Mastoparan activates calcium spiking analogous to Nod factor-induced responses in *Medicago truncatula* root hair cells.

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**SUMMARY**

The rhizobial derived signalling molecule Nod factor is essential for the establishment of the *Medicago truncatula/Sinorhizobium meliloti* symbiosis. Nod factor perception and signal transduction in the plant involves calcium spiking and leads 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 *DMI3*, a calcium and calmodulin dependent protein kinase (CCaMK) that is required for Nod factor signalling. 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 *NFP* and *DMI2* and in the putative cation channel...
that 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.

INTRODUCTION

Calcium is a common secondary messenger that functions in a diverse array of signalling pathways. The 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 signalling cascades. In plants, oscillatory calcium has been seen in guard cells in response to ABA, 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 signalling 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 (IP₃), nicotinic acid adenine dinucleotide phosphate (NAADP), cyclic ADP-ribose (cADPR) and calcium itself can function as secondary messengers directly or indirectly modifying calcium channels to activate calcium release (Sanders et al., 2002). Animal cells show IP₃, NAADP and cADPR-induced calcium release from the reticulum network including the ER and the nuclear envelope (Gerasimenko et al., 1995; Gerasimenko et al., 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-mobilising 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; Navazio et al., 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.

Proliferation of cytosolic IP$_3$ is a function of phospholipase C (PLC) that degrades plasma membrane phosphatidylinositol 4,5-bisphosphate to generate IP$_3$ 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 the mobilisation of IP$_3$. 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 the mobilisation 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 requires the activity of $DMI3$ (Charron et al., 2004) 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 signalling pathway upstream of calcium spiking. This activity of Mastoparan was taken to indicate the presence of heterotrimeric G-proteins in the Nod factor signalling 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 the nodulation gene expression studies Mas7 activation of calcium spiking does not require $NFP$, $DMI1$ or
DMI2 that 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 *M. truncatula* root hair cells.

The requirement for DMI3/CCaMK in Mastoparan and Mas7 (Mastoparan synthetic analog) 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 *Medicago truncatula*. It has previously been shown that the concentration of Mas7 (0.5 μM) required for induction of *ENOD11-GUS* (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 ratiometric measurements of calcium changes within individual cells (Miyawaki et al., 1997; Miyawaki et al., 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 1). The inactive analog Mas17 that contains a single amino acid change in the peptide showed no calcium oscillations (Table 1). Treatment with 0.2 μM Mas7 activated calcium oscillations, but in only 13.5% of root hair cells analysed, while treatment with 2 μM Mas7 was predominantly lethal. The calcium oscillations induced by Mas7 appear broadly similar to Nod factor induced calcium spiking, but the period between oscillations is longer (Fig. 1; Fig. 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, 10-20 minutes, than has ever been observed in Nod factor responses. These cells showing very long periods between oscillations still maintain a 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.

The structure of the 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 and this is consistent with pharmacological studies (Engstrom et al., 2002). A 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. In order 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 (Fig. S2).

**The spatial resolution of Mas7 induced calcium oscillations**

Nod factor induced calcium spiking is mostly restricted to the cytosol associated with the nucleus (Fig. 4b), with minor changes occurring at the root hair tip (Shaw and Long, 2003). Because of the interference from the florescence of neighbouring cells, we can only assess the hair region of root hair cells in the cameleon transformed plants. Therefore, in order 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 MW) and the unresponsive dye Texas red dextran (10,000 MW). Injection of the two dyes allows pseudo-ratio imaging of calcium changes (Shaw and Long, 2003), with Texas red providing a control for cytoplasmic content within the zone being analysed. We found that in contrast to the nuclear restricted nature of Nod factor induced calcium spiking (Fig. 4d-f), Mas7 induced calcium oscillations occur throughout the cell (Fig. 4a-c). The 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 analysed the ability of Mas7 to induce calcium responses in plants mutated in components of the Nod factor signalling pathway. Nod factor signalling 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 calcium/calmodulin dependent protein kinase 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 the cameleon 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 2). The mutant alleles chosen are presumed to be null alleles since 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), that inhibits type IIA calcium ATPases, and n-butanol, that 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. W. and A. D. unpublished data). In order 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 (Fig. S3)

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 signalling 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. S. and G. O. unpublished data), but we have been unsuccessful in activating 40 calcium spikes, that 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: a slower initial release of calcium in the Mas7 response; greater variability in the lag and period in Mas7 induced calcium oscillations and an 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* is dependent on *DMI3* (Charron et al., 2004), whose mutant phenotypes suggest a specific role in symbiosis signalling (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 signalling pathway.

The Mas7 induction of calcium oscillations and ENOD11 expression (Charron et al., 2004) do not require NFP, DMI1 or DMI2, M. truncatula Nod factor signalling 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 Nod factor signalling 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 channel(s) that are 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 indicates that Mas7 and Nod factor act on different sets of calcium channel(s) or differentially activate equivalent calcium channel(s).

Mastoparan/Mas7 has been shown to activate G-proteins by catalysing 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 that modulates calcium release from the ryanodine receptor (Hirata et al., 2000; Hirata et al., 2003). As such Mastoparan can activate calcium release independent of G protein activation. This is consistent with observations in G protein
mutants of *Arabidopsis* 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 phospholipase C and D 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 that 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 directly or indirectly to generate a secondary signal that activates the calcium channel, or whether Mastoparan modulates other components of the calcium signalling machinery that require the activity of phospholipases. Defining the mode of action of Mastoparan could provide insights into mechanisms of the Nod factor signalling pathway and the induction of calcium spiking.

**Materials and Methods**

*Plant material and growth conditions*

*M. truncatula* seeds were scarified for 5 minutes in concentrated sulfuric acid, washed twice in sterile water, surface sterilized for 3 minutes in undiluted Clorox bleach, then washed 5 times in sterile water. Seeds were imbibed for 1-3 hours, 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 (BNM) (Ehrhardt et al., 1992) agar at pH 6.5 containing the ethylene inhibitor 0.1μM L-α-(2-aminoethoxyvinyl)-glycine and grown overnight at 25°C, with the roots shaded by wrapping the plates in aluminium foil. The root tips of the seedlings were removed to avoid disturbance during imaging and the seedlings were then transferred to a liquid BNM bath contained on a large coverslip. 0.5 μM Mas7 (Sigma), 0.5 μM Mas17 (Sigma) or 1nM *Sinorhizobium meliloti* Nod factor were added to the bath during imaging. 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 the 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 Inc) and Texas red dextran (10,000 MW, Molecular Probes Inc). 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 monochrometer (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). The data was 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 CFP or YFP emissions prior to exposure on the ccd chip. The ratio of CFP:YFP was assayed using Metaflor (Universal Imaging).

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Figure legends

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

Figure 2. Quantification of the lag and period of Mas7 induced calcium oscillations. 0.5 μM Mas7 treated cells 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). A 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. 13 cells were analysed for the Mas7 treatment and 15 cells were analysed for the Nod factor treatment.

Figure 3. The structure of Mas7 induced calcium oscillations are slightly different to Nod factor induced calcium spiking. (a), Nod factor (NF) induced calcium spiking and Mas7 induced calcium oscillations, note that Mas7 induced oscillations show a 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 a Mas7 induced calcium oscillation is equivalent to a Nod factor induced calcium spiking, but the first phase of the spike is longer and the second phase shorter in Mas7 induced calcium spiking. 26 cells were analysed for Mas7 (0.5 μM) treatments and 30 cells were analysed in the Nod factor
(1 nM) treatment. The black bar indicates the first phase of the spike, the upward slope, while the white bar indicates the second phase of the spike, the downward slope. The error bars represent the standard deviation. The y axis in (a) is the ratio of YFP:CFP in arbitrary units.

**Figure 4.** Mas7 induced calcium changes occur throughout the cell. (a-c), a cell microinjected with the fluorescent dyes Oregon Green/Texas red and treated with 0.5 μM Mas7, (a), the cell prior to a calcium spike, (b), the same cell at the peak of a spike 15 seconds 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-f), a cell microinjected with the fluorescent dyes treated with 1 nM Nod factor, (d) the cell prior to a calcium spike, (e), the same cell at the peak of a spike 15 seconds later. (f). The 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/Texas red in arbitrary units. The domains and traces in (a), (c), (d) and (f) are colour 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 μM) induced responses in the Nod factor signalling mutants. Cells on wild type and mutant lines were microinjected with the calcium responsive dye Oregon green and the non-responsive dye Texas red. Calcium changes were measured as the ratio of Oregon green/Texas red allowing pseudoratiometric imaging of calcium changes in Mas7 treated cells. The y axis represents the ratio of Oregon green/Texas red in arbitrary units. WT: wild type

**Figure 6.** Mas7 induced calcium oscillations are inhibited by CPA and n-butanol, assayed using cameleon transformed cells. (a). A cell treated with 1 nM Nod factor and secondarily treated with 10 μM CPA shows rapid inhibition of calcium spiking following CPA addition. (b). 0.5 μM Mas7 induced calcium oscillations are rapidly inhibited
following treatment with 0.5% (68 mM) n-butanol. (c). 10 μM CPA treatment inhibits 0.5 μM 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:CFP in arbitrary units.

Table 1: Calcium spiking, assayed in wild type plants transformed with cameleon and treated with 0.5 μM Mas7 or 0.5 μM Mas17.

| Number of 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                     |

Table 2: 0.5 μM Mas7-induced calcium spiking assayed in wild type and mutant plants microinjected with the fluorescent dyes Oregon green/Texas red

| Number of plants | Cells spiking/total cells |
|------------------|---------------------------|
| WT               | 5                         | 2/6                      |
| nfp-1            | 4                         | 3/10                     |
| dmi1-1           | 3                         | 2/7                      |
| dmi2-1           | 4                         | 2/8                      |
| dmi3-1           | 7                         | 2/15                     |
