Calcium Spiking Patterns and the Role of the Calcium/Calmodulin-Dependent Kinase CCaMK in Lateral Root Base Nodulation of *Sesbania rostrata* □□

Ward Capoen,ᵃᵇ,c,¹ Jeroen Den Herder,ᵃᵇ,¹ Jongho Sun,⁺ Christa Verplancke,ᵃᵇ Annick De Keyser,ᵃᵇ Riet De Rycke,ᵃᵇ Sofie Goormachtig,ᵃᵇ Giles Oldroyd,⁺¹ and Marcelle Holstersᵃᵇ,¹,²

ᵃ Department of Disease and Stress Biology, John Innes Centre, Norwich NR4 7UH, United Kingdom
ᵇ Department of Plant Biotechnology and Genetics, Ghent University, 9052 Gent, Belgium
ᶜ Department of Plant Systems Biology, Flanders Institute for Biotechnology, 9052 Gent, Belgium

Nodulation factor (NF) signal transduction in the legume-rhizobium symbiosis involves calcium oscillations that are instrumental in eliciting nodulation. To date, Ca²⁺ spiking has been studied exclusively in the intracellular bacterial invasion of growing root hairs in zone I. This mechanism is not the only one by which rhizobia gain entry into their hosts; the tropical legume *Sesbania rostrata* can be invaded intercellularly by rhizobia at cracks caused by lateral root emergence, and this process is associated with cell death for formation of infection pockets. We show that epidermal cells at lateral root bases respond to NFs with Ca²⁺ oscillations that are faster and more symmetrical than those observed during root hair invasion. Enhanced jasmonic acid or reduced ethylene levels slowed down the Ca²⁺ spiking frequency and stimulated intracellular root hair invasion by rhizobia, but prevented nodule formation. Hence, intracellular invasion in root hairs is linked with a very specific Ca²⁺ signature. In parallel experiments, we found that knockdown of the calcium/calmodulin-dependent protein kinase gene of *S. rostrata* abolished nodule development but not the formation of infection pockets by intercellular invasion at lateral root bases, suggesting that the colonization of the outer cortex is independent of Ca²⁺ spiking decoding.

INTRODUCTION

Leguminous plants have coevolved with nitrogen-fixing rhizobia to establish a sophisticated root endosymbiosis. In a developmental process guided by reciprocal signal exchange, the plants form new organs, nodules, to house bacteria that reduce molecular dinitrogen and feed their host with ammonia. In most studied interactions, nodulation occurs in a susceptible root zone with developing root hairs (zone I). This process is activated by bacterial signals, the lipochitoooligosaccharide nodulation (Nod) factors (NFs) (D’Haeze and Holsters, 2002; Jones et al., 2007). In response to NFs, root hairs form a curl that provides a pocket for a microbial colony. Concomitantly, cortical cell division is triggered for organ formation. Purified NFs elicit several physiological responses in susceptible root hair cells, such as calcium influx, membrane depolarization, and rhythmic Ca²⁺ oscillations (spiking) in and around the nucleus (Oldroyd and Downie, 2004).

The molecular basis of legume symbiosis has been particularly well studied in *Medicago truncatula* and *Lotus japonicus*, in which essential nodulation genes have been identified through forward genetics and map-based cloning. Characterization of plant mutants that are affected in NF signal perception or transduction revealed that NFs are perceived by LysM domain-containing receptor-like kinases, such as lysine motif receptor-like kinase3 (LYK3)-LYK4/Nod factor perception (NFP) of *M. truncatula* and Nod factor receptor1 (NFR1)/NFR5 of *L. japonicus* (Ben Amor et al., 2003; Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003, 2007; Arrigoni et al., 2006). Other plant nodulation functions are common with the more ancient symbiosis with arbuscular mycorrhiza and include putative cation transporters (does not make infection1 [DMI1] of *M. truncatula* and CASTOR/POLLUX of *L. japonicus*) (Ané et al., 2004; Imaizumi-Anraku et al., 2005), a leucine-rich repeat-type receptor-like kinase (DMI2) of *M. truncatula*, symbiosis receptor-like kinase (SYMRK) of *L. japonicus* (Kanamori et al., 2006; Saito et al., 2007), and calcium/calmodulin-dependent kinase (CCaMK) of *M. truncatula* (Lévy et al., 2004; Mitra et al., 2004). Transcription factors of the GRAS (gibberellin-insensitive, repressor of gal-3, and SCARECROW) family nodulation signaling pathway (NSP1 and NSP2) of *M. truncatula* and the ethylene response factor required for nodulation (ERN) family of *M. truncatula* are involved in NF-specific gene expression. Together with the transcriptional regulator nodulation inception (NIN) identified in *L. japonicus* and *M. truncatula*, they regulate downstream responses, such as cytokinin signaling for primordium initiation (Schauser et al., 1999; Kaló et al., 2005; Smit et al., 2007).

¹ These authors contributed equally to this work.
² Address correspondence to marcelle.holsters@psb.vib-ugent.be.
³ The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: marcelle.holsters@psb.vib-ugent.be.
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Ca$^{2+}$ oscillations have been shown to directly affect gene expression in animal systems (Gu and Spitzer, 1995; Dolmetsch et al., 1997; De Koninck and Schulman, 1998). In plants, Ca$^{2+}$ oscillations are believed to be involved in abscisic acid signaling in guard cells, in pollen tube elongation, and in the NF signal transduction pathway of legumes (Holdaway-Clarke et al., 1997; Li et al., 1998; Allen et al., 2001; Oldroyd et al., 2001b; Oldroyd and Downie, 2006). NF-induced Ca$^{2+}$ spiking is correlated with nodulin gene expression, and is influenced by the hormones ethylene and jasmonate (JA) and by the developmental context in the root, and is blocked by inhibitors of Ca$^{2+}$ pumps and channels (Pingret et al., 1998; Oldroyd et al., 2001a; Engstrom et al., 2002; Miwa et al., 2006a, 2006b; Sun et al., 2006).

The nonnodulating mutants dmi3 of M. truncatula and sym9 of pea (Pisum sativum) show normal Ca$^{2+}$ spiking and are affected in a CCaMK-encoding gene (Lévy et al., 2004; Mitra et al., 2004), which is presumably required for the interpretation of the Ca$^{2+}$ signature (Oldroyd and Downie, 2004). CCaMK has a kinase domain, a calmodulin (CaM) binding domain with strong homology to CaM-dependent kinase II in animals, and a C-terminal Ca$^{2+}$ binding domain with three EF hands homologous to the neuronal Ca$^{2+}$ binding protein visinin (Patil et al., 1995). Auto-phosphorylation of CCaMK after EF hand binding of Ca$^{2+}$ ions enhances the Ca$^{2+}$/CaM association that subsequently suppresses autoinhibition, thus activating the kinase domain and allowing substrate phosphorylation (Patil et al., 1995; Gleason et al., 2006; Tirichine et al., 2006). CCaMK is expressed in roots and in developing nodules (Lévy et al., 2004), and transcripts have been detected in a few cell layers directly adjacent to the meristem of indeterminate nodules. CCaMK localizes to the nucleus of root cells, corresponding to the site where the Ca$^{2+}$ spiking intensity is the highest and the possible target proteins NSP1 and NSP2 accumulate (Erhardt et al., 1996; Kaô et al., 2005; Limpens et al., 2005; Smit et al., 2005). Functional analysis has indicated that the CCaMK proteins are important for nodule organogenesis and root hair invasion and that gain of function has indicated that the CCaMK proteins are important for nodule formation, the situation is different for each type of bacterial invasion. Indeed, RHC infection is strongly correlated with Ca$^{2+}$ oscillations during RHC nodulation. Modulation of the ethylene or JA levels slowed down the Ca$^{2+}$ spiking frequency and stimulated RHC invasion but was incompatible with nodule development. In parallel, we studied the function of CCaMK of S. rostrata during LR nodulation using RNA interference (RNAi).

Our data show that, although Ca$^{2+}$ spiking is a common component of the signaling pathways for RHC and LR nodule formation, the situation is different for each type of bacterial invasion. Indeed, RHC infection is strongly correlated with Ca$^{2+}$ oscillations of the appropriate frequency, while intercellular rhizobial infection at LRFs probably functions independently of Ca$^{2+}$ spiking and CCaMK.

RESULTS

Comparison of NF-Induced Ca$^{2+}$ Oscillations at LRFs and in Root Zone I

As a first approach to investigate the role of Ca$^{2+}$ spiking during LR nodulation, we studied NF-induced Ca$^{2+}$ oscillations in zone I and at LRFs. In aeroponic roots of S. rostrata, growing root hairs respond to azorhizobia with curling, infection thread invasion, and concomitant formation of nodules in zone I (Goormachtig et al., 2004a). To visualize Ca$^{2+}$ spiking, we microinjected the Ca$^{2+}$-sensitive dye Oregon Green into zone I root hairs after the root had been briefly submerged in liquid medium (see Methods). The root hairs did not respond to NFs as far as Ca$^{2+}$ responses.
were concerned, an observation in accordance with the quick inhibition of RHC nodulation by submergence (Goormachtig et al., 2004a).

To circumvent this limitation, roots were grown hydroponically in medium supplemented with 7 μM L-α-(2-aminoethoxyvinyl)-glycine (AVG), an inhibitor of ethylene synthesis. Such roots have normal root hairs and RHC nodulation (Goormachtig et al., 2004a). Growing root hairs in zone I were microinjected with Oregon Green and subsequently exposed to NFs. Approximately 50% of the cells responded and showed Ca^{2+} oscillations with an average period of 179.8 ± 77.6 s and an asymmetric spike shape: the upward and downward phases corresponded to 21 and 79% of the spike duration, respectively (Figures 1A and 1D), which is similar to the spike shape in *M. truncatula* zone I root hairs treated with *Sinorhizobium meliloti* NFs (Figures 1B and 1E). The *M. truncatula* spikes showed a rapid increase in Ca^{2+} that constituted 16% of the spike duration and a slow reduction in Ca^{2+} levels (Figure 1E). In *S. rostrata*, the average period was

![Figure 1. NF-Induced Ca^{2+} Spiking Patterns in *S. rostrata* Compared with a *M. truncatula* Reference.](image-url)

(A) to (C) Representative Ca^{2+} spiking traces; the percentage of visualized cells that initiated Ca^{2+} spiking upon exposure to NFs is shown, and the number of cells tested is given in parentheses.

(A) Ca^{2+} spiking traces in zone I root hairs of *S. rostrata*.

(B) Ca^{2+} spiking traces in zone I root hairs of *M. truncatula*.

(C) Ca^{2+} spiking traces in root hair initials at LRBs of *S. rostrata*.

(D) to (F) Schematic representation of the average spike shape of traces depicted in (A) to (C), respectively. The numbers in the curve are averages of the proportion of upward and downward parts of the spike.

(G) Statistical analysis of Ca^{2+} spiking shapes. Average times taken from at least 30 Ca^{2+} spikes are presented for several traces under each condition. The upward phases (dark gray) were identical, but the downward phases (light gray) were significantly different (P < 0.001). Error bars indicate SD. Statistically significant differences in comparison with *S. rostrata* LRBs are indicated with an asterisk as well as the P value.
intracellularly. Antagonists of ethylene synthesis or perception as well as inhibitors of reactive oxygen species production inhibit both NF-dependent root hair outgrowth and LRB nodulation (D’Haeze et al., 2003). As these root hair initials located at LRBs were markers for NF responses during LRB nodulation and were amenable to microinjection, we screened them for NF-induced Ca\(^{2+}\) spiking. NF application induced a distinct spiking signature in these root hair initials: 87% of the cells responded with an average spiking period of 55.5 ± 20.8 s (Figures 1C and 1F). The LRB-associated spikes were more symmetrical than the zone I–associated spikes in *M. truncatula* and *S. rostrata*, with upward and downward phases representing 39 and 61% of the spike duration, respectively (Figures 1F and 1G).

A drawback of Oregon Green as a calcium indicator is that it cannot be used to measure changes in the amplitude of Ca\(^{2+}\) oscillations. To address this issue, we injected the different cell types with a ratiometric calcium indicator, dextran-linked 1-[6-amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy) ethane-N,N,N’,N’-tetraacetic acid, penta-potassium salt (Fura-2). Amplitudes were measured in zone I cells of *M. truncatula* (*n = 3*) and in LRB cells of *S. rostrata* under hydroponic conditions without (*n = 6*) and with 7 \(\mu M\) AVG (*n = 5*). All spikes analyzed confirmed the nuclear localization of NF-induced Ca\(^{2+}\) spiking and an amplitude (200 to 250 nM) similar to published data (Figure 2A) (Walker et al., 2000). Thus, the NF-induced Ca\(^{2+}\) oscillations had similar amplitudes in all tested tissues (Figure 2B).

Phospholipase D (PLD) and Ca\(^{2+}\)-ATPases have been implicated in NF-induced Ca\(^{2+}\) spiking and gene expression in root hairs of *M. truncatula* (den Hartog et al., 2001, 2003; Engstrom et al., 2002). We used the Ca\(^{2+}\)-ATPase antagonists cyclopiazonic acid (CPA) and 2,5-di-t-1,4-benzohydroquinone (BHQ) and the PLD antagonist \(\text{n-butanol}\) to assess whether similar mechanisms are involved in the generation of LRB-associated Ca\(^{2+}\) spiking. Both CPA and BHQ inhibited NF-induced LRB Ca\(^{2+}\) spiking in *S. rostrata* (see Supplemental Figure 1 online). Suppression by BHQ was transient, with spontaneous reversion of the Ca\(^{2+}\) spiking within ~10 min after application, similarly to what was observed in *M. truncatula* (Engstrom et al., 2002). The primary alcohol \(\text{n-butanol}\) can accept the phosphatidyl moiety produced by PLD, resulting in a decreased production of phosphatidic acid, in contrast with tert-butanol that provides a valuable control (Parmentier et al., 2003). Addition of 0.5\% \(\text{n-butanol}\) reversibly inhibited LRB-associated Ca\(^{2+}\) spiking, whereas tert-butanol had no effect (see Supplemental Figure 1 online). These data suggest that similar mechanisms generate Ca\(^{2+}\) spiking in zone I root hairs and in root hair initials at LRBs.
truncatula (Figures 3A to 3C). Ca\(^{2+}\) spiking was strongly affected by 150 \(\mu\)M AVG and totally abolished by 100 \(\mu\)M JA (Figure 3A). Addition of 1-aminocyclopropane-1-carboxylic acid in concentrations ranging from 40 to 1000 \(\mu\)M had no effect.

Hence, Ca\(^{2+}\) spiking at LRBs was suppressed or slowed down by JA, while increased ethylene levels did not affect the LRB signature; by contrast, interference with ethylene biosynthesis by the addition of AVG retarded the Ca\(^{2+}\) spiking frequency. Treatment with 7 \(\mu\)M AVG also changed the shape of the Ca\(^{2+}\) spike, by expanding the second phase, resulting in more asymmetrical spikes that resembled those in *M. truncatula* or *S. rostrata* zone I root hairs (Figure 3D).

Because application of AVG or JA shifts the Ca\(^{2+}\) spiking signature at LRBs, we wanted to investigate the effect of the hormones on nodule formation and on rhizobial invasion. For a quantitative analysis, hydroponic roots were pretreated for 24 h with different concentrations of AVG or JA before inoculation with *A. caulinodans* strain ORS571 (pRG960SD-32) that expresses the \(\beta\)-glucuronidase (GUS) reporter gene *uidA*. Three days after inoculation (DAI), roots were stained for GUS and LRBs were excised for stereomicroscopy observation. Ten plants were used per treatment, and four or five LRBs per root were scored for numbers of intercellular infection pockets and intracellular infection threads. The data (Figures 4A and 4B) demonstrate that pretreatment with low concentrations of AVG or JA stimulated intracellular invasion of the root hairs. In control roots, infection threads were observed only very occasionally. Treatment with 1 \(\mu\)M AVG or 12.5 \(\mu\)M JA significantly increased the number of infection threads in root hairs and decreased the number of intercellular infection pockets (Figures 4A and 4B). Both infection thread and infection pocket formation were reduced by higher concentrations of AVG and JA and completely inhibited by 150 or 100 \(\mu\)M JA.

Previously, 7 \(\mu\)M AVG had been shown to block LRB nodulation completely (D’Haese et al., 2003). Similar observations were made upon JA treatment. Treatment with 25 \(\mu\)M JA reduced the number of LRB nodules by 75\% (on average 11.4 nodules on untreated roots versus 2.6 nodules on treated roots).

The concentrations of AVG and JA that affected bacterial invasion and nodule formation were in general lower than those required for the Ca\(^{2+}\) spiking switch. Presumably, the 24-h pretreatment in the nodulation experiments improved penetration of the compounds, whereas in the Ca\(^{2+}\) spiking analysis, the effects were measured 10 min after addition of AVG and JA. To examine this possible explanation, we pretreated hydroponic roots for 24 h at the low concentrations used in the infection assays (Figures 4C and 4D) and visualized Ca\(^{2+}\) spiking upon application of NFs. Indeed, 24 h of pretreatment with 1 \(\mu\)M AVG (n = 7) and 12.5 \(\mu\)M JA (n = 7) significantly extended the spiking period compared with untreated cells (n = 10) (Figure 4C) and shifted the spike shape to one resembling that after 10-min high-concentration treatment (Figures 3D and 4D). Hence, 24-h pretreatments at low concentrations and minute-long treatments at high concentrations have similar effects on Ca\(^{2+}\) spiking, suggesting that penetration of the compounds is not immediate and is dose dependent.

A semithin section through a control (Figure 4E) and an AVG-pretreated (Figure 4F) sample illustrate the differences in the LRB responses to *A. caulinodans* inoculation at the microscopic level. Whereas in the control a nodule develops opposite infection pockets (Figure 4E), pretreatment with 7 \(\mu\)M AVG interfered with nodule development and infection pocket formation, but led to inflated root hairs (Figure 4F). Limited cell division was occasionally seen (as in Figure 4F), but nodules never developed.

Together, these observations suggest that intracellular root hair invasion is associated with a particular NF-induced Ca\(^{2+}\) signature. Indeed, shifting the fast spiking pattern at LRBs toward slower frequencies, resembling the default signature in susceptible zone I root hairs, correlated with intracellular infection threads; however, the same conditions were incompatible with nodule initiation opposite these root hair infection sites. *S. rostrata* CCaMK Expression during LRB Nodulation

CCaMK is a critical component of the NF signaling pathway and presumably functions in decoding NF-induced Ca\(^{2+}\) spiking. To assess whether Ca\(^{2+}\) spiking decoding is relevant for LRB nodulation, we investigated the role of CCaMK. To identify *S. rostrata* CCaMK, the CCaMK cDNA sequences of *M. truncatula*, pea, rice (*Oryza sativa*), and tobacco (*Nicotiana tabacum*) were aligned. PCR primers were designed to amplify a well-conserved CCaMK region from a *S. rostrata* nodule cDNA library. A full-length cDNA clone was isolated by rapid amplification of cDNA ends. The corresponding protein of 522 amino acids was most homologous to *L. japonicus* CCaMK and *M. truncatula* DMI3, with 90.4 and 89.0\% amino acid similarity, respectively. The putative protein had a kinase domain with a conserved Thr (Thr-270) for autophosphorylation, a CaM binding domain, and three EF hand domains for Ca\(^{2+}\) binding at the C terminus (see Supplemental Figure 2A online). DNA gel blot hybridization with a 652-bp probe containing the CaM binding domain showed only one band after genomic DNA digestion with different restriction enzymes, indicating that *S. rostrata* CCaMK is a single gene. A 1824-bp promoter region upstream of the start codon was isolated from genomic DNA of *S. rostrata* and fused to the cDNA sequence. The resulting construct was introduced into roots of the nodulation-defective *M. truncatula* dmi3-1 mutant by *Agrobacterium rhizogenes* transformation. Transgenic roots were inoculated with *S. melloti* 1021, and functional nodules were observed (see Supplemental Figure 2B online) in five out of six transgenic roots. The complementation nodules had a normal morphology, with an apical meristem, infection zone, and a large fixation zone with cells fully occupied by symbiosomes (see Supplemental Figure 2B online). Hence, *S. rostrata* CCaMK is the functional ortholog of the *M. truncatula* CCaMK.

*S. rostrata* CCaMK transcripts were present in adventitious rootlets, and the transcript level gradually increased during stem nodulation, with a maximum at 4 to 5 DAI (Figure 5A). To localize the expression of *CCaMK*, the 1824-bp promoter region was used to drive the transcription of the *uidA* reporter gene. In uninoculated transgenic roots, GUS staining was observed in zones that are potentially responsive to nodulation, i.e., at the LRBs (Figure 5B) and in root zone I (Figure 5C). At 2 DAI with *A. caulinodans*, the promoter was active in the nodule primordia at LRBs (Figure 5D). One day later, when zonation was initiated, CCaMK was still expressed in the
**Figure 3.** Hormonal Influences on NF-Induced Ca²⁺ Spiking Signatures.

(A) Representative examples of altered traces observed in root hair cells at LRBs after treatments with AVG and JA. Concentrations are indicated above the relevant trace; the top trace is a reference, showing NF-induced Ca²⁺ spiking in untreated roots. The frequency is reduced without inhibition of Ca²⁺ spiking by 7 μM AVG and 50 μM JA. Spiking is strongly affected by 150 μM AVG and is totally inhibited by 100 μM JA. The percentage of visualized cells that initiated Ca²⁺ spiking is shown, and the number of cells tested is given in parentheses.

(B) Effect of treatment with 7 μM AVG and 50 μM JA on the average period of Ca²⁺ spiking. Error bars indicate SD. Statistically significant differences between treatment and control are indicated with an asterisk as well as the P value.

(C) Shift in distribution of Ca²⁺ spiking periods at LRBs toward the distribution found in zone I root hairs after treatment with 7 μM AVG or 50 μM JA. For each treatment, the percentage of cells with a period in the indicated range was plotted against their spiking periods, and the spiking period was compared between the different treatments.

(D) Schematic representation of spike shapes derived from several representative traces. The numbers in the curves are average proportions of upward and downward segments of the spikes. A statistically significant difference was observed for the downward phase of AVG-treated compared with untreated root hairs at LRBs (indicated with an asterisk; the P value is also shown).

[See online article for color version of this figure.]
Figure 4. Effects of AVG on LRB Nodulation and Bacterial Invasion.

(A) Graphs showing the effect of 24-h pretreatment at several AVG concentrations on infection pocket (IP) and infection thread (IT) formation. The dark- and light-gray bars show the number of IPs and ITs per LRB, respectively, as observed under stereomicroscopy. A significant increase in ITs can be seen after pretreatment with 1 μM AVG when compared with the untreated control plants (indicated with an asterisk; the P value is also given). Error bars indicate SD.

(B) As for (A) but for JA pretreatment. A significant increase in ITs can be seen after pretreatment with 12.5 μM JA when compared with the untreated control plants (indicated with an asterisk; the P value is also shown). Error bars indicate SD.

(C) Effect of 24-h pretreatment with 1 μM AVG and 12.5 μM JA on the average period of Ca²⁺ spiking. Error bars indicate SD. A significant change can be seen after pretreatment with 1 μM AVG and 12.5 μM JA when compared with the untreated control plants (indicated with an asterisk; the P value is also shown).

(D) Statistical analysis of the change in Ca²⁺ spike shapes after 24-h pretreatment with either 1 μM AVG or 12.5 μM JA. The dark and light bars represent the upward and downward part of the spike, respectively. Error bars indicate SD. A significant change can be seen in the 1 μM AVG and 12.5 μM JA pretreatments compared with the untreated control plants (indicated with an asterisk; the P value is also shown).

(E) and (F) Semithin sections of a developing LRB nodule at 3 DAI with *A. caulinodans* (E) and pretreated for 24 h with 7 μM AVG before inoculation with *A. caulinodans* (F). Typical features of nodule development and the changes that occur upon hormonal pretreatment are noticeable at the light microscopic level. Arrowhead and arrow indicate infection pocket and infected root hair, respectively. Bars = 100 μm.
nodule primordium (Figure 5E). Sectioning indicated that GUS was found mainly in the developing nodule and was low in cortical cells of the infection center (Figures 5F and 5G). In maturing nodules, CCaMK was associated with the infection zone and, to a lesser extent, the fixation zone (Figures 5H to 5J).

A similar pattern of CCaMK expression was observed in adventitious root nodules on S. rostrata stems by means of RNA in situ analysis. At the early stages of nodule development, cells of the nodule primordium accumulated CCaMK transcripts (Figures 5K and 5L), with the strongest transcript accumulation...
near the meristem and weakest expression in the infection zone and in nitrogen-fixing cells (fixation zone). Very low CCaMK expression was observed around bacterial infection pockets or progressing intercellular infection threads in the outer cortex (Figures 5K and 5L) or in the infection center (Figure 5M). This observation revealed that CCaMK expression correlated strongly with nodule development and was low in cortical cells during intercellular rhizobial invasion.

**Knockdown of S. rostrata CCaMK Arrests Nodule Development but Not Intercellular Colonization**

To investigate the function of CCaMK during LRB nodulation, we reduced CCaMK transcript levels in transgenic S. rostrata roots by RNAi. Two constructs covering regions of ~200 bp (CCaMK-KO2 and CCaMK-KO3) were introduced into S. rostrata by transformation with A. rhizogenes 2659 (Van de Velde et al., 2003). Plants with transgenic roots, identified by green fluorescent protein (GFP) cotransformation, were transferred to tubes with nitrogen-deprived Norris medium and inoculated with A. caulinodans ORS571 (pBHReSREDT3) after 9 to 14 d. In control transgenic roots transformed with the empty vector (n = 34), developing nodules at LRBs were observed at 4 DAI; mature determinate nodules formed by 7 DAI (Figures 6A and 6B). In lines transformed with either of the two knockdown constructs (n = 10), mere bumps (n = 8) and in control lines (n = 6) (Figure 6J). A strong correlation was found between the degree of transcript reduction and the severity of the phenotype. Lines with the strongest decrease in expression (lower than 20%; e.g., KO2#a7) had small nodule-like structures (Figures 6G and 6H). Hence, a decrease in CCaMK transcript numbers in transgenic RNAi roots had a dosage-dependent effect on the nodule size, meaning that nodule development was hampered.

Light and transmission electron microscopy (TEM) showed that the central tissue of nodules in lines with a moderately reduced CCaMK expression (such as KO2#a7) had a few infected cells at 7 DAI, most of which were not completely filled with symbiosomes (cf. Figures 6F and 6C). Some infection threads in the infection center were abnormally broad (Figure 6F). TEM analysis indicated that these infection threads had aberrant shapes with bulged outgrowths and often a rim of low electron-dense material at the walls (Figures 6L and 6M, in comparison with Figure 6K). Nodules that appeared on an incidental GFP-negative root of these lines provided an internal control: they had a normal size and a central tissue with many fixing cells that were completely filled with bacteroids, identical to lines transformed with an empty vector (Figure 6C). In transgenic roots with strongly reduced CCaMK expression, only a few cortical cells divided at 7 DAI, but the cortex at the LRBs was colonized by bacteria in infection pockets (Figure 6l) and aberrant infection threads occurred.

**DISCUSSION**

Several lines of evidence link Ca2+ spiking in zone I root hairs of legume roots to NF signaling for nodulation. First, NFs from incompatible rhizobia or NFs lacking key modifications are unable to induce proper Ca2+ oscillations (Ehrhardt et al., 1996; Oldroyd et al., 2001a, 2001b). Second, plant mutants that are blocked at the earliest stages of the symbiosis are impaired in NF-induced Ca2+ spiking, including mutations in genes coding for the putative LysM-type NF receptors, putative ion channels, a membrane-bound leucine-rich-repeat receptor-like kinase, and components of the nucleoporin complex, functions presumably involved in generating or regulating spiking downstream of the NF perception (Catoira et al., 2000; Ben Amor et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Imaizumi-Anraku et al., 2005; Kanamori et al., 2006; Miwa et al., 2006a; Saito et al., 2006). Third, one of the essential nodulation genes encodes a CCaMK protein (Catoira et al., 2000; Miwa et al., 2006a), a strong candidate for decoding the Ca2+ signal that leads to transcriptional changes. Moreover, gain-of-function mutations of CCaMK result in spontaneous nodule formation (Gleason et al., 2006; Tirichine et al., 2006). Besides these genetic data, several physiological lines of evidence are available. For instance, inhibitors of Ca2+ spiking suppress the induction of nodulin genes, such as early nodulin 11 (ENOD11) (Pingret et al., 1998; Engstrom et al., 2002; Miwa et al., 2006b), and, on average, ~36 uninterrupted spikes are required to allow ENOD11 expression (Miwa et al., 2006a).

Until now, the role of Ca2+ spiking in symbiosis had been studied exclusively in legumes with zone I nodulation and intracellular invasion in growing root hairs. S. rostrata presents an alternative nodulation that differs in position, physiological environment, invasion mode, and the need for ethylene and has less stringent NF structural requirements (Goormachtig et al., 2004a). During LRB nodulation in S. rostrata, bacterial invasion skips the epidermis, allowing the study of the role of NF signaling components in the intercellular colonization of the cortex.

Here, we examined Ca2+ spiking patterns at LRBs and in root zone I of S. rostrata, and, in parallel, we studied the role of S. rostrata CCaMK in LRB nodulation. The outer cortical cells, where invasion takes place, were not amenable for microinjection with a Ca2+-sensitive dye. The only accessible targets were root hair initials present at LRBs of the otherwise hairless hydropnic roots. These epidermal cells respond to NFs and to bacterial inoculation by outgrowth and deformation, but they do not
become invaded. Just like LRB nodulation, this response depends on NFs, H$_2$O$_2$, and ethylene (D’Haeze et al., 2003). As these root hair initial responses have the same signaling requirements as nodulation at LRBs, we used these cells to analyze Ca$^{2+}$ spiking. We found that spike shape and frequency were different in LRB cells and zone I root hairs. The Ca$^{2+}$ spiking at LRBs was faster than in zone I, and the shape of the spikes was more symmetrical (Figure 1). Despite these differences, a pharmacological analysis suggests a similar underlying mechanism for the generation of RHC- and LRB-associated Ca$^{2+}$ spiking.
patterns, involving phospholipid signaling and Ca\textsuperscript{2+}-ATPases (see Supplemental Figure 1 online).

The LRB Ca\textsuperscript{2+} spiking pattern could be modulated by altering hormone levels. As observed in *M. truncatula* (Sun et al., 2006), JA extended the period of Ca\textsuperscript{2+} spikes. In contrast with the situation in *M. truncatula* (Oldroyd et al., 2001a), excess ethylene had no effect on LRB Ca\textsuperscript{2+} spiking; on the contrary, inhibition of ethylene production either blocked it, or, at low concentrations, increased its period. Increased JA levels or reduced ethylene levels also affected the spike shape, rendering it more asymmetric, thus shifting the signature toward the pattern observed in zone I root hairs. Modulating the levels of these hormones prominently influenced the downward phase of the Ca\textsuperscript{2+} spike, which is associated with the resequestration of Ca\textsuperscript{2+} into the internal store (Figure 3). Therefore, the target for ethylene and JA might be the Ca\textsuperscript{2+}-ATPase that functions in the reuptake of calcium.

Interestingly, the hormonal modulations that conferred zone I identity to the Ca\textsuperscript{2+} spiking pattern at LRBs stimulated infection thread formation. In the absence of AVG or JA, intracellular infection threads were rarely observed. At low concentrations, AVG or JA promoted intracellular invasion in root hairs, whereas at high concentrations they negatively affected LRB nodulation and reduced the number of both infection pockets and infected root hairs. With Fura-2 as a ratiometric dye, no significant amplitude differences were found between the cell types, indicating that shape and period are the determining factors for the shift in infection strategy (Figures 2 and 4). These findings correlate a defined Ca\textsuperscript{2+} spiking profile with the capacity for intracellular root hair invasion, be it in a zone I or in a LRB developmental context. The hormonal changes negatively affected LRB nodule development and infection pocket formation (Figures 4E and 4F), which could be caused either via the altered spiking signature or directly by the modified hormone levels.

Additional data were obtained from the study of the role of CCaMK in LRB nodulation. CCaMK proteins are plausible candidates to interpret the NF-triggered Ca\textsuperscript{2+} signature and to transmit information for gene expression, nodule formation, and infection thread progression. A *S. rostrata* CCaMK cDNA clone, corresponding to a unique gene, complemented the *DMI3-1* mutation of the *M. truncatula* CCaMK gene. qRT-PCR demonstrated that expression of CCaMK is upregulated during nodulation at adventitious root bases. A promoter-GUS reporter construct and in situ hybridization revealed expression in nodule primordia and in the proximal cells of the meristematic zone of developing nodules (Figure 5). In *M. truncatula* nodules, CCaMK, DMI1, and DMI2 transcripts are localized in the apical preinfection zone (Bersoult et al., 2005; Limpens et al., 2005; Rieley et al., 2007), and DMI2 and NFP promoter activity has been observed in the nodule primordium prior to penetration by infection threads (Bersoult et al., 2005; Arrighi et al., 2006).

Interestingly, downregulation of CCaMK expression by RNAi severely interfered with nodule formation but hardly affected the primary intercellular cortical invasion. The degree of transcript reduction and the impairment of nodule development correlated well. Lines with <20% residual transcripts merely developed small bumps at the LRBs, implying that CCaMK is important for nodulation. All suppressed lines had large infection pockets and intercellular infection threads, suggesting that signaling via CCaMK is not essential to initiate these structures (Figure 6). However, intracellular cortical infection threads were abnormal, resembling the lumpy structures observed in *S. rostrata* SYMRK knockdown lines and wild-type plants upon invasion with NF-deficient bacteria (Figure 6) (Capoen et al., 2005; Den Herder et al., 2007). Thus, proper infection thread progression in *S. rostrata* requires cell-autonomous NF perception with signal transduction that involves both CCaMK and, as shown previously, SYMRK (Capoen et al., 2005).

Our data show that initiation and development of nodule primordia at LRBs require CCaMK and that CCaMK plays a role in the progression of infection threads (Figure 6). The similar requirements for CCaMK in RHC nodulation in *M. truncatula* (Lévy et al., 2004) allow us to conclude that Ca\textsuperscript{2+} oscillations are a basic component in nodule primordia initiation and infection thread progression both in an RHC and LRB context. Different Ca\textsuperscript{2+} signatures are associated with RHC and LRB nodulation, but, at present, we cannot conclude whether the Ca\textsuperscript{2+} spiking pattern observed in root hair initials is representative of the pattern in cortical cells during LRB nodulation.

Rhizobial invasion via RHC depends on CCaMK (Catoira et al., 2000; Tirichine et al. 2006). Here, we report that this type of invasion correlates with a specific Ca\textsuperscript{2+} spiking pattern, suggesting that a frequency-dependent phosphorylation of CCaMK targets might be an important step in this process. By contrast, infection pocket formation seems independent of CCaMK (Figure 6), implying a mechanism for the initiation of cortical cell death that does not depend on Ca\textsuperscript{2+} oscillations.

Based on these observations, we propose a model with a dual pathway downstream of the primary NF perception at LRBs. NF perception by specific receptors would cause Ca\textsuperscript{2+} spiking, of which the decoding by CCaMK is essential for nodule formation. In parallel, NF perception generates secondary signals that, independently of Ca\textsuperscript{2+} spiking, are essential for the outer cortex colonization with infection pocket formation. Plausible mediators of these primary intercellular invasion events are H\textsubscript{2}O\textsubscript{2} and ethylene (D’Haeze et al., 2003).

Our data also confirm the key role for ethylene in submergence-adapted nodulation in *S. rostrata*: under waterlogged conditions, when ethylene accumulates, RHC nodulation in zone I is suppressed (D’Haeze et al., 2003), while LRB nodulation that requires ethylene is prevalent. We demonstrate that the root hairs present on hydroponic roots can perceive NFs but cannot mount a calcium response with the appropriate period for root hair invasion, presumably because of inhibiting hormones that accumulate in hydroponic roots. This problem is circumvented via a Ca\textsuperscript{2+}-independent intercellular infection pathway with infection pocket formation allowing the accumulation of sufficient numbers of rhizobia to generate a greatly amplified signal for further nodule invasion.

In conclusion, we have shown that ethylene and JA modulate the Ca\textsuperscript{2+} oscillations that are activated by rhizobial NFs at LRBs of *S. rostrata* and that a specific Ca\textsuperscript{2+} signature correlates well with the intracellular invasion mode. The physiological context and, in particular, the ethylene concentration, influences Ca\textsuperscript{2+} spiking and the choice of the developmental pathway that is activated by NF signaling in *S. rostrata*, thus contributing to the
phenotypic plasticity that is characteristic of nodulation of this tropical legume.

METHODS

Biological Material
Sesbania rostrata: Brem seedlings were germinated and grown in tubes with liquid medium (hydropionic roots) or in Leonard jars (aeroponic roots) according to the procedures described (Fernández-López et al., 1998). Medicago truncatula A17 seedlings were germinated and grown as described (Sun et al., 2006). Azorhizobium caulinodans ORS571 labeled with the red fluorescent protein (DsRed; Clontech) was obtained by introducing the plasmid pBHRdsRED3 (Smit et al., 2005) into A. caulinodans ORS571 by electroporation. The A. caulinodans ORS571 (pBHRdsRED3), ORS571 (pBBRS-hem-gfp6-S65T) (D’Haeze et al., 2004), and ORS571 (pRG960SD-32) (D’Haeze et al., 1998) strains were grown on yeast extract broth medium with appropriate antibiotics (Van den Eede et al., 1987). NFs were purified from A. caulinodans cultures as previously described (Mergaert et al., 1993).

Ca2+ Spiking Analysis
For Ca2+ spiking analysis (Ehrhardt et al., 1996; Wais et al., 2000), micropipettes were made from borosilicate capillaries with an electrode puller (model 773; Campden Instruments). Needles were preloaded with either dextran-linked Fura-2 or Oregon Green and Texas Red (10,000 molecular weight; Molecular Probes). Cells were injected via iontophoresis with a cell amplifier (model Intra 767; World Precision Instruments) and a stimulus generator (World Precision Instruments).

Plants were mounted on slides containing buffered nodulation medium (Ehrhardt et al., 1992) for microinjection of suitable root hairs and subsequent imaging. After microinjection, cells were visualized for 10 min to verify viability; only cells that still showed cytoplasmic streaming were challenged with 10 pM purified A. caulinodans or Sinorhizobium meliloti NFs. For visualization, an inverted epifluorescence microscope (TE2000; Nikon) equipped with a monochromator (Optoscan; Cairn Research) was used. Cells were imaged with a CCD camera (ORCA-ER; Hamamatsu) and an image splitter (Optosplitt; Cairn Research). Subsequently, data were analyzed with MetaFluor software (Universal Imaging). All traces shown are the fluorescence intensity ratio of Oregon Green to Texas Red; although not considered a ratiometric method, all Imaging). All traces shown are the fluorescence intensity ratio of Oregon Green to Texas Red; although not considered a ratiometric method, all

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Diaplan bright-field microscope (Leitz). GFP and dsRED analyses (Van de Velde et al., 2003) and TEM (D’Haeze et al., 1998) were done as described.

Identification of the S. rostrata CCaMK Promoter and Open Reading Frame
Nestled primers were chosen in conserved regions of CCaMK proteins from other plant species. RT-PCR on S. rostrata nodule cDNA revealed a 652-bp fragment. The full-length sequence was obtained by 5’ and 3’ rapid amplification of cDNA ends (Smart RACE cDNA amplification kit; Clontech), and the complete open reading frame was amplified with primers SrDMi3FLS (5’-GGGAGATTCAAAAGACCTGTAG-3’) and SrDMi3FLS (5’-GATGGCAATAAAAAAATTTTGGAAAG-3’) and recombined in the pCRII-TOPO vector (Invitrogen). The full-length open reading frame was transferred to the pDONR221 entry vector (Invitrogen). The S. rostrata CCaMK promoter was identified with the Universal GenomeWalker kit (Clontech) applied to genomic DNA, and the 1824-bp region upstream of the start codon was amplified with primers attB4-SrDMi3promFuls (5’-GGGGACAAACTTTTATAGAAAATTTTGTGAG-3’) and attB1-SrDMi3promFuls (5’-GGGAGCTTTTTTTGTCAAAAATTTTGGTGAC-3’) and recombined in the pDONR P4-1 (Invitrogen).
Expression Analysis of *S. rostrata* CCaMK

qRT-PCR was performed with primers Dmi3-Q2S (5'-TTTCAT-TGTCGCCCTACTATGCC-3') and Dmi3-Q2AS (5'-GCTTTGCTGTATGG-GAAAATGCC-3') or Dmi3-Q3S (5' -AAACAAAGGTTGAGAAAAGC-3') and Dmi3-Q3AS (5'-ACAGGACCATGAGACTGAAC-3') on the Lightcycler 480 (Roche Diagnostics) with the SYBR Green I master mix (Roche Diagnostics) according to the manufacturer’s instructions. The constitutively active *S. rostrata* LUB1 gene was amplified with primers SrUB1Q-S (5'-GGGAAGACGTTGGAGTGAG-3') and SrUB1Q-AS (5'-AGAGCGCA-GAAACAGTGAAGG-3') (Capoen et al., 2007) to determine relative values with the qBase software (Hellemans et al., 2007). One representative result from three biological repeats is shown.

To analyze the promoter-GUS activity, the Multisite Gateway three-fragment vector construction kit (Invitrogen) was used to fuse the promoter with the uidA gene (in pDONR207-GUS) and the T3SS terminator (in pENTR-R2-T3SS-L3) into vector pKm43GW-rolD for coexpression with GFP. To obtain transgenic roots, *S. rostrata* embryonic axes were transformed (Van de Velde et al., 2003). A 652-bp region was used as template to produce a 3'2S-labeled antisense probe for the in situ hybridization that was performed according to Goormachtig et al. (1997).

**RNAi of *S. rostrata* CCaMK**

Knockdown constructs (SrCCaMK-KO2 and SrCCaMK-KO3) were produced by recombining two regions of *CCaMK* in the pK7GWIWG2-D binary GATEWAY vector (Invitrogen) (Karimi et al., 2002). For the SrCCaMK-KO2 and SrCCaMK-KO3 constructs, the primes Dmi3KO2-Sense (5'-CAAAAAGAGGCTTCACACTAACAAGAAAAG-3') and Dmi3KO2-Asense (5'-AGAGCGCCAGTAAATTGGAAGAGTTG-3'); and Dmi3KO3-Sense (5'-CAAAAAGAGGCTTCACACTAACAAGAAAAG-3') and Dmi3KO3-Asense (5'-AGAGCGCCAGTAAATTGGAAGAGTTG-3') were used, respectively. To obtain transgenic roots, *S. rostrata* embryonic axes were transformed as described, and composite plants carrying transgenic roots were selected by GFP (Van de Velde et al., 2003). The empty vector pPZP200-egfp was used as control. The knockdown level was determined from transgenic roots cultured in vitro on a plate by qRT-PCR as described above.

**Dmi3-1 Complementation**

For complementation of the *M. truncatula* dmi3-1 mutant, the *S. rostrata* CCaMK promoter and open reading frame were recombined with Multistatic Gateway recombination into the vector pKm43GW-rolD (Invitrogen). The generation of transgenic roots on *S. rostrata* CCaMK promoter and open reading frame were recombined with Multistatic Gateway recombination into the vector pKm43GW-rolD (Invitrogen). The generation of transgenic roots on *S. rostrata* CCaMK promoter and open reading frame were recombined with Multistatic Gateway recombination into the vector pKm43GW-rolD (Invitrogen).

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EU622875 (S. rostrata CCaMK, complete coding sequence) and EU622876 (S. rostrata CCaMK, promoter region and 5' untranslated region).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Mechanisms of Ca^{2+} Spiking in *S. rostrata* LRB-Associated Root Hair Cells.

**Supplemental Figure 2.** CCaMK Sequence Alignment and Functional Complementation.

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