconstitution buffer (Tris-HCl 150 mM, EGTA 4 mM, supplemented with 0.8 mM phenylmethylsulfonyl fluoride, pH 7.0).
Bacterial light emission was measured in a purpose-built luminometer. Bacteria (50 μl) were placed, after aequorin reconstitution, in the luminometer chamber in close proximity to a low-noise photomultiplier, with a built-in amplifier discriminator. The output of the discriminator was captured by a THORN-EMI photon counting board (Electron Tubes Limited, Middlesex, UK) and the luminescence data were converted off-line into Ca\textsuperscript{2+} concentration values by using a computer algorithm based on the Ca\textsuperscript{2+} response curve of aequorin [40]. All stimuli were administered to cells by using a light-tight syringe through the luminometer port. The experiments were terminated by lysing the cells with 15% ethanol in a Ca\textsuperscript{2+}-rich solution (0.5 M CaCl\textsubscript{2} in H\textsubscript{2}O) to discharge the remaining aequorin pool. For experiments performed in the presence of different external Ca\textsuperscript{2+} concentrations, cells were extensively washed and resuspended in buffer A (25 mM Hepes, 125 mM NaCl, 1 mM MgCl\textsubscript{2}, pH 7.5), as described by [16]. When needed, cells were pretreated for 10 min with 5 mM EGTA.

**Bacterial cell viability assay**

Bacterial cell viability was monitored by the LIVE/DEAD\textsuperscript{®} BacLight\textsuperscript{™} Bacterial Viability kit (Molecular Probes), according to manufacturer’s instructions. This fluorescence-based assay use a mixture of SYTO 9 and propidium iodide stains to distinguish live and dead bacteria. Bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. Samples were observed with a Leica 5000B fluorescence microscope. Images were acquired with a Leica 300F digital camera using the Leica Application Suite (LAS) software.

**Semi-quantitative RT-PCR experiments**

*M. loti* cells grown to mid-exponential phase and treated as for Ca\textsuperscript{2+} measurement experiments (see above) were incubated for 1 h with plant root exudates, tetronic acid or cell culture medium only (as control). To stabilize RNA, bacteria were treated with the RNA protect Bacteria Reagent (Qiagen). Bacterial cell wall was then lysed with 1 μg/ml lysozyme (Sigma) in TE buffer. Total RNA was first extracted using RNeasy Mini kit (Qiagen) and, after DNase I treatment (Promega), quantified. RNA (5 μg) was primed with Random Decamers (Ambion), reverse transcribed with PowerScript Reverse Transcriptase (Clontech) and diluted 1:5. 5 μl of diluted first-strand cDNA were used as a template in a 50 μl PCR reaction solution. Reverse transcription (RT)-PCR was performed with 5 μl diluted first-strand cDNA. The oligonucleotide primers were designed against *nodA*, *nodB*, *nodC* and glutamine synthetase II (GSII) sequences from *M. loti* [43] and the aequorin gene (*aeq*) from *Aequorea victoria* [44], using Primer 3 software. To amplify 16S rRNA gene, Y1 and Y2 primers were used [45].

The thermal cycler was programmed with the following parameters: 20 s at 94°C, 30 s at 68°C and Advantage 2 Polymerase mix (Clontech) was used as Taq polymerase. PCR reactions were allowed to proceed for different number of cycles to determine the exponential phase of amplification. Densitometric analysis of ethidium bromide-stained agarose gels (0.5 μg/ml) was performed using QuantityOne software (Bio-Rad). RT-PCR experiments were conducted in triplicate on three independent experiments. The primer sequences used to obtain amplions were: 5‘-TATGAGCCGACCCGGACCCCTTTAAT-3‘ and 5‘-CCGTATAGACCGAGTTCAAGCGACAA-3‘ for *nodA*, 5‘-
ATACTCGATGTGCTGGCGAAAT-3' and 5'-GCCCTGGTTGGCCTCACAATACCTTC-3' for nodB, 5'-CCACCTACGATCTGAGCCTGCGCCATAA-3' and 5'-CCACCTACGATCTGAGCCTGCGCCATAA-3' for nodC, 5'-ACCCAGCTACTGGGACATC-GACATC-3' and 5'-GCCACGCATAGCTAAAACCTTGTTCC-3' for GSI, 5'-TAAACCTGAGCAACACTGTGAGCAA-3'.

5'-ATACCGGATGAGCCTGTTGTCGTTTT-3' for aequorin, Y1 (5'-TGGCTCAGAACGAACGCTGGCGGC-3') and Y2 (5'-CCACCTACGATCTGAGCCTGCGCCATAA-3') for 16S rRNA. Amplicons were sequenced by BMR Genomics (Padova, Italy).

Authors' contributions
RM cloned the apoaequorin gene, carried out the RT-PCR experiments and participated in the Ca2+ measurement experiments. SA and AS introduced the apoaequorin gene into E. coli and M. loti. LN performed the nodulation studies, prepared the plant root exudates and was involved in acquisition and interpretation of Ca2+ measurement data. MP and LN conceived of the study, designed the experiments and wrote the paper. AS helped with manuscript discussion and participated in its editing. All authors read and approved the final manuscript.

Additional material

Additional file 1
Map of the apoaequorin-expressing plasmid pAEQ80. Abbreviations: P, IPTG-inducible synthetic promoter (Pório); HA1-AEQ, cloned apoaequorin cDNA with hemagglutinin epitope; KmR, kanamycin resistance gene; lacIv, constitutive lac repressor gene. Relevant restriction endonuclease sites are also shown. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2180-9-206-S1.tiff]

Additional file 2
Validation of the aequorin-expressing M. loti experimental system. A. Analysis of aequorin expression in M. loti based on an in vitro reconstruction assay. Data are the means ± SEM of three experiments. B. Effect of pAEQ80 plasmid and expressed recombinant apoaequorin on M. loti cell growth. Data are means of two independent experiments. C. Nodulated root of L. japonicus with rhizobial infection in legumes. Annu Rev Plant Biol 2008, 59:519-546.

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References
1. Oldroyd GED, Downie JA: Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu Rev Plant Biol 2008, 59:519-546.
2. Garg N, Geetanjali : Symbiotic nitrogen fixation in legume nodules: process and signaling. A review. Agron Sustain Dev 2007, 27:59-68.
3. Cooper JE: Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. J Appl Microbiol 2007, 103:1355-1365.
4. Norris V, Grant S, Freestone P, Canvin J, Sheikh FN, Toth I, Trinei M, Modha K, Norman RI: Calcium signalling in bacteria. J Bacteriol 1996, 178:3677-3682.
5. Domínguez DC: Calcium signalling in bacteria. Mol Microbiol 2004, 54:291-297.
6. Case RM, Eisner D, Gurney A, Jones O, Mualem S, Verkhratsky A: Evolution of calcium homeostasis: from birth of the first cell to an omnipresent signalling system. Cell Calcium 2007, 42:345-350.
7. Kung C, Blount P: Channels in microbes: so many holes to fill. Mol Microbiol 2004, 53:373-380.
8. Yang K: Prokaryotic calmodulins: recent developments and evolutionary implications. J Mol Microbiol Biotechnol 2001, 3:457-459.
9. Michiels J, Xi C, Verhaert J, Vanderleyden J: The functions of Ca2+ in bacteria: a role for EF-hand proteins? Trends Microbiol 2002, 10:87-93.
10. Mithöfer A, Mazars C: Aequorin-based measurements of intracellular Ca2+-signatures in plant cells. Biochim Biophys Acta 2006, 1691:1-10.
20. Torrecilla I, Leganés F, Bonilla I, Fernández-Piñas F. A calcium signal is involved in heterocyst differentiation in the cyanobacterium Anabaena sp. PCC7120. Microbiology 2004, 150:3771-3779.

21. Torrecilla I, Leganés F, Bonilla I, Fernández-Piñas F. Light-to-dark transitions trigger a transient increase in intracellular Ca²⁺ modulated by the redox state of the photosynthetic electron transport chain in the cyanobacterium Anabaena sp. PCC7120. Plant Cell Environ 2004, 27:810-819.

22. Alberghini S, Filippini R, Marchetti E, Dindo ML, Shevelev AB, Battist A, Squarini A. Construction of a Pseudomonas sp. derivative carrying the cry9AA gene from Bacillus thuringiensis and a proposal for new standard criteria to assess entomocidal properties of bacteria. Res Microbiol 2005, 156:690-699.

23. Sanders D, Brownlee C, Harper JF. Communicating with calcium. Plant Cell 1999, 11:691-706.

24. Falcitore A, d’Alcalà MR, Croot P, Bowler C. Different and new Nod factors produced by Rhizobium tropici CIA1899 following Na⁺ stress. FEMS Microbiol Lett 2009, 306:220-231.

25. Estévez J, Sorias-Díaz ME, de Córdoba FF, Morón B, Manyani H, Gil A, Thomas-Oates J, van Brussel AA, Dardennei MS, Sousa C, Megías M. Different and new Nod factors produced by Rhizobium tropici CIA1899 following Na⁺ stress. FEMS Microbiol Lett 2009, 306:220-231.

26. Suzuki H, Sasaki R, Ogata Y, Nakamura Y, Sakurai N, Kitajima H, Kanaya S, Aoki K, Shibata D, Saito K. Construction of a transgenic Arabidopsis line that overexpresses a calcium-sensing receptor and displays altered calcium responses. Proc Natl Acad Sci USA 2009, 106:220-231.

27. López-Lara IF, Berg JD, van der, Thomas-Oates JE, Glushka J, Lugtenberg BJJ, Spaink HP. Structural identification of the lipo-chitin oligosaccharide nodulation signals of Rhizobium tropici. Mol Microbiol 2004, 53:627-638.

28. Saei K, Kouchi H. The Lotus symbiont, Mesorhizobium loti: molecular genetic techniques and applications. J Plant Res 2000, 113:457-465.

29. Gagnon H, Ibrahim RK. Aldonic acids: a novel family of nod gene inducers of Mesorhizobium loti, Rhizobium lupini, and Sinorhizobium meliloti. Mol Plant-Microbe Interact 1998, 11:988-998.

30. De-la-Peña C, Lei Z, Watson BS, Summer LW, Vivanco JM. Structural identification of the lipo-chitin oligosaccharide nodulation signals of Rhizobium tropici. Mol Microbiol 2004, 53:627-638.

31. Zhu Y, Pierson LS III, Hawes MC. Extracellular proteins in pea root tip and border cell exudates. Plant Physiol 2007, 143:773-783.

32. Zhu Y, Pierson LS III, Hawes MC. Induction of microbial genes for pathogenesis and symbiosis by chemicals from root border cells. Plant Physiol 1997, 115:1691-1698.

33. Brini M, Pinton P, Pozzan T, Rizzuto R. Targeted recombinant aequorins: tools for monitoring [Ca²⁺] in the various compartments of a living cell. Microsc Res Tech 1999, 46:380-389.

34. Berridge MJ. Calcium oscillations. J Biol Chem 1990, 265:5383-5386.

35. Dodd AN, Jakobsen MK, Baker AJ, Telzerow A, Sul-Wen Hou SW, Laplaze L, Barrot L, Poethig RS, Haseloff J, Webb AAR. Time of day modulates low-temperature Ca²⁺ signals in Arabidopsis. Plant J 2006, 48:962-973.

36. McAinsh MR, Pitman JK. Shaping the calcium signature. New Phytol 2009, 181:275-294.

37. Wolf DM, Arkin AP. Motifs, modules and games in bacteria. Curr Opin Microbiol 2003, 6:125-134.

38. Schlaman HRM, Okker RJH, Lugtenberg BJJ. Regulation of nodulation gene expression by NodD in rhizobia. J Bacteriol 1992, 174:5177-5182.

39. Dazzo FB. Leguminous root nodules. In Experimental Microbial Ecology Edited by: Burns R, Slater J. Oxford: Blackwell Scientific Publications, 1992:431-446.

40. Brini M, Marsault R, Bastianutto C, Alvarez J, Pozzan T, Rizzuto R. Transfected aequorin in the measurement of cytosolic Ca²⁺ concentration ([(Ca²⁺)m]). J Biol Chem 1995, 270:9886-9903.

41. Figurski DH, Helmink DR. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci USA 1979, 76:1648-1652.

42. Barbrola A, Chiurazzi M. A procedure for Lotus japonicus in vitro nodulation studies. In Lotus japonicus Handbook Edited by: Márquez AJ, Stougaard J, Udvardi M, Parniske M, Spank H, Saibach G, Webb J, Chiurazzi M. Berlin: Springer; 2005:83-86.

43. Kaneko T, Nakamura Y, Satô S, Asamizu E, Kato T, Sasamoto S, Watanabe A, Isewasa K, Ishikawa K, Kawashima K, Kimura T, Kishida Y, Kiyokawa C, Kohara M, Matsumoto M, Matsuno A, Mochizuki Y, Nakayama S, Nakazaki N, Shimo S, Sugimoto M, Takeuchi C, Yamada M, Tabata S. Complete genome structure of the nitrogen-fixing symbiotic bacterium Mesorhizobium loti. DNA Res 2000, 7:331-338.

44. Inouye S, Noguchi M, Sakai Y, Takagi Y, Miyata T, Iwanga S, Miyata T, Tsuji F. Cloning and sequence analysis of cDNA for the luminescent protein aequorin. Proc Natl Acad Sci USA 1985, 82:3154-3158.

45. Young JPW, Downer HL, Eardly BD: Phylogeny of the phototrophic rhizobium strain BTA-I by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. J Bacteriol 1991, 173:2271-2277.

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