

Mastoparan Activates Calcium Spiking Analogous to Nod Factor-Induced Responses in *Medicago truncatula* Root Hair Cells^{1[W][OA]}

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The rhizobial-derived signaling molecule Nod factor is essential for the establishment of the *Medicago truncatula*/*Sinorhizobium meliloti* symbiosis. Nod factor perception and signal transduction in the plant involve calcium spiking and lead to the induction of nodulation gene expression. It has previously been shown that the heterotrimeric G-protein agonist mastoparan can activate nodulation gene expression in a manner analogous to Nod factor activation of these genes and this requires *DOESN'T MAKE INFECTIONS3* (*DMI3*), a calcium- and calmodulin-dependent protein kinase (CCaMK) that is required for Nod factor signaling. Here we show that mastoparan activates oscillations in cytosolic calcium similar but not identical to Nod factor-induced calcium spiking. Mastoparan-induced calcium changes occur throughout the cell, whereas Nod factor-induced changes are restricted to the region associated with the nucleus. Mastoparan-induced calcium spiking occurs in plants mutated in the receptor-like kinases *NOD FACTOR PERCEPTION* and *DMI2* and in the putative cation channel *DMI1*, which are all required for Nod factor induction of calcium spiking, indicating either that mastoparan functions downstream of these components or that it uses an alternative mechanism to Nod factor for activation of calcium spiking. However, both mastoparan and Nod factor-induced calcium spiking are inhibited by cyclopiazonic acid and *n*-butanol, suggesting some common mechanisms underpinning these two calcium agonists. The fact that mastoparan and Nod factor both activate calcium spiking and can induce nodulation gene expression in a *DMI3*-dependent manner strongly implicates CCaMK in the perception and transduction of the calcium signal.

Calcium is a common secondary messenger that functions in a diverse array of signaling pathways. Maintenance of specificity for such a ubiquitous signal is likely to be a feature of the calcium signature, which is defined by both spatial and temporal components of the calcium response (Sanders et al., 2002). One of the most complex calcium signatures is repetitive oscillations in calcium and these have been identified in a number of plant and animal signaling cascades. In plants, oscillatory calcium has been seen in guard cells in response to abscisic acid, cold, and external calcium (McAinsh et al., 1995), in growing pollen tubes (Iwano et al., 2004), and in legume root hair cells in response to the rhizobial signaling molecule Nod factor (Ehrhardt et al., 1996). In guard cells, the frequency and number

of oscillations dictates the long-term closure of the stomate (Allen et al., 2001) and this is consistent with calcium oscillation frequency dictating the level and spectrum of gene induction in animal cell lines (Dolmetsch et al., 1997).

In both plant and animal systems, inositol-1,4,5-trisphosphate (IP_3), nicotinic acid adenine dinucleotide phosphate (NAADP), cADP-Rib (cADPR), and calcium itself can function as secondary messengers directly or indirectly modifying calcium channels to activate calcium release (Sanders et al., 2002). In animal cells, IP_3 , NAADP, and cADPR induce calcium release from the reticulum network, including the endoplasmic reticulum (ER) and the nuclear envelope (Gerasimenko et al., 1995, 1996; Galione and Churchill, 2000; Churchill and Galione, 2001; Leite et al., 2003; Marius et al., 2006). In addition, NAADP appears to function on a non-ER store and recent evidence indicates lysosome-like vesicles as a target of NAADP action (Churchill et al., 2002). The vacuole acts as a major internal store for calcium in plant cells (Sanders et al., 2002) and both IP_3 and cADPR calcium-mobilizing activity has been shown to exist on the tonoplast membrane (Allen et al., 1995). However, there is also evidence for IP_3 , cADPR, and NAADP activatable channels on plant ER membranes (Martinec et al., 2000; Navazio et al., 2000, 2001). cADPR, IP_3 , and NAADP can act in concert or individually and the diverse combinatorial nature of these three messengers partly explains the diverse calcium signatures that are produced.

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Proliferation of cytosolic IP₃ is a function of phospholipase C (PLC), which degrades plasma membrane phosphatidylinositol-4,5-bisphosphate to generate IP₃ and diacylglycerol. PLC is activated by heterotrimeric G proteins that, in turn, are induced by G-protein-coupled receptors (Singer et al., 1996; Assmann, 2002). In this way, ligand perception at the plasma membrane can be linked to calcium changes in the cytosol by mobilization of IP₃. In animal systems, G-protein agonists, such as the wasp venom peptide mastoparan, can activate PLC independent of ligand perception via activation of the heterotrimeric G proteins and thus induce calcium changes (Ross and Higashijima, 1994; Sukumar et al., 1997). Mastoparan has been shown to activate a number of responses in plant cells, including mobilization of calcium (Tucker and Boss, 1996; Pingret et al., 1998). However, recent evidence indicates that mastoparan effects in plants can occur independently of the heterotrimeric G proteins (Miles et al., 2004) and, to date, the target for mastoparan in plant cells has not been defined.

Mastoparan has previously been shown to activate nodulation gene expression in a manner analogous to Nod factor activation of these genes (Pingret et al., 1998). This induction is absent in the *doesn't make infections3* mutant (*dmi3*; Charron et al., 2004) and so requires a calcium- and calmodulin-dependent protein kinase (CCaMK; Levy et al., 2004; Mitra et al., 2004), but does not require *DMI1* or *DMI2*, two components of the Nod factor signaling pathway upstream of calcium spiking. This activity of mastoparan was taken to indicate the presence of heterotrimeric G proteins in the Nod factor signaling pathway (Pingret et al., 1998). Here, we show that the synthetic analog of mastoparan (Mas7) activates oscillations in calcium similar to Nod factor-induced calcium spiking. Analogous to nodulation gene expression studies, Mas7 activation of calcium spiking does not require *NOD FACTOR PERCEPTION (NFP)*, *DMI1*, or *DMI2*, which are necessary for Nod factor activation of calcium spiking. The fact that CCaMK is required to transduce the signal from two independent activators of calcium oscillations strongly implicates this protein in decoding the oscillatory calcium signal.

RESULTS

The Mastoparan Synthetic Analog Mas7 Activates Calcium Oscillations in *Medicago truncatula* Root Hair Cells

The requirement for *DMI3/CCaMK* in mastoparan and Mas7 activation of the Nod factor reporter *ENOD11-GUS* (Charron et al., 2004) suggested that mastoparan and Mas7 were able to activate calcium responses in root hair cells of *M. truncatula*. It has previously been shown that the concentration of Mas7 (0.5 μM) required for induction of *ENOD11-GUS* (Supplemental Fig. S1) induces lethality in approximately 50% of *M. truncatula* root hair cells (Charron et al.,

2004). We therefore chose to assess calcium responses using an *M. truncatula* line stably transformed with the calcium reporter cameleon YC2.1 (Miwa et al., 2006), which allows a large number of cells to be assayed. Cameleon also has the advantage of allowing ratio-metric measurements of calcium changes within individual cells (Miyawaki et al., 1997, 1999). Similar to previous reports, we also saw a high degree of lethality of root hair cells treated with 0.5 μM Mas7 as indicated by loss of cytoplasmic streaming. However, among the cells that survived this treatment (indicated by maintenance of cytoplasmic streaming), a significant proportion (27%) showed calcium oscillations (Fig. 1; Table I). The inactive analog Mas17, which contains a single amino acid change in the peptide, showed no calcium oscillations (Table I). Treatment with 0.2 μM Mas7 activated calcium oscillations, but in only 13.5% of root hair cells analyzed, whereas treatment with 2 μM Mas7 was predominantly lethal. Calcium oscillations induced by Mas7 appear broadly similar to Nod

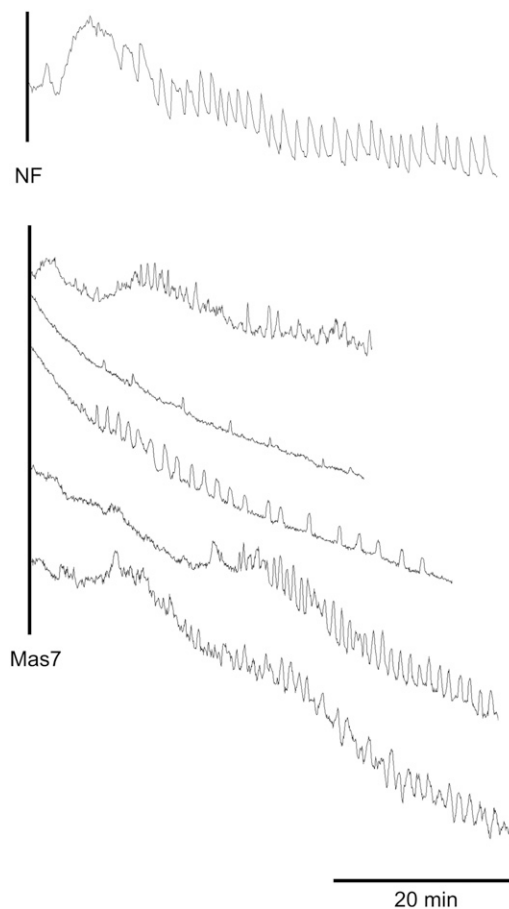


Figure 1. Single cells showing calcium oscillations following Nod factor and Mas7 addition. Cells expressing cameleon were analyzed for their calcium responses to 1 nM Nod factor (NF) and 0.5 μM Mas7. A selection of cells treated with Mas7 is shown, revealing the variety of calcium oscillation frequencies observed. The lines indicate the point of addition of either Nod factor or Mas7. The y axis is the ratio of YFP to CFP in arbitrary units.

Table 1. Calcium spiking assayed in wild-type plants transformed with cameleon and treated with Mas7 or Mas17

	No. Plants	Cells Spiking/Total Cells
Mas7 (0.5 μM)	7	22/82
Mas7 (0.2 μM)	6	7/52
Mas17 (0.5 μM)	3	0/38

factor-induced calcium spiking, but the period between oscillations is longer (Figs. 1 and 2A) and the lag from treatment to induction of oscillations is also longer (Fig. 2B) with Mas7-induced calcium oscillations. However, for both period and lag, Mas7-induced oscillations are much more variable than Nod factor-induced calcium spiking. The variability in period is mostly the result of variability between cells with Mas7-induced calcium oscillations showing a much broader range of periods (Fig. 2C). Indeed, Mas7-induced calcium oscillations can show a much longer period (10 to 20 min) than has ever been observed in Nod factor responses. These cells showing very long periods between oscillations still maintain continuity of period (Fig. 1), indicating a mechanism for rhythmicity even over these long periods. We have shown that Nod factor-induced calcium spiking is not restricted to those cells that activate *ENOD11* (Miwa et al., 2006) and, in an analogous manner, we observed Mas7-induced calcium oscillations in both growing and mature root hair cells.

Structure of Mas7-Induced Calcium Oscillations

Nod factor-induced calcium spiking shows a very rapid increase in calcium followed by a more gradual decline (Fig. 3; Ehrhardt et al., 1996). This can be interpreted to indicate the opening of calcium channels on internal stores and the rapid movement of calcium down its concentration gradient, followed by the slower active uptake of calcium into calcium stores. This uptake would depend on calcium ATPases, which is consistent with pharmacological studies (Engstrom et al., 2002). Careful comparison between Nod factor-induced calcium spiking and Mas7-induced calcium oscillations indicates subtle differences between these two responses. The overall time from initiation of the calcium increase to a return to basal calcium levels is equivalent between Nod factor- and Mas7-induced responses (Fig. 3). However, Mas7 shows a slower initial increase in calcium levels such that the first phase of the spike represents 34% of the overall spike time for Mas7 compared with 17% in Nod factor-induced spiking. The initial phase of the spike will be dependent on the activation dynamics of a population of calcium channels. We interpret the Mas7 spike structure to indicate that either the dynamics of calcium channel opening differ at the individual channel level compared with Nod factor activation of these channels or that Mas7 is less effective at activating the population of channels at an

equivalent time. To ensure that the differences we observed in Mas7-induced calcium oscillations were not a function of unrelated actions of Mas7, we treated cells undergoing Nod factor-induced calcium spiking with different concentrations of Mas7. Treatment with Mas7 did not affect the structure of Nod factor-induced calcium spiking until the application of higher concentrations of Mas7 caused cell death (Supplemental Fig. S2).

Spatial Resolution of Mas7-Induced Calcium Oscillations

Nod factor-induced calcium spiking is mostly restricted to the cytosol associated with the nucleus (Fig. 4, D–F), with minor changes occurring at the root hair tip (Shaw and Long, 2003). Because of the interference from the fluorescence of neighboring cells, we can only assess the hair region of root hair cells in the cameleon-transformed plants. Therefore, to assess the spatial nature of the Mas7-induced calcium changes, we micro-injected root hair cells with the calcium-responsive dye Oregon green dextran (10,000 molecular weight [MW]) and the unresponsive dye Texas red dextran (10,000 MW). Injection of the two dyes allows pseudoratio imaging of calcium changes (Shaw and Long,

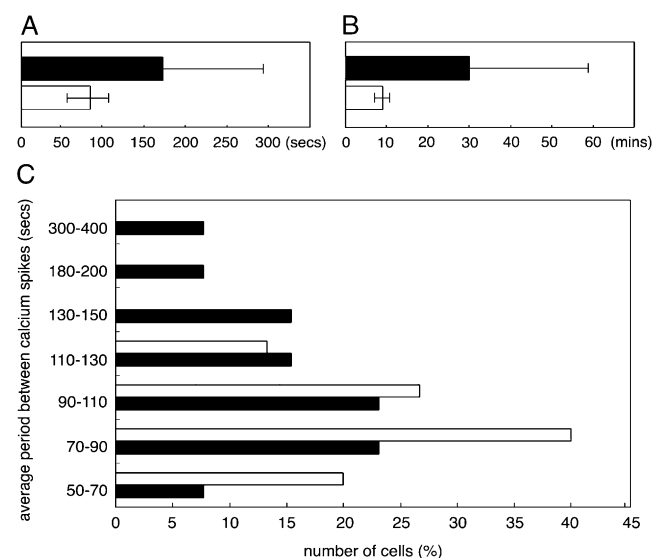


Figure 2. Quantification of the lag and period of Mas7-induced calcium oscillations. Cells treated with 0.5 μM Mas7 show a longer and more variable period between calcium oscillations (A) and a longer and more variable lag from addition of Mas7 to induction of oscillations (B) compared with Nod factor (1 nM)-induced calcium spiking. C, Histogram showing the average period per cell for Mas7- and Nod factor-treated cells. Cells treated with Mas7 show a consistent period between spikes, but the average period per cell is more variable for Mas7-treated cells than Nod factor-treated cells. However, the predominant period is consistent between Mas7- and Nod factor-treated cells. Black bars = cells treated with 0.5 μM Mas7; white bars = cells treated with 1 nM Nod factor. Thirteen cells were analyzed for the Mas7 treatment and 15 cells were analyzed for the Nod factor treatment.

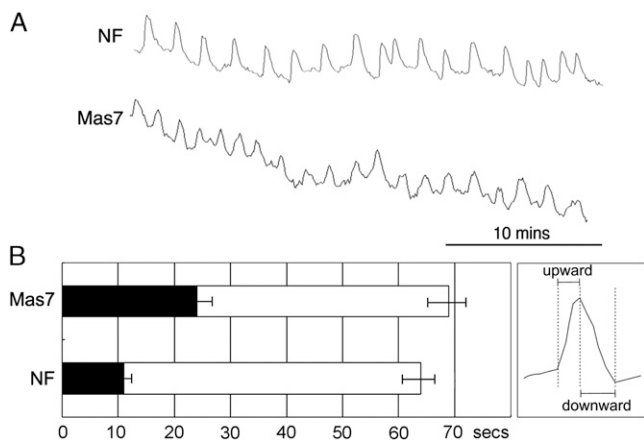


Figure 3. The structure of Mas7-induced calcium oscillations is slightly different from Nod factor-induced calcium spiking. A, Nod factor (NF)-induced calcium spiking and Mas7-induced calcium oscillations (note that Mas7-induced oscillations show slower calcium increase compared with Nod factor-induced calcium spiking). B, Quantification of the two phases of the spike reveal that the overall timing of Mas7-induced calcium oscillation is equivalent to Nod factor-induced calcium spiking, but the first phase of the spike is longer and the second phase shorter in Mas7-induced calcium spiking. Twenty-six cells were analyzed for Mas7 (0.5 μM) treatments and 30 cells were analyzed in the Nod factor (1 nM) treatment. Black bar = first phase of the spike, the upward slope; white bar = the second phase of the spike, the downward slope. Error bars = SD. The y axis in A is the ratio of YFP to CFP in arbitrary units.

2003), with Texas red providing a control for cytoplasmic content within the zone being analyzed. We found that, in contrast to the nuclear-restricted nature of Nod factor-induced calcium spiking (Fig. 4, D–F), Mas7-induced calcium oscillations occur throughout the cell (Fig. 4, A–C). Mas7-induced calcium changes are very apparent in regions distal to the nucleus, where Nod factor calcium changes are not observed (Fig. 4C). This suggests that Mas7 is able to activate calcium channels in a region of the cell that Nod factor cannot and so these data reveal some differences between Nod factor- and Mas7-induced calcium oscillations.

Mas7 Induction of Calcium Oscillations Does Not Require *NFP*, *DMI1*, and *DMI2*

To assess the relationship between Nod factor- and Mas7-induced calcium oscillations, we analyzed the ability of Mas7 to induce calcium responses in plants mutated in components of the Nod factor signaling pathway. Nod factor signaling is initiated by receptor-like kinases with sugar-binding motifs that are strong candidates for the Nod factor receptor and are represented by *NFP* in *M. truncatula* (Amor et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006). Acting downstream of *NFP* is a second receptor-like kinase, *DMI2* (Endre et al., 2002; Stracke et al., 2002), and a putative cation channel, *DMI1* (Ane et al., 2004), and all three genes are required for Nod factor

activation of calcium spiking (Wais et al., 2000). The CCaMK *DMI3* (Levy et al., 2004; Mitra et al., 2004) is also required for Nod factor signal transduction, but is not required for the activation of calcium spiking (Wais et al., 2000), indicating a function downstream of this calcium response. At the time of these experiments, we did not have these mutant lines containing theameleon reporter and therefore we chose to microinject root hair cells of *nfp*, *dmi1-1*, *dmi2-1*, and *dmi3-1* with Oregon green dextran and Texas red dextran. We found that all mutant lines were able to initiate calcium oscillations following treatment with Mas7 (Fig. 5; Table II). The mutant alleles chosen are presumed to be null alleles because they contain premature stop codons (Endre et al., 2002; Ane et al., 2004; Levy et al., 2004; Mitra et al., 2004). This work indicates that, unlike Nod factor, Mas7 does not require *NFP*, *DMI1*, or *DMI2* to activate calcium responses.

Inhibitors of Mas7-Induced Calcium Oscillations

To further assess the relationship between Mas7- and Nod factor-induced calcium oscillations, we analyzed the effects of a number of inhibitors known to abolish Nod factor-induced calcium spiking. Both cyclopiazonic acid (CPA), which inhibits type IIA calcium ATPases, and *n*-butanol, which inhibits phospholipase D (PLD), have been shown to inhibit Mas7 induction of *ENOD11-GUS* (Charron et al., 2004). Both of these inhibitors block Nod factor-induced calcium spiking without obvious detrimental effects on cellular viability (Engstrom et al., 2002; Charron et al., 2004; H. Miwa and A. Downie, unpublished data). To assess the effect of these inhibitors on Mas7 responses, we treated cells that showed robust and highly repetitive Mas7-induced calcium oscillations. Three cells on three independent plants showed inhibition of Mas7-induced calcium oscillations following treatment with 0.5% *n*-butanol (Fig. 6B). Treatment with 10 μM CPA did cause inhibition of Mas7-induced calcium oscillations (Fig. 6C), but was less severe than has been observed with Nod factor-induced calcium spiking (Fig. 6A). We saw an equivalent effect of CPA on three Mas7-treated cells from three independent plants. Occasionally, we have seen a similar pattern of CPA inhibition on Nod factor-induced calcium spiking, particularly in cells showing a rapid frequency of spiking (Supplemental Fig. S3).

Table II. 0.5 μM Mas7-induced calcium spiking assayed in wild-type and mutant plants microinjected with the fluorescent dyes Oregon green/Texas red

	No. Plants	Cells Spiking/Total Cells
Wild type	5	2/6
<i>nfp-1</i>	4	3/10
<i>dmi1-1</i>	3	2/7
<i>dmi2-1</i>	4	2/8
<i>dmi3-1</i>	7	2/15

DISCUSSION

Calcium oscillations are a component of the Nod factor signal transduction pathway and probably act as a secondary messenger to transduce the perception of Nod factor at the plasma membrane to activate gene expression in the nucleus (Oldroyd and Downie, 2004). Gain-of-function mutations in *DMI3/CCaMK* lead to induction of nodule development in the absence of Nod factor (Gleason et al., 2006; Tirichine et al., 2006), indicating the essential nature of the calcium signal. Work in both animal cells and plant guard cells has revealed the relevance of oscillatory calcium signals through the use of systems that can activate cellular calcium oscillations in the absence of signaling ligands (Dolmetsch et al., 1998; Allen et al., 2001). This work has revealed that calcium oscillations can activate downstream responses and the period between oscillations dictates the nature of the downstream response. We have attempted to recapitulate this work in *M. truncatula* root hair cells (J. Sun and G. Oldroyd, unpublished data), but we have been unsuccessful in activating 36 sequential calcium spikes, which have been shown to be required for nodulation gene expression (Miwa et al., 2006). Here, we show that in addition to Nod factor, we can activate calcium oscillations in *M. truncatula* root hair cells using the G-protein agonist Mas7. This provides a valuable tool for assessing the relevance of calcium oscillations for the activation of nodulation responses.

Mas7 activates calcium oscillations broadly similar to Nod factor-induced calcium spiking, but with a

number of differences: slower initial release of calcium in the Mas7 response; greater variability in the lag and period in Mas7-induced calcium oscillations; and expansion in the cellular location of Mas7-induced calcium changes. Despite the differences observed between Mas7- and Nod factor-induced calcium spiking, both calcium agonists induce *ENOD11* and *ENOD12* expression with a similar spatial pattern to Nod factor activation of these genes (Pingret et al., 1998). Both Nod factor and Mas7 induction of *ENOD11* are dependent on *DMI3* (Charron et al., 2004), whose mutant phenotypes suggest a specific role in symbiosis signaling (Catoira et al., 2000). These data indicate that Mas7-induced calcium oscillations mimic Nod factor-induced calcium spiking and this highlights the importance of an oscillatory calcium signal for the induction of *CCaMK* and its activation of nodulation gene expression. In the absence of a forced calcium oscillatory system, the fact that two calcium agonists, Mas7 and Nod factor, induce calcium oscillations and both activate appropriate nodulation gene expression represents the best evidence we have to show the importance of calcium oscillations in this symbiosis signaling pathway.

Mas7 induction of calcium oscillations and *ENOD11* expression (Charron et al., 2004) do not require *NFP*, *DMI1*, or *DMI2*, *M. truncatula* Nod factor signaling genes required for Nod factor-induced calcium spiking (Wais et al., 2000). This can be interpreted in two ways: either Mas7-induced calcium oscillations are mechanistically unrelated to Nod factor-induced calcium spiking or Mas7 activates a component of the

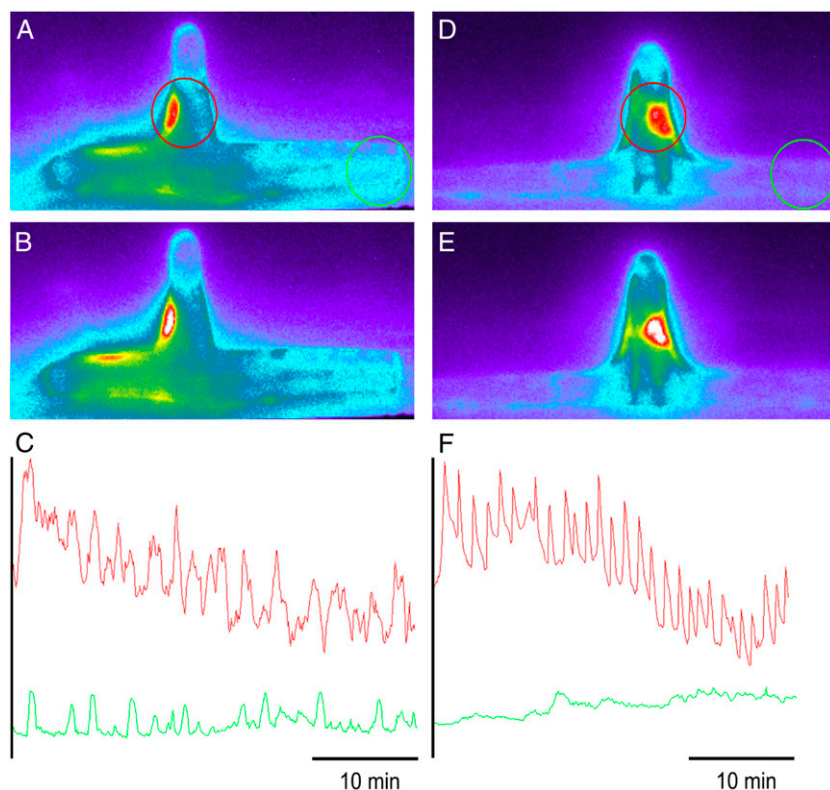


Figure 4. Mas7-induced calcium changes occur throughout the cell. A to C, Cell microinjected with the fluorescent dyes Oregon green/Texas red and treated with $0.5 \mu\text{M}$ Mas7. A, Cell prior to a calcium spike. B, The same cell at the peak of a spike 15 s later. Note that calcium increases, indicated by red and white coloration, occur both in the nucleus and distant to the nucleus. C, Calcium changes in the cell assessed in the domains indicated in A. D to F, Cell microinjected with the fluorescent dyes treated with 1 nM Nod factor. D, Cell prior to a calcium spike. E, The same cell at the peak of a spike 15 s later. F, Quantification of calcium changes in the regions denoted in D. Note that Nod factor-induced calcium changes are restricted to the nuclear region and do not occur in regions in the basal part of the root hair cell. The y axis in C and F represents the ratio of Oregon green to Texas red in arbitrary units. The domains and traces in A, C, D, and F are color coded such that the red trace is the quantification of the domain indicated in red and the equivalent is true for the green domain and trace.

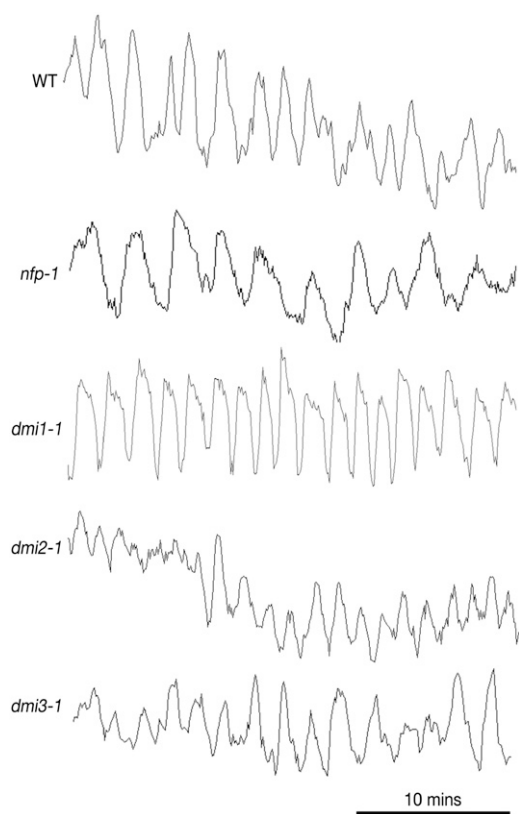


Figure 5. Representative traces showing the Mas7 ($0.5 \mu\text{M}$)-induced responses in the Nod factor signaling mutants. Cells on wild-type and mutant lines were microinjected with the calcium-responsive dye Oregon green and the nonresponsive dye Texas red. Calcium changes were measured as the ratio of Oregon green to Texas red allowing pseudoratiometric imaging of calcium changes in Mas7-treated cells. The y axis represents the ratio of Oregon green to Texas red in arbitrary units. WT, Wild type.

Nod factor signaling pathway downstream of *NFP*, *DMI1*, and *DMI2*. If we presume that mastoparan- and Nod factor-induced calcium oscillations are mechanistically related, then the fact that mastoparan can activate calcium oscillations in *DMI1* mutants indicates that this putative cation channel is not the calcium channel responsible for Nod factor-induced calcium spiking. Two inhibitors that are known to abolish Nod factor-induced calcium spiking, the calcium ATPase inhibitor CPA and the PLD inhibitor *n*-butanol, inhibit Mas7-induced calcium oscillations, indicating common mechanisms between these two responses. However, this is not definitive proof that the calcium channels involved in Nod factor- and Mas7-induced calcium spiking are equivalent. Indeed, the differences observed between the structure and cellular location of these two calcium responses indicate that Mas7 and Nod factor act on different sets of calcium channels or differentially activate equivalent calcium channels.

Mastoparan/Mas7 has been shown to activate G proteins by catalyzing GDP/GTP exchange similar to

the action of G-protein-coupled receptors (Sukumar et al., 1997). However, mastoparan has also been shown to have additional sites of action in animal studies, including inhibition of calcium ATPases (Longland et al., 1999) and modifying glycogen phosphorylase, which modulates calcium release from the ryanodine receptor (Hirata et al., 2000, 2003). As such, mastoparan can activate calcium release independent of G-protein activation. This is consistent with observations in G-protein mutants of *Arabidopsis thaliana* that show mastoparan activation of mitogen-activated protein kinases (Miles et al., 2004), indicating a mastoparan site of action independent of plant G proteins. Mastoparan activates PLC and PLD in legume roots (den Hartog et al., 2001) and, based on pharmacological analysis, it appears that PLD is required for Mas7 induction of *ENOD11* (Charron et al., 2004). This indicates that mastoparan leads to the activation of PLD, which, in turn, induces calcium oscillations and such a model is supported by the inhibition of Mas7-induced calcium oscillations by *n*-butanol, a known inhibitor of plant PLD. However, it is also possible that the site of mastoparan action requires the activity of PLD; for instance, the protein that is modulated by mastoparan may require phosphatidic acid for activity. Hence, it is currently unclear whether mastoparan modulates phospholipases that directly or indirectly generate a secondary signal that activates the calcium channel or whether mastoparan modulates other components of the calcium signaling machinery that require the activity of phospholipases. Defining the mode of action of mastoparan could provide insight into mechanisms of the Nod factor signaling pathway and the induction of calcium spiking.

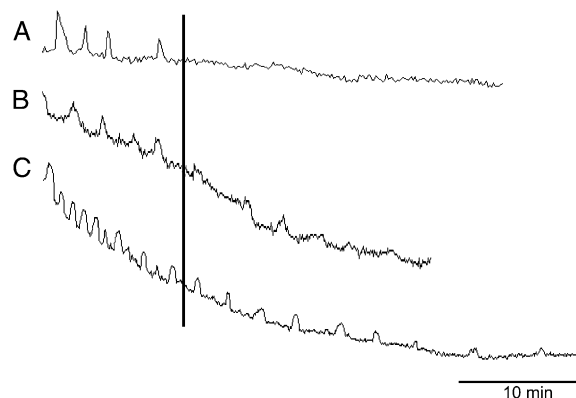


Figure 6. Mas7-induced calcium oscillations are inhibited by CPA and *n*-butanol, assayed using cameleon-transformed cells. A, Cell treated with 1 nM Nod factor and secondarily treated with $10 \mu\text{M}$ CPA shows rapid inhibition of calcium spiking following CPA addition. B, $0.5 \mu\text{M}$ Mas7-induced calcium oscillations are rapidly inhibited following treatment with 0.5% (68 mM) *n*-butanol. C, $10 \mu\text{M}$ CPA treatment inhibits $0.5 \mu\text{M}$ Mas7-induced calcium oscillations, but this inhibition is less severe than CPA inhibition of Nod factor-induced calcium spiking (A). All traces are representative of three independent experiments. The y axis is the ratio of YFP to CFP in arbitrary units.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Medicago truncatula seeds were scarified for 5 min in concentrated sulfuric acid, washed twice in sterile water, surface sterilized for 3 min in undiluted commercial bleach, then washed five times in sterile water. Seeds were imbibed for 1 to 3 h, transferred to petri plates with damp filter papers, and germinated overnight in the dark at room temperature. Germinated seedlings were transferred to buffered nodulation media (Ehrhardt et al., 1992) agar, pH 6.5, containing the ethylene inhibitor 0.1 μM L- α -(2-aminoethoxyvinyl)-Gly and grown overnight at 25°C, with the roots shaded by wrapping the plates in aluminum foil. The root tips of the seedlings were removed to avoid disturbance during imaging and the seedlings were then transferred to a liquid buffered nodulation media bath contained on a large coverslip. During imaging, 0.5 μM Mas7 (Sigma), 0.5 μM Mas17 (Sigma), or 1 nM *Sinorhizobium meliloti* Nod factor were added to the bath. For the calcium measurements using cameleon, we used an *M. truncatula* R108 line that was stably transformed with cameleon YC2.1 regulated by the 35S promoter (Miwa et al., 2006). We used mutant lines that had at minimum been backcrossed once. Nod factor preparations were isolated as described by Ehrhardt et al. (1996).

Calcium Imaging

For analysis of mutants, we microinjected root hair cells with calcium-responsive dyes as described by Ehrhardt et al. (1996), with slight modifications as described by Wais et al. (2000). Micropipettes were pulled from filamented capillaries on a pipette puller (model 773; Campden Instruments). These were loaded with Oregon green dextran (10,000 MW; Molecular Probes) and Texas red dextran (10,000 MW; Molecular Probes). Injections were performed using iontophoresis with currents generated from a cell amplifier (model Intra 767; World Precision Instruments) and a stimulus generator made to our specifications (World Precision Instruments). Cells were analyzed on an inverted epifluorescence microscope (model TE2000; Nikon) using a monochromator (model optoscan; Cairns Research) to generate specific wavelengths of light. During image capture, the image was split using the Optosplit (Cairn Research), and each image passed through a filter for either Oregon green or Texas red emissions prior to exposure on the CCD chip (model ORCA-ER; Hamamatsu). Data were analyzed using Metaflor (Universal Imaging).

Lines expressing the calcium reporter cameleon YC2.1 were generated as described (Miwa et al., 2006). These lines were analyzed on the same inverted epifluorescent microscope (model TE2000, Nikon). During image capture, the image was split using the Optosplit (Cairn Research), and each image passed through a filter for either cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) emissions prior to exposure on the CCD chip. The ratio of CFP to YFP was assayed using Metaflor (Universal Imaging).

Supplemental Data

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

Supplemental Figure S1. Mas7 induction of *ENOD11-GUS*.

Supplemental Figure S2. Mas7 treatment does not affect Nod factor-induced calcium spiking.

Supplemental Figure S3. CPA treatment of a cell undergoing rapid Nod factor-induced calcium spiking.

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LITERATURE CITED

- Allen GJ, Chu SP, Harrington CL, Schumacher K, Hoffmann T, Tang YY, Grill E, Schroeder JI (2001) A defined range of guard cell calcium oscillation parameters encodes stomatal movements. *Nature* **411**: 1053–1057
- Allen GJ, Muir SR, Sanders D (1995) Release of Ca^{2+} from individual plant vacuoles by both InsP_3 and cyclic ADP-ribose. *Science* **268**: 735–737
- Amor BB, Shaw SL, Oldroyd GE, Maillet F, Penmetsa RV, Cook D, Long SR, Denarie J, Gough C (2003) The *NFP* locus of *Medicago truncatula* controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation. *Plant J* **34**: 495–506
- Ane JM, Kiss GB, Riely BK, Penmetsa RV, Oldroyd GE, Ayax C, Levy J, Debelle F, Baek JM, Kalo P, et al (2004) *Medicago truncatula* DM11 required for bacterial and fungal symbioses in legumes. *Science* **303**: 1364–1367
- Arrighi JF, Barre A, Ben Amor B, Bersoult A, Soriano LC, Mirabella R, de Carvalho-Niebel F, Journet EP, Gherardi M, Huguet T, et al (2006) The *Medicago truncatula* lysine motif-receptor-like kinase gene family includes NFP and new nodule-expressed genes. *Plant Physiol* **142**: 265–279
- Assmann SM (2002) Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. *Plant Cell (Suppl)* **14**: S355–373
- Catoira R, Galera C, de Billy F, Penmetsa RV, Journet E, Maillet F, Rosenberg C, Cook D, Gough C, Denarie J (2000) Four genes of *Medicago truncatula* controlling components of a Nod factor transduction pathway. *Plant Cell* **12**: 1647–1665
- Charron D, Pingret JL, Chabaud M, Journet EP, Barker DG (2004) Pharmacological evidence that multiple phospholipid signaling pathways link rhizobium nodulation factor perception in *Medicago truncatula* root hairs to intracellular responses, including Ca^{2+} spiking and specific ENOD gene expression. *Plant Physiol* **136**: 3582–3593
- Churchill GC, Galione A (2001) NAADP induces Ca^{2+} oscillations via a two-pool mechanism by priming IP₃- and cADPR-sensitive Ca^{2+} stores. *EMBO J* **20**: 2666–2671
- Churchill GC, Okada Y, Thomas JM, Genazzani AA, Patel S, Galione A (2002) NAADP mobilizes Ca^{2+} from reserve granules, lysosome-related organelles, in sea urchin eggs. *Cell* **111**: 703–708
- den Hartog M, Musgrave A, Munnik T (2001) Nod factor-induced phosphatidic acid and diacylglycerol pyrophosphate formation: a role for phospholipase C and D in root hair deformation. *Plant J* **25**: 55–65
- Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI (1997) Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. *Nature* **386**: 855–858; erratum Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI (1997) *Nature* **388**: 308
- Dolmetsch RE, Xu K, Lewis RS (1998) Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* **392**: 933–936
- Ehrhardt DW, Atkinson EM, Long SR (1992) Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. *Science* **256**: 998–1000
- Ehrhardt DW, Wais R, Long SR (1996) Calcium spiking in plant root hairs responding to *Rhizobium* nodulation signals. *Cell* **85**: 673–681
- Endre G, Kereszt A, Kevei Z, Mihacea S, Kalo P, Kiss GB (2002) A receptor kinase gene regulating symbiotic nodule development. *Nature* **417**: 962–966
- Engstrom EM, Ehrhardt DW, Mitra RM, Long SR (2002) Pharmacological analysis of Nod factor-induced calcium spiking in *Medicago truncatula*: evidence for the requirement of type IIA calcium pumps and phosphoinositide signaling. *Plant Physiol* **128**: 1390–1401
- Galione A, Churchill GC (2000) Cyclic ADP-ribose as a calcium-mobilizing messenger. *Sci STKE* **2000**: PE1
- Gerasimenko OV, Gerasimenko JV, Belan PV, Petersen OH (1996) Inositol trisphosphate and cyclic ADP-ribose-mediated release of Ca^{2+} from single isolated pancreatic zymogen granules. *Cell* **84**: 473–480
- Gerasimenko OV, Gerasimenko JV, Tepikin AV, Petersen OH (1995) ATP-dependent accumulation and inositol trisphosphate- or cyclic ADP-ribose-mediated release of Ca^{2+} from the nuclear envelope. *Cell* **80**: 439–444
- Gleason C, Chaudhuri S, Yang T, Munoz A, Poovaiah BW, Oldroyd GED (2006) Nodulation independent of rhizobia induced by a calcium-activated kinase lacking autoinhibition. *Nature* **441**: 1149–1152
- Hirata Y, Atsumi M, Ohizumi Y, Nakahata N (2003) Mastoparan binds to glycogen phosphorylase to regulate sarcoplasmic reticular Ca^{2+} release in skeletal muscle. *Biochem J* **371**: 81–88
- Hirata Y, Nakahata N, Ohizumi Y (2000) Identification of a 97-kDa mastoparan-binding protein involving in Ca^{2+} release from skeletal muscle sarcoplasmic reticulum. *Mol Pharmacol* **57**: 1235–1242
- Iwano M, Shiba H, Miwa T, Che FS, Takayama S, Nagai T, Miyawaki A, Isogai A (2004) Ca^{2+} dynamics in a pollen grain and papilla cell during pollination of *Arabidopsis*. *Plant Physiol* **136**: 3562–3571
- Leite MF, Thrower EC, Echevarria W, Koulen P, Hirata K, Bennett AM, Ehrlich BE, Nathanson MH (2003) Nuclear and cytosolic calcium are regulated independently. *Proc Natl Acad Sci USA* **100**: 2975–2980

- Levy J, Bres C, Geurts R, Chalhoub B, Kulikova O, Duc G, Journet EP, Ane JM, Lauber E, Bisseling T, et al (2004) A putative Ca²⁺ and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. *Science* **303**: 1361–1364
- Longland CL, Mezna M, Michelangeli F (1999) The mechanism of inhibition of the Ca²⁺-ATPase by mastoparan: mastoparan abolishes cooperative Ca²⁺ binding. *J Biol Chem* **274**: 14799–14805
- Madsen EB, Madsen LH, Radutoiu S, Olbryt M, Rakwalska M, Szczyglowski K, Sato S, Kaneko T, Tabata S, Sandal N, et al (2003) A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. *Nature* **425**: 637–640
- Marius P, Guerra MT, Nathanson MH, Ehrlich BE, Leite MF (2006) Calcium release from ryanodine receptors in the nucleoplasmic reticulum. *Cell Calcium* **39**: 65–73
- Martinez J, Felzl T, Scanlon CH, Lumsden PJ, Machackova I (2000) Subcellular localization of a high affinity binding site for D-myo-inositol-1,4,5-trisphosphate from *Chenopodium rubrum*. *Plant Physiol* **124**: 475–483
- McAinsh MR, Webb AAR, Taylor JE, Hetherington AM (1995) Stimulus-induced oscillations in guard cell cytosolic free calcium. *Plant Cell* **7**: 1207–1219
- Miles GP, Samuel MA, Jones AM, Ellis BE (2004) Mastoparan rapidly activates plant MAP kinase signaling independent of heterotrimeric G proteins. *Plant Physiol* **134**: 1332–1336
- Mitra RM, Gleason CA, Edwards A, Hadfield J, Downie JA, Oldroyd GE, Long SR (2004) A Ca²⁺/calmodulin-dependent protein kinase required for symbiotic nodule development: gene identification by transcript-based cloning. *Proc Natl Acad Sci USA* **101**: 4701–4705
- Miwa H, Sun J, Oldroyd G, Downie A (2006) Analysis of calcium spiking using aameleon calcium sensor reveals that nodulation gene expression is regulated by calcium spike number and the developmental status of the cell. *Plant J* **48**: 883–894
- Miyawaki A, Griesbeck O, Heim R, Tsien RY (1999) Dynamic and quantitative Ca²⁺ measurements using improved cameleons. *Proc Natl Acad Sci USA* **96**: 2135–2140
- Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, Ikura M, Tsien RY (1997) Fluorescent indicators for Ca²⁺ based on green fluorescent proteins and calmodulin. *Nature* **388**: 882–887
- Navazio L, Bewell MA, Siddiqua A, Dickinson GD, Galione A, Sanders D (2000) Calcium release from the endoplasmic reticulum of higher plants elicited by the NADP metabolite nicotinic acid adenine dinucleotide phosphate. *Proc Natl Acad Sci USA* **97**: 8693–8698
- Navazio L, Mariani P, Sanders D (2001) Mobilization of Ca²⁺ by cyclic ADP-ribose from the endoplasmic reticulum of cauliflower florets. *Plant Physiol* **125**: 2129–2138
- Oldroyd GE, Downie JA (2004) Calcium, kinases and nodulation signaling in legumes. *Nat Rev Mol Cell Biol* **5**: 566–576
- Pingret JL, Journet EP, Barker DG (1998) Rhizobium nod factor signaling: evidence for a G-protein-mediated transduction mechanism. *Plant Cell* **10**: 659–672
- Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Gronlund M, Sato S, Nakamura Y, Tabata S, Sandal N, et al (2003) Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* **425**: 585–592
- Ross EM, Higashijima T (1994) Regulation of G-protein activation by mastoparans and other cationic peptides. *Methods Enzymol* **237**: 26–37
- Sanders D, Pelloux J, Brownlee C, Harper JF (2002) Calcium at the crossroads of signaling. *Plant Cell* **14**: S401–417
- Shaw SL, Long SR (2003) Nod factor elicits two separable calcium responses in *Medicago truncatula* root hair cells. *Plant Physiol* **131**: 976–984
- Singer WD, Brown HA, Jiang X, Sternweis PC (1996) Regulation of phospholipase D by protein kinase C is synergistic with ADP-ribosylation factor and independent of protein kinase activity. *J Biol Chem* **271**: 4504–4510
- Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J, Szczyglowski K, et al (2002) A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature* **417**: 959–962
- Sukumar M, Ross EM, Higashijima T (1997) A Gs-selective analog of the receptor-mimetic peptide mastoparan binds to Gs alpha in a kinked helical conformation. *Biochemistry* **36**: 3632–3639
- Tirichine L, Imaizumi-Anraku H, Yoshida S, Murakami Y, Madsen LH, Miwa H, Nakagawa T, Sandel N, Albrechtsen AS, Kawaguchi M, et al (2006) Deregulation of a Ca²⁺/calmodulin-dependent kinase leads to spontaneous nodule development. *Nature* **441**: 1153–1156
- Tucker EB, Boss WF (1996) Mastoparan-induced intracellular Ca²⁺ fluxes may regulate cell-to-cell communication in plants. *Plant Physiol* **111**: 459–467
- Wais RJ, Galera C, Oldroyd G, Catoira R, Penmetsa RV, Cook D, Gough C, Denarie J, Long SR (2000) Genetic analysis of calcium spiking responses in nodulation mutants of *Medicago truncatula*. *Proc Natl Acad Sci USA* **97**: 13407–13412