RESEARCH ARTICLE

# *Rhizobium* Nod Factor Signaling: Evidence for a G Protein–Mediated Transduction Mechanism

Jean-Luc Pingret, Etienne-Pascal Journet, and David G. Barker[1]

Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, INRA-CNRS UMR215, BP 27, 31326 Castanet-Tolosan Cedex, France

*Rhizobium* nodulation (Nod) factors are lipochitooligosaccharide signals that elicit key symbiotic developmental responses in the host legume root. In this study, we have investigated Nod factor signal transduction in the *Medicago* root epidermis by using a pharmacological approach in conjunction with transgenic plants expressing the Nod factor–responsive reporter construct *pMtENOD12–GUS*. Evidence for the participation of heterotrimeric G proteins in Nod factor signaling has come from three complementary observations: (1) the amphiphilic peptides mastoparan and Mas7, known G protein agonists, are able to mimic Nod factor–induced epidermal *MtENOD12* expression; (2) growth of plants in nodulation-inhibiting conditions (10 mM $NH_4NO_3$) leads to a dramatic reduction in both Nod factor– and mastoparan-elicited gene expression; and (3) bacterial pertussis toxin, a well-characterized G protein antagonist, blocks the activities of both the Nod factor and mastoparan. In addition, we have found that antagonists that interfere with phospholipase C activity (neomycin and U73122) and $Ca^{2+}$ influx/release (EGTA, $La^{3+}$, and ruthenium red) block Nod factor/mastoparan activity. Taken together, these results are consistent with a Nod factor signal transduction mechanism involving G protein mediation coupled to the activation of both phosphoinositide and $Ca^{2+}$ second messenger pathways.

## INTRODUCTION

Nitrogen fixation in legumes takes place in highly specialized root organs (nodules) that result from the association between the host plant and endosymbiotic soil bacteria known as rhizobia. During early stages of this association, molecular signals are synthesized by rhizobia that are essential for initiating both morphogenetic and organogenetic responses in the appropriate host plant. These so-called nodulation (Nod) factors are decorated lipochitooligosaccharides whose structures are species dependent and contribute to host specificity (reviewed in Dénarié et al., 1996; Long, 1996). Nodulation of *Medicago* species, such as alfalfa or *M. truncatula*, is mediated by *Rhizobium meliloti* Nod factors (NodRm) that possess a sulfate moiety attached to the reducing end of the chitin tetrasaccharide backbone, in addition to an acetyl group and an unsaturated lipid chain (predominantly C16:2) attached to the nonreducing sugar (Lerouge et al., 1990). In alfalfa, hair deformation responses can be elicited at subnanomolar Nod factor concentrations (Roche et al., 1991), whereas significantly higher levels ($10^{-7}$ to $10^{-9}$ M) are required to evoke nodule organogenesis in inner cortical tissues (Truchet et al., 1991).

The exquisite sensitivity of the plant root to exogenous Nod factors suggests that these molecules are perceived by receptor-mediated mechanisms. Biochemical studies have characterized Nod factor binding activities in *Medicago* cell extracts (Bono et al., 1995; Niebel et al., 1997), although it remains to be shown that these activities correspond to functional receptors. Because of its physical accessibility, the root epidermis has been the focus of various studies designed to reveal early steps in Nod factor signal transduction. Nod factors elicit both a transient membrane depolarization (Ehrhardt et al., 1992; Felle et al., 1995; Kurkdjian, 1995) and a rapid intracellular alkalinization (Felle et al., 1996) in alfalfa root hairs. Ehrhardt et al. (1996) demonstrated that Nod factors can trigger intracellular cytosolic calcium oscillations in alfalfa root hairs, and Gehring et al. (1997) observed plateau-like increases in intracellular calcium in cowpea root hairs in response to Nod factors. Although all of these responses are dependent on the structural characteristics of the Nod factor, it is not yet established whether they are part of a signal transduction pathway leading to symbiotic downstream events, such as root hair deformation or specific gene expression.

A number of plant genes have been identified whose transcription can be specifically elicited in root tissues in response to applications of Nod factors (reviewed in Heidstra

and Bisseling, 1996). One of the best studied of these genes, *ENOD12*, encoding a repetitive proline-rich protein, has been characterized in pea (Scheres et al., 1990), vetch (Vijn et al., 1995b), alfalfa (Allison et al., 1993), and the model legume *M. truncatula* (Pichon et al., 1992). Although no function has yet been assigned to the *ENOD12* gene product, it has been proposed that this protein is a cell wall component modifying the infectibility of the root hair with respect to *Rhizobium* (Pichon et al., 1992). Reverse transcriptase–polymerase chain reaction (RT-PCR) experiments have detected rapid Nod factor–dependent *ENOD12* expression in roots of pea (Horvath et al., 1993), vetch (Vijn et al., 1995a; Heidstra et al., 1997), and alfalfa (Bauer et al., 1994). Complementary spatiotemporal studies using transgenic alfalfa plants expressing a fusion between the *MtENOD12* promoter and the reporter gene encoding β-glucuronidase (*GUS*) have shown that gene transcription can be triggered in epidermal cells within hours of *Rhizobium* inoculation or Nod factor addition (Pichon et al., 1992; Journet et al., 1994). This response can be elicited by NodRm concentrations down to $10^{-12}$ to $10^{-13}$ M, and GUS activity is maximal in the differentiating region of the root between the growing root tip and the zone of root hair emergence, where the root nodulation process is initiated. *R. meliloti* Nod factors lacking the sulfate moiety are $10^3$-fold less active in eliciting *pMtENOD12–GUS* transcription in the epidermis, showing that this rapid molecular response is sensitive to host specificity determinants (Journet et al., 1994).

In this study, we have made use of transgenic plants expressing the *pMtENOD12–GUS* gene fusion to investigate the transduction of the Nod factor signal leading to specific gene expression in the root epidermis by using a pharmacological approach. We found that the amphiphilic peptide mastoparan is capable of triggering *MtENOD12* expression in the absence of Nod factors (agonist activity), whereas pertussis toxin acts to block gene expression (antagonist activity), thus providing evidence that Nod factor signal transduction in legumes may be mediated by heterotrimeric G proteins. Results with pharmacological antagonists that inhibit phospholipase C activity further suggest that G protein–dependent activation of the phosphoinositide second messenger pathway might be part of the signal transduction cascade. Finally, the use of a variety of antagonists that interfere with increases in cytosolic $Ca^{2+}$ concentration leads us to propose roles for both intracellular $Ca^{2+}$ release and extracellular $Ca^{2+}$ influx in transducing the Nod factor signal.

## RESULTS

One of the most powerful approaches currently available for studying intracellular signal transduction in higher organisms involves the use of a pharmacological arsenal designed to perturb different components of the transduction pathway. We chose to use transgenic *Medicago* plants express-

ing *pMtENOD12–GUS* for a pharmacological analysis of *Rhizobium* Nod factor signal transduction at the root epidermis. This bioassay is extremely sensitive, offers spatiotemporal resolution at the cellular level, and is particularly convenient for testing an extensive array of potential effectors at a wide range of concentrations.

Previous studies had shown that reporter gene activity could first be detected in epidermal cells of transgenic alfalfa 2 to 3 hr after the addition of NodRm (Journet et al., 1994), and similar results were subsequently obtained with *M. truncatula* expressing the same transgene (Chabaud et al., 1996). Before using this material for signal transduction studies, we wished to confirm, using RT-PCR, that these findings were also valid for the endogenous *MtENOD12* gene. Figure 1 shows that in the responsive zone of *M. truncatula* roots, *MtENOD12* transcripts were first detected 1 hr after treatment with $10^{-8}$ M NodRm, with steady state levels reaching a maximum value after 2 to 3 hr. This correlates well with promoter activation as deduced from the transgenic plant studies. Use of RT-PCR also allowed us to determine whether de novo protein synthesis is required for Nod factor–elicited expression of *MtENOD12*, as has recently been shown for the vetch *ENOD12* gene (Vijn et al., 1995a; Heidstra et al., 1997). When NodRm was added to *M. truncatula* roots in the presence of 5 μM cycloheximide, transcriptional activation of *MtENOD12* was totally inhibited (Figure 1), thus demonstrating that de novo protein synthesis is also a prerequisite for the symbiotic induction of the *M. truncatula ENOD12* gene in epidermal cells.

## Heterotrimeric G Protein Peptide Agonists Elicit *pMtENOD12–GUS* Transcription in the Differentiating Root Epidermis

Mastoparan is a cationic amphipathic tetradecapeptide isolated from wasp venom (Table 1) and acts as a generic acti-
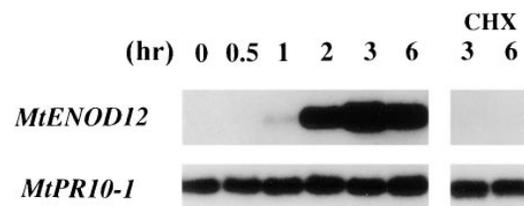


**Figure 1.** Transcriptional Activation of *MtENOD12* in *M. truncatula* Roots Treated with the Nod Factor.

*M. truncatula* roots were treated with $10^{-8}$ M NodRm, and responsive zones were harvested at the indicated times. Total RNA was extracted, and *MtENOD12* transcript levels were evaluated by RT-PCR analysis (see Methods). Similar experiments (3 and 6 hr) were performed in the presence of the protein synthesis inhibitor cycloheximide (CHX). *MtPR10-1* RNA was amplified as a control for the quality and quantity of the RNA samples.

**Table 1.** Sequences of G Protein Peptide Effectors[a]

| Peptide | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mastoparan | Ile | Asn | Leu | Lys | Ala | Leu | Ala | Ala | Leu | Ala | Lys | Lys | Ile | Leu-NH$_2$[b] |
| Mas7 | Ile | Asn | Leu | Lys | Ala | Leu | Ala | Ala | Leu | Ala | Lys | Ala[c] | Leu[c] | Leu-NH$_2$[b] |
| Mas17 | Ile | Asn | Leu | Lys | Ala | Lys[c] | Ala | Ala | Leu | Ala | Lys | Lys | Leu[c] | Leu-NH$_2$[b] |

[a] Numbers above the columns indicate the amino acid position relative to the peptide N terminus.
[b] The C-terminal amino acid is an amide.
[c] Residues with amino acid substitutions in the two mastoparan analogs.

vator of animal heterotrimeric GTP binding regulatory proteins (G proteins) (Ross and Higashijima, 1994). Because G proteins are also believed to be key components of signal transduction pathways in plants, we asked whether mastoparan was capable of acting as an agonist by eliciting transcription of *MtENOD12* in the absence of the Nod factor signal. Figure 2A shows the typical pattern of GUS staining observed when roots of transgenic alfalfa plants are treated for 6 hr with 10$^{-9}$ M NodRm. Under these conditions, gene expression is centered around a region in which root hairs are emerging and is first detectable in a zone preceding hair emergence. Brief (2 to 3 hr) treatments with NodRm had previously shown that it is precisely this region of the root that is most sensitive to Nod factors (Journet et al., 1994). When 1 µM mastoparan was substituted for the Nod factor, GUS activity was also induced in epidermal cells and localized
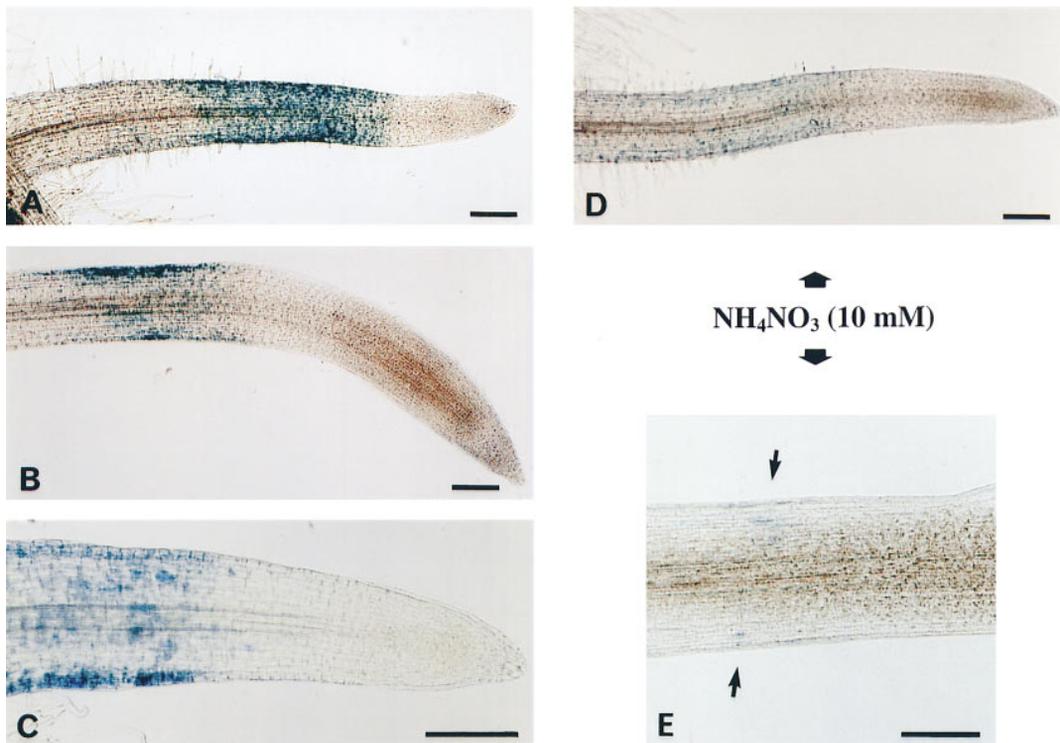


**Figure 2.** Histochemical Localization of GUS Activity in Roots of Transgenic Alfalfa after Treatment with Either the Nod Factor or Mastoparan.

**(A)** Secondary root treated for 6 hr with 10$^{-9}$ M NodRm.
**(B)** Primary root treated with 1 µM mastoparan for 6 hr.
**(C)** Secondary root treated with 0.2 µM Mas7 for 24 hr.
**(D)** and **(E)** Roots of transgenic plants grown for 72 hr in the presence of 10 mM NH$_4$NO$_3$ and then treated for 6 hr with either 10$^{-9}$ M NodRm **(D)** or 1 µM mastoparan **(E)**. Arrows in **(E)** indicate the few GUS-staining cells in the magnified root segment located just behind the root tip.
Bars in **(A)** to **(E)** = 350 µm.

to a region of the root behind the tip and preceding root hair emergence (Figure 2B). The results presented in Table 2 show that mastoparan elicits epidermal *MtENOD12* transcription in the range 0.2 to 2.0 $\mu$M. However, when the mastoparan concentration was increased to 10 $\mu$M, agonist activity could no longer be observed because the plant response fell to background levels. Note that in the absence of elicitor, a small percentage ($<$10%) of transgenic plants showed detectable GUS staining in a few cells in the responsive root zone (classified as a very weak response; Table 2 and see Methods).

The maximal plant response to mastoparan was observed at a concentration of 1 $\mu$M (visual evaluation) and was comparable in intensity to treatments with relatively low concentrations ($10^{-11}$ to $10^{-13}$ M) of NodRm. Unfortunately, the levels of endogenous *MtENOD12* transcripts elicited by mastoparan (or by $10^{-11}$ M NodRm) in *M. truncatula* plants were not sufficient to give clear signals in RT-PCR experiments using total RNA from root segments, as described above (data not shown). Finally, it should be noted that the omission of $Ca^{2+}$ ions from the incubation medium resulted in a dramatic reduction in mastoparan-elicited gene activation (Table 2).

To provide further evidence that mastoparan is acting as a G protein agonist, we tested a number of synthetic mastoparan analogs with modified agonist activities. The peptide Mas7, with enhanced hydrophobicity at the C-terminal end (replacement of Lys-12 by Ala; Table 1), is generally considered to be more active as a G protein agonist than is the native mastoparan (Higashijima et al., 1990). Mas7 also elicited *MtENOD12* transcription in the submicromolar range (Table 2 and Figure 2C), and visual comparison of stained roots indeed suggested an optimal response to Mas7 at lower concentrations (0.2 $\mu$M) than for mastoparan. At 2 $\mu$M (fivefold lower than for mastoparan), Mas7 ceases to be an

agonist for early nodulin gene induction. Again, the presence of 1 mM $Ca^{2+}$ appears essential for optimal Mas7 activity (Table 2). We also tested an analog of mastoparan, known as Mas17, which is unable to activate G proteins (Higashijima et al., 1990). This peptide contains a charged residue (replacement of Leu-6 by Lys; Table 1) located in the middle of the hydrophobic stretch required for the $\alpha$-helical membrane conformation apparently essential for G protein agonist activity (see Discussion). Significantly, Mas17 (0.2 to 5 $\mu$M) totally lacked the capacity to elicit *pMtENOD12–GUS* expression above background levels (Table 2).

## Pharmacological Activities of Cholera and Pertussis Toxins

The bacterial cholera and pertussis toxins are well-characterized specific modulators of animal heterotrimeric G proteins in vitro and in vivo (reviewed in Simon et al., 1991; Conklin and Bourne, 1993). Cholera toxin is known to activate certain classes of G proteins by ADP ribosylation of an arginine residue belonging to the $G_{\alpha}$ subunit, thus preventing GTP hydrolysis and maintaining the G protein in its active GTP-bound form. On the other hand, pertussis toxin blocks the activation of a different subset of G proteins by catalyzing the ADP ribosylation of a $G_{\alpha}$ cysteine residue, thus interfering with the interaction with the receptor. The results presented in Table 3 show that cholera toxin (up to 100 $\mu$g/mL) is unable to act as agonist and trigger *pMtENOD12–GUS* expression in the *Medicago* root in the absence of the Nod factor. Pertussis toxin, on the other hand, appears to be an efficient antagonist with respect to Nod factor–elicited gene expression. Partial inhibition can already be observed at 2 $\mu$g/mL, as shown by a reduction in both the percentage of positively staining plants and the intensity of the response (Table 3). When the toxin concentration is raised to 8 $\mu$g/mL, the Nod factor response falls to near background levels. These results show that Nod factor signal transduction at the epidermis is pertussis toxin sensitive, thus providing additional evidence for the participation of heterotrimeric G protein(s) in this signaling pathway. Finally, it is significant that pertussis toxin (2.5 $\mu$g/mL) is also able to block gene expression elicited by mastoparan. The lower concentration of toxin required for efficient inhibition presumably reflects the relatively low level of expression induced by the peptide agonist.

## Inhibitory Effect of $NH_4NO_3$ on Both Nod Factor– and Mastoparan-Elicited Epidermal Gene Expression

The inhibitory effect of combined nitrogen is one of the most typical characteristics of the symbiotic legume–*Rhizobium* association, although the mechanism by which this negative control operates is currently not known. We wished to determine whether the triggering of *MtENOD12* gene expression

**Table 2.** G Protein Peptide Effector Activities

| Peptide | Concentration ($\mu$M) | % GUS-Staining Plants |
|---|---|---|
| —[a] | — | 7[b] |
| Mastoparan | 0.2 | 81 |
| | 1 | 93 |
| | 2 | 78 |
| | 10 | 11[b] |
| Minus $Ca^{2+}$ [c] | 1 | 29[b] |
| Mas7 | 0.2 | 93 |
| | 1 | 40 |
| | 2 | 6[b] |
| Minus $Ca^{2+}$ [c] | 0.2 | 38[b] |
| Mas17 | 0.2 | 0 |
| | 1 | 5[b] |
| | 5 | 6[b] |

[a] Control in the absence of peptide.
[b] Very weak response.
[c] Incubation medium lacking calcium.

in the epidermis by Nod factors is dependent on nitrogen status, and if so, whether the mastoparan-elicited response is also subject to similar regulation. For this purpose, 10 mM $NH_4NO_3$ was added to the growth medium of transgenic alfalfa plants 72 hr before treatment with either of the elicitors. A comparison of Figures 2A and 2D illustrates the striking reduction in $10^{-9}$ M NodRm-elicited *GUS* expression observed after growth of transgenic alfalfa roots in the presence of combined nitrogen. Semiquantitative data derived from such experiments (using both $10^{-9}$ and $10^{-11}$ M NodRm) are presented in Table 4. The very weak responses observed for combined nitrogen–grown plants treated with $10^{-11}$ M NodRm reflect the lower level of *GUS* expression elicited at this concentration under normal growth conditions (Journet et al., 1994). Significantly, growth in 10 mM $NH_4NO_3$ also resulted in a very pronounced attenuation in the level of gene transcription in response to treatment with 1 μM mastoparan (cf. Figures 2B and 2E). The residual level of *GUS* expression obtained with the peptide agonist is comparable to that observed for $10^{-11}$ M NodRm (Table 4), in line with the levels of mastoparan-elicited expression, as discussed earlier. Thus, both Nod factor– and mastoparan-activated expression of *MtENOD12* in the root epidermis are downregulated after growth in combined nitrogen.

## Phospholipase C Antagonists Inhibit Nod Factor–Elicited *MtENOD12* Expression

The cleavage of phosphatidylinositol (4,5)-bisphosphate ($PIP_2$) to yield inositol (1,4,5)-trisphosphate ($IP_3$) and diacylglycerol (DAG) is a common feature of signal transduction pathways in animal cells and most probably also in plants. Because this reaction is frequently catalyzed by G protein–mediated activation of specific phospholipase C (PLC)

**Table 4.** Inhibitory Effect of Combined Nitrogen Growth on *MtENOD12* Induction

| Growth in Presence of 10 mM $NH_4NO_3$ | Elicitor | Concentration (M) | % GUS-Staining Plants |
|---|---|---|---|
| No | NodRm | $10^{-9}$ | 100 |
| Yes | | $10^{-9}$ | 76[a] |
| No | | $10^{-11}$ | 100 |
| Yes | | $10^{-11}$ | 61[b] |
| No | Mastoparan | $10^{-6}$ | 93 |
| Yes | | $10^{-6}$ | 33[b] |

[a] Clearly reduced response.
[b] Very weak response.

isoenzymes, we attempted to identify PLC antagonists that are capable of blocking the Nod factor–elicited response in the root epidermis. Neomycin, a positively charged aminoglycoside, is a widely used eukaryotic PLC antagonist (reviewed in McDonald and Mamrack, 1995). We found that 50 μM neomycin sulfate partially blocked epidermal expression of the *pMtENOD12–GUS* fusion, with total inhibition at 100 to 200 μM (Table 5). Inhibition was observed whether neomycin was added 1 hr before Nod factor (our standard conditions for antagonist treatment; see Methods) or concomitantly, implying that neomycin is a rapidly acting antagonist. On the other hand, when neomycin was added either 15 or 60 min after the Nod factor, reporter gene expression was clearly observed at the end of the 6-hr treatment (Table 5). Bearing in mind that in the absence of antagonist, GUS activity can first be detected no earlier than 2 to 3 hr after addition of the Nod factor (Journet et al., 1994), this result implies that neomycin is unable to block cellular processes necessary for completion of the Nod factor–elicited response. Therefore, we can conclude that the inhibitory action of neomycin is unlikely to be a consequence of nonspecific cellular toxicity. This result also implies that short (15-min) exposures to Nod factor are sufficient to elicit downstream gene expression, in line with previous results for root hair deformation (Heidstra et al., 1994).

To obtain further evidence for the role of PLC-dependent polyphosphoinositide hydrolysis during Nod factor signal transduction, we made use of an aminosteroid PLC inhibitor known as U73122 that is well characterized in animal studies (Smith et al., 1990). Partial inhibition of the epidermal Nod factor response by U73122 could be observed at a concentration of 1 μM, with total inhibition at 5 to 10 μM (Table 5). A structurally related aminosteroid, U73343, which does not suppress PLC activity, is routinely used as a control for the specificity of U73122 action (Smith et al., 1990). Significantly, we found that U73343 had no inhibitory activity with respect to Nod factor signal transduction (Table 5). Finally, if G proteins are indeed involved in PLC activation,

**Table 3.** Effect of Cholera (CTX) and Pertussis (PTX) Toxins on *MtENOD12* Induction

| Elicitor | Effector | Concentration (μg/mL) | % GUS-Staining Plants |
|---|---|---|---|
| —[a] | —[a] | — | 7[b] |
| | CTX | 0.05 | 0 |
| | | 2.0 | 8[b] |
| | | 100 | 7[b] |
| NodRm ($10^{-9}$ M) | —[a] | — | 100 |
| | PTX | 0.4 | 100 |
| | | 2.0 | 73[c] |
| | | 8.0 | 21[b] |
| Mastoparan ($10^{-6}$ M) | —[a] | — | 93 |
| | PTX | 2.5 | 8[b] |

[a] Control in the absence of elicitor and/or effector.
[b] Very weak response.
[c] Clearly reduced response.

**Table 5.** Effect of the PLC Antagonists Neomycin and U73122 on *MtENOD12* Induction

| Elicitor | Effector | Concentration (μM) | % GUS-Staining Plants |
|---|---|---|---|
| NodRm | —[a] | — | 100 |
| ($10^{-9}$ M) | Neomycin | 10 | 100 |
| | | 50 | 67[b] |
| | | 100 | 10[c] |
| | | 200 | 0 |
| | CA[d] | 100 | 17[c] |
| | +15 min[e] | 100 | 61[b] |
| | +60 min[e] | 100 | 90 |
| | U73122 | 1 | 79[b] |
| | | 5 | 0 |
| | | 10 | 0 |
| | U73343 | 1 | 100 |
| | | 5 | 100 |
| | | 10 | 100 |
| Mastoparan | —[a] | — | 93 |
| ($10^{-6}$ M) | Neomycin | 100 | 6[c] |
| | U73122 | 5 | 0 |
| | U73343 | 5 | 93 |

[a] Control in the absence of effector.
[b] Clearly reduced response.
[c] Very weak response.
[d] CA, concomitant addition of elicitor and effector.
[e] Timing of effector addition with respect to elicitor.

then phosphoinositide pathway antagonists should also block *pMtENOD12–GUS* expression elicited by the G protein agonist mastoparan. Our results clearly show that neomycin and U73122 are efficient inhibitors of mastoparan activity, whereas the negative control analog U73343 is without antagonist activity (Table 5).

## Ca²⁺ Release/Influx Antagonists Inhibit Nod Factor–Elicited *MtENOD12* Expression

Transient rises in cytoplasmic Ca²⁺ concentration have been implicated in the responses of higher plants to a wide variety of external signals. We have examined whether the Nod factor signaling pathway leading to *MtENOD12* epidermal gene expression requires Ca²⁺ release from intracellular stores and/or inward flux across the plasma membrane. Ruthenium red is considered a diagnostic antagonist of ryanodine receptors (RYR) in animal cells and has also been used to block calcium release from internal stores in plant cells (e.g., Knight et al., 1992; Subbaiah et al., 1994). We found that ruthenium red is an efficient antagonist with respect to the epidermal Nod factor response at concentrations as low as 10 μM (Table 6), suggesting that transduction of the Nod factor signal requires the mobilization of intracellular Ca²⁺ stores (see Discussion). By varying the timing of addition of ruthenium red with respect to Nod factor, we were able to show

(as for neomycin) that this particular antagonist acts very rapidly and that inhibition of epidermal gene expression is unlikely to result from nonspecific cellular toxicity (Table 6). Finally, we tested whether ruthenium red is capable of blocking peptide agonist activity. Our results clearly show that 10 μM ruthenium red is an efficient antagonist with respect to mastoparan-elicited gene expression (Table 6).

Two complementary approaches were used to study the potential role of extracellular Ca²⁺ influxes. First, we used the calcium chelator EGTA to remove free Ca²⁺ ions from the external medium and the apoplast. The results presented in Table 7 show that the Nod factor response was totally blocked in the presence of 2 mM EGTA. The concomitant addition of 5 mM Ca²⁺ (but not Mg²⁺) restored gene expression, showing that this inhibition is specifically due to Ca²⁺ chelation. A normal epidermal response could also be recovered when 5 mM Ca²⁺ was added to the medium after a 3-hr incubation with Nod factors in the presence of 2 mM EGTA (Table 7), indicating that the EGTA-induced inhibition was reversible. In contrast to the G protein peptide agonists, *pMtENOD12–GUS* gene expression can be efficiently triggered by Nod factors even in the absence of added Ca²⁺ in the incubation medium. The reason for this difference is currently unclear. The second approach involved the use of the trivalent lanthanum ion La³⁺, an efficient competitor for Ca²⁺ influx via plasma membrane channels in plant cells (e.g., Huang et al., 1994). In the absence of added Ca²⁺, La³⁺ partially inhibited Nod factor–elicited gene expression at 5 μM and totally blocked the response at 50 to 100 μM (Table 7). Significantly, the concomitant addition of increasing concentrations of Ca²⁺ progressively relieved inhibition of the Nod factor response (Table 7).

The importance of extracellular sources of Ca²⁺ for mastoparan activity is illustrated by the fact that *MtENOD12* ac-

**Table 6.** Effect of the RYR Antagonist Ruthenium Red on *MtENOD12* Induction

| Elicitor | Effector | Concentration (μM) | % GUS-Staining Plants |
|---|---|---|---|
| NodRm | —[a] | — | 100 |
| ($10^{-9}$ M) | Ruthenium red | 2 | 93[b] |
| | | 10 | 12[c] |
| | | 50 | 0 |
| | CA[d] | 10 | 10[c] |
| | +15 min[e] | 10 | 50[b] |
| | +60 min[e] | 10 | 100 |
| Mastoparan | —[a] | — | 93 |
| ($10^{-6}$ M) | Ruthenium red | 10 | 5[c] |

[a] Control experiment in the absence of effector.
[b] Clearly reduced response.
[c] Very weak response.
[d] CA, concomitant addition of elicitor and effector.
[e] Timing of effector addition with respect to elicitor.

**Table 7.** Effect of $Ca^{2+}$ Influx Antagonists EGTA and $La^{3+}$ on *MtENOD12* Induction

| Elicitor | Effector | Concentration | [$Ca^{2+}$] (mM) / | Incubation Time with Elicitor (hr) | % GUS-Staining Plants |
|---|---|---|---|---|---|
| NodRm | —[a] | — | 0 or 5 / | 3 | 95 |
| ($10^{-9}$ M) | —[a] | — | 0 or 5 / | 6 | 100 |
| | EGTA | 2 mM | 0 / | 3 or 6 | 6[b] |
| | | 2 mM | 5 / | 6 | 100 |
| | | 2 mM | 5 ($Mg^{2+}$)[c] / | 6 | 7[b] |
| | | 2 mM | 0/3, followed by 5/3 | | 94 |
| | $La^{3+}$ | 5 μM | 0 / | 6 | 77[d] |
| | | 50 μM | 0 / | 6 | 11[b] |
| | | 100 μM | 0 / | 6 | 6[b] |
| | | 50 μM | 1 / | 6 | 91[d] |
| | | 50 μM | 10 / | 6 | 100 |
| | | 50 μM | 10 ($Mg^{2+}$)[c] / | 6 | 8[b] |
| Mastoparan | —[a] | — | 0 / | 6 | 29[b] |
| ($10^{-6}$ M) | EGTA | 2 mM | 0 / | 6 | 0 |
| | $La^{3+}$ | 50 μM | 0 / | 6 | 0 |

[a] Control in the absence of effector.
[b] Very weak response.
[c] $Ca^{2+}$ replaced by $Mg^{2+}$.
[d] Clearly reduced response.

tivation falls to a very low level when $Ca^{2+}$ ions are omitted from the reaction medium (Tables 2 and 7). The addition of the $Ca^{2+}$-influx antagonists EGTA and $La^{3+}$ further inhibits mastoparan activity (Table 7). Taken together, these findings are consistent with a role for calcium ion influx/release during Nod factor signal transduction at the root epidermis and argue that changes in cytosolic $Ca^{2+}$ concentration act downstream of the mastoparan-sensitive step.

## DISCUSSION

In this study, we report the use of transgenic alfalfa plants carrying the *MtENOD12* early nodulin promoter fused to the *GUS* reporter gene for studying the *Rhizobium* Nod factor transduction pathway. Root epidermal *MtENOD12* expression is a rapid cell-specific response to lipochitooligosaccharide Nod factors, and we have shown by using RT-PCR that endogenous gene transcription can first be detected within 1 hr of exposure to the symbiotic signal, reaching maximal levels after 2 to 3 hr. This correlates remarkably well with the timing of *GUS* expression in transgenic alfalfa in response to Nod factors (Journet et al., 1994), although we cannot rule out the possibility that there exist subtle differences in expression between the promoter fusion and the intact native gene. We have demonstrated that transcriptional activation of *MtENOD12* requires de novo protein synthesis and that gene expression is strongly inhibited when plants are grown in the presence of 10 mM $NH_4NO_3$. These results are similar to those reported for Nod factor–induced

expression of the vetch *ENOD5* and *ENOD12* genes (Vijn et al., 1995a; Heidstra et al., 1997).

### Is Nod Factor Signal Transduction at the Root Epidermis Mediated by G Proteins?

Mastoparan and its more potent synthetic analog Mas7 are commonly used as diagnostics for the participation of G proteins in vertebrate signal transduction pathways, and more recently they have also been used in signal transduction studies in both plant cell cultures (Legendre et al., 1993) and plant tissues (Armstrong and Blatt, 1995; Munnik et al., 1995; Kim et al., 1996). Mastoparan assumes a helical conformation upon transfer to a lipid environment (Wakamatsu et al., 1992), and it has been proposed that this amphipathic cationic α-helix mimics the action of the third intracellular loop of specific membrane receptors in activating associated G proteins (Ross and Higashijima, 1994). Our results are consistent with G protein–mediated Nod factor signal transduction because submicromolar concentrations of both mastoparan and Mas7 are capable of mimicking Nod factor activity by triggering *MtENOD12* transcription in the differentiating root epidermis. Furthermore, the synthetic peptide analog Mas17, predicted not to form an amphipathic helix at the lipid interface because of the replacement of Leu-6 by Lys, is totally devoid of agonist activity. Pharmacological agents that interfere with putative downstream components of the Nod factor transduction pathway, such as neomycin and U73122 (PLC antagonists) and ruthenium red, EGTA, and $La^{3+}$ (calcium flux antagonists), have similar

inhibitory activities on mastoparan-elicited gene expression. Finally, growth of plants in the presence of high concentrations of combined nitrogen, conditions known to inhibit nodulation, dramatically reduces gene expression in response to both Nod factors and mastoparan.

In animal cell studies, it has been reported that mastoparan concentrations ≥10 μM can modify the activity of C-type phospholipases (Wallace and Carter, 1989; Drøbak and Watkins, 1994) and can even disrupt cell membranes (Tanimura et al., 1991). Such alternative activities have not yet been evaluated in the context of plant cells. However, 10 μM mastoparan and 2 μM Mas7 are no longer able to elicit epidermal *MtENOD12* transcription, and preliminary experiments suggest that both of these peptides actually inhibit the Nod factor response at these elevated doses (data not shown). This could be due to secondary effects as outlined above and might explain why the maximum level of gene expression elicited by mastoparan/Mas7 is relatively low. In addition, it is probable that these peptide agonists are less efficient in initiating signal transduction as compared with normal ligand-activated receptor coupling.

We have also discovered that pertussis toxin, a well-characterized G protein antagonist, is able to block expression of Nod factor–elicited *pMtENOD12–GUS* expression. In addition, this bacterial exotoxin blocks gene expression induced by mastoparan, which is entirely consistent with analogous G protein agonist/antagonist experiments performed in animal cell studies (Higashijima et al., 1988). Pertussis toxin is known to ribosylate a C-terminal cysteine residue common to a subset of mammalian $G_\alpha$ subunits, thereby uncoupling the receptor from its G protein (reviewed in Conklin and Bourne, 1993). When pertussis toxin is applied externally to intact cells, the G protein–ribosylating component of pertussis toxin (A subunit protomer) is internalized after membrane binding of the B subunit oligomer. Recently, it has been shown that the B oligomer itself is also capable of eliciting direct cellular responses in animal cells, implying that caution needs to be exercised in interpreting antagonist activities under these conditions (reviewed in Wong and Rosoff, 1996). Nevertheless, the complementary agonist and antagonist activities of mastoparan/Mas7 and pertussis toxin, respectively, together provide strong evidence favoring a role for G protein mediation in Nod factor signal transduction.

Mammalian heterotrimeric G proteins have been divided into four major classes ($G_s$, $G_i$, $G_q$, and $G_{12}$) based on the structural and functional characteristics of the $G_\alpha$ subunits (reviewed in Simon et al., 1991). Mastoparan and Mas7 are believed to interact preferentially with members of the $G_i$ class (including $G_o$), with a lower sensitivity for $G_q$ (Ross and Higashijima, 1994). Pertussis toxin–sensitive $G_\alpha$ subunits usually belong to the $G_{\alpha i}$ class, and the corresponding G proteins are generally insensitive to cholera toxin. Finally, it is known that PLC activity can be regulated by G proteins that belong to either $G_i$ or $G_q$ classes (Simon et al., 1991). Although intact heterotrimeric G proteins have not yet been

isolated in plants, a small number of $G_\alpha$ and $G_\beta$ subunit genes have been cloned from a variety of plant species (reviewed in Millner and Causier, 1996), and the first putative $G_\alpha$ subunits have been biochemically characterized (e.g., Wise et al., 1997). Sequence analysis shows that these first $G_\alpha$ subunits do not possess the pertussis-sensitive C-terminal ribosylation site, suggesting that other families of heterotrimeric G proteins most probably exist in plants. Sequence comparisons also show that plant $G_\alpha$ subunits are evolutionarily distant from their animal counterparts and do not fit into the mammalian classification (Gotor et al., 1996). Thus, despite certain functional analogies to the $G_i$ class of mammalian heterotrimeric G proteins, it would be premature at this stage to predict the precise nature of the G protein that our results suggest to be involved in Nod factor signal transduction.

## Nod Factor Signaling and the Phosphoinositide Pathway

The aminoglycoside neomycin, believed to inhibit PLC action by binding $PIP_2$, has been widely used as an antagonist to investigate the role of phosphoinositide cycle metabolites in signaling cascades in higher eukaryotes (McDonald and Mamrack, 1995). We found that 100 to 200 μM neomycin efficiently blocked Nod factor–elicited *MtENOD12* transcription in the *Medicago* root, and our results further show both that neomycin is a rapidly acting antagonist and that its inhibitory action is not the consequence of nonspecific cellular toxicity. Studies with plants have shown that 100 to 300 μM neomycin inhibits PLC activity in plant plasma membranes and pollen microsomes (Chen and Boss, 1991; Franklin-Tong et al., 1996) and very rapidly inhibits $IP_3$ production in both Chlamydomonas and soybean cell suspensions (Quarmby et al., 1992; Legendre et al., 1993). Alternative sites of action have been reported for neomycin, although in general only when the effector is applied at significantly higher millimolar concentrations (reviewed in Kim et al., 1996).

We have also made use of a second PLC antagonist, the aminosteroid U73122, reported to specifically inhibit PLC activity in a variety of animal cell types (see Hansen et al., 1995). Interestingly, there is evidence that the target for U73122 action is G protein–mediated PLC activation (Thompson et al., 1991). Although, to our knowledge, the use of U73122 has not yet been described in plants, inhibition of epidermal *MtENOD12* expression was obtained with antagonist concentrations (1 to 10 μM) similar to those routinely used in inactivating mammalian PLCs (Hansen et al., 1995; Rodriguez et al., 1995). Significantly, the near-identical analog U73343, differing by a single unsaturated bond and used as a control for nonspecific inhibition in animal cell experiments (e.g., see Hansen et al., 1995), did not inhibit transduction of the Nod factor signal. Taken together, these results are consistent with the participation of PLC-dependent $PIP_2$ hydrolysis during Nod factor signal transduction in the root epidermis.

## $Ca^{2+}$ as a Putative Second Messenger in Nod Factor Signaling

Calcium-mediated intracellular signaling is ubiquitous in all higher eukaryotes, and changes in cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_{cyt}$) are implicated in the responses of plants to environmental, hormonal, pathogenic, and developmental signals (reviewed in Bush, 1995). Increases in $[Ca^{2+}]_{cyt}$ can potentially arise either from $Ca^{2+}$ release from intracellular organelles, such as the endoplasmic reticulum or the vacuole, or via influx from the extracellular medium across the plasma membrane.

Ruthenium red is a membrane-permeable diagnostic antagonist for RYR intracellular $Ca^{2+}$ channels, originally characterized in animal cell studies (see Galione, 1994). Subbaiah et al. (1994) demonstrated rapid penetration of ruthenium red into maize cells and correlated its antagonist activity with the blocking of intracellular $Ca^{2+}$ release in response to anoxia. Our results show that ruthenium red inhibits Nod factor–elicited *pMtENOD12–GUS* expression at concentrations in the range of 10 to 50 $\mu$M, which is entirely consistent with typical antagonist levels used in plant studies (Knight et al., 1992; Subbaiah et al., 1994; Allen et al., 1995). We have also been able to demonstrate the rapidity of ruthenium red action and exclude the possibility that inhibition results from nonspecific cellular toxicity.

A possible role for calcium influx across the plasma membrane in Nod factor signal transduction has been examined using both the $Ca^{2+}$ chelator EGTA and the putative calcium channel blocker $La^{3+}$. Our results show that EGTA totally represses *MtENOD12* induction when added to the external medium. This inhibition can be reversed by the subsequent addition of excess $Ca^{2+}$, thus arguing that EGTA treatment does not prejudice cell viability. The $La^{3+}$ ion, a membrane-impermeable $Ca^{2+}$ channel blocker, inhibits the epidermal Nod factor response when applied in the concentration range 5 to 100 $\mu$M. This is in line with typical concentrations used in plant studies (e.g., Huang et al., 1994; Malhó et al., 1994). $La^{3+}$ inhibition can be progressively reversed by the concomitant addition of increasing concentrations of $Ca^{2+}$, suggesting a direct competition between these two ions for influx channels. Finally, it should be emphasized that these results do not necessarily imply a direct role for $Ca^{2+}$ influx during Nod factor signal transduction, because these antagonists might be acting to prevent recharging of intracellular stores and hence indirectly running down signaling resulting from repetitive release from these stores (reviewed in Meldolesi, 1993).

## A Model for Nod Factor Signal Perception/Transduction at the Root Epidermis

The conclusions that can be drawn from our pharmacological experiments are consistent with the simplified model for Nod factor signal transduction illustrated in Figure 3, which

is largely based on known transduction pathways characterized in animal cells. However, it must be made clear that in terms of positioning the various signal transduction elements, our combined agonist/antagonist results only allow us to place PLC activation and $Ca^{2+}$ influx/release downstream of the putative G protein–mediated step. In addition, certain components or second messengers (e.g., changes in cytosolic $Ca^{2+}$ concentration) may be invoked more than once in the pathway leading from signal perception to transcription of the epidermal marker gene *MtENOD12*. In light of our findings, it is important to investigate possible roles in Nod factor signaling for the putative membrane-bound second messenger DAG as well as the soluble messenger $IP_3$, which are both generated by $PIP_2$ hydrolysis. Although DAG is known to activate $Ca^{2+}$-dependent protein kinase C in animal cells, a role for DAG in plant signal transduction has yet to be demonstrated. If $IP_3$ indeed functions as a secondary messenger, it is likely that the transduction pathway involves intracellular $Ca^{2+}$ release from both $IP_3$-responsive receptor channels ($IP_3$R) and RYR channels. Finally, although not indicated in the model, it is also conceivable that cytosolic $Ca^{2+}$ fluxes could be mediated directly via G protein regulation of plasma membrane $Ca^{2+}$ channels (reviewed in Jan and Jan, 1997).

To what extent is this model coherent with other experimental data relating to Nod factor perception/transduction
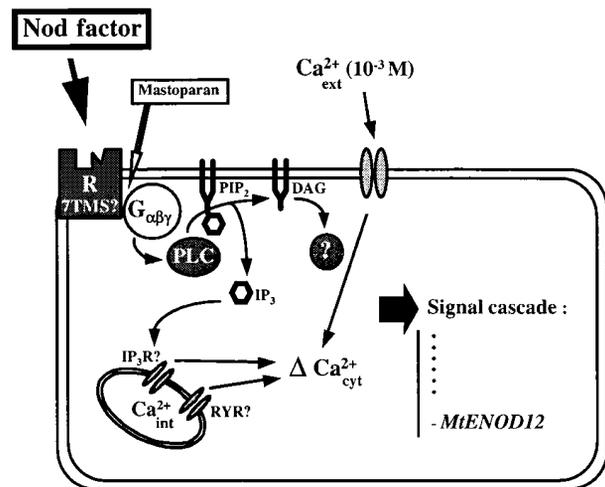


**Figure 3.** Putative Model for the Nod Factor Signal Transduction Pathway.

This model is based on analogies with known signal transduction pathways in animal cell systems and the results presented in this article. $Ca^{2+}_{int}$ and $Ca^{2+}_{ext}$, intracellular (organelle) and extracellular (apoplastic) calcium stores, respectively; $G_{\alpha\beta\gamma}$, heterotrimeric G protein; and R, putative Nod factor receptor. $\Delta Ca^{2+}_{cyt}$ represents changes in cytosolic calcium concentration. The putative site of action of the G protein agonist peptides mastoparan and Mas7 is indicated.

in root epidermal cells? Both plasma membrane depolarization (Ehrhardt et al., 1992; Felle et al., 1995; Kurkdjian, 1995) and intracellular alkalinization (Felle et al., 1996) can be elicited by Nod factor application to alfalfa root hairs. Both of these rapid responses could in theory be coupled to changes in intracellular calcium concentration and might even be mediated directly by G protein regulation of ion channels. It has also been reported that Nod factors can elicit rapid plateau-like increases in intracellular calcium in root hairs of cowpea (Gehring et al., 1997) and regular calcium spiking in alfalfa root hairs (Ehrhardt et al., 1996). Although the precise mechanisms controlling intracellular calcium oscillations are currently unknown, it is believed that this process involves negative and positive feedback and the interplay of second messengers (such as $IP_3$ and $Ca^{2+}$ itself) coupled to $Ca^{2+}$ influx/release (reviewed in Berridge and Dupont, 1994). In light of our results, it will be particularly interesting to examine whether the various signal transduction antagonists that we have identified (interfering with G protein action, PLC activity, and $Ca^{2+}$ influx/release) are also capable of blocking these different cellular responses.

Ardourel et al. (1994) have proposed, based on studies with *R. meliloti* mutants, that at least two types of Nod factor receptor are present in the root epidermis: a specific "signaling" receptor for initiating early events required for subsequent infection and a second "entry" receptor, with even greater stringency for Nod factor structure, necessary for the infection process itself. Based on its spatiotemporal expression pattern (Pichon et al., 1992) and Nod factor stringency requirements (Journet et al., 1994), epidermal expression of the *MtENOD12* gene clearly falls into the signaling receptor category of responses. In mammalian cells, G proteins are almost invariably coupled to a family of architecturally related seven-transmembrane-span (7TMS) receptors (Dohlman et al., 1991). Thus, the Nod factor signaling receptor may be related to this ubiquitous family of 7TMS receptors. A high-affinity Nod factor binding activity has recently been characterized in the microsomal fraction of *Medicago* cell extracts (Niebel et al., 1997), although the molecular nature of this putative receptor remains to be determined.

In conclusion, the results presented in this article provide a working model for Nod factor signal transduction in root epidermal cells and a collection of pharmacological effectors of potential use in addressing questions relating to lipochitooligosaccharide signaling. For example, we can ask whether similar transduction pathways are used for all varieties of Nod factors as well as for other epidermal Nod factor–inducible genes. We can also ask whether there are mechanistic similarities between Nod factor signaling in legumes and the responses elicited by synthetic lipochitooligosaccharides in tobacco protoplast cultures (Röhrig et al., 1995). Finally, these pharmacological effectors, and in particular the peptide agonists, should also prove useful in the characterization of legume mutants with altered nodulation responses resulting from modifications in Nod factor perception/transduction.

## METHODS

### Assay for *pMtENOD12–GUS* Activation in the Transgenic Alfalfa Root Epidermis

Transgenic plants were grown from seedlings in the growth pouch system, as described in Journet et al. (1994). For pharmacological studies, 10-day-old plants were removed from pouches and the root systems were immersed in 30 mL of a buffered solution (20 mM Mes–NaOH, 0.5 mM $MgSO_4$, 1 mM KCl, and 1 mM $CaCl_2$, pH 5.9) maintained at 25°C and containing the appropriate combinations of nodulation (Nod) factors and/or pharmacological effectors. For certain experiments (see Table 7), the $Ca^{2+}$ concentration in the buffer was either modified or replaced by additional $Mg^{2+}$. At the end of the incubation period (6 hr unless otherwise specified), roots were stained for β-glucuronidase (GUS) activity and observed as described previously (Journet et al., 1994). Epidermal responses to Nod factors were essentially identical (in both localization and timing) to those previously described when factors were added directly to the growth pouch (Journet et al., 1994), except that root immersion generally enhanced sensitivity and significantly reduced plant-to-plant variability. As a result of this improved sensitivity, a low-level background response (classified as very weak; see below) was observed in a small percentage of plants (ranging from 0 to 10%) in the absence of elicitor treatment (Table 2). Thus, only experiments leading to positive responses in >10 to 20% of the plants tested were considered to be above background. For each elicitor/effector treatment, a total of 10 to 50 plants were examined, and all experiments were performed at least twice.

In antagonist experiments, roots were pretreated for 1 hr (unless otherwise stated) before the addition of Nod factor or mastoparan to the incubation medium. For the purposes of evaluating antagonist (and combined nitrogen) effects, the levels of GUS staining for individual plants were visually assigned to one of the following four categories: (1) normal intensity response; (2) response of clearly reduced intensity (e.g., see Figure 2D); (3) very weak response (only a few positively staining cells; e.g., see Figure 2E); or (4) no detectable response. A reduction in the overall percentage of positive plants was always accompanied by a reduction in the number of GUS-staining cells per plant. Thus, responses are always very weak when only a small percentage of plants responds. After agonist treatments, plants were assigned to one of three categories: (1) clear positive response; (2) very weak response; or (3) no detectable response. Because no differences were observed between transgenic *Medicago varia* (alfalfa) and *M. truncatula* and because adequate stocks of transgenic *M. truncatula* seed were not available when these studies were initiated, the pharmacological experiments presented here were performed with the transgenic alfalfa line expressing *pMtENOD12–GUS* described previously (Journet et al., 1994).

### Nodulation Factors and Pharmacological Effectors

Preparations of purified *Rhizobium meliloti* NodRm factors (majority of species NodRmIV(C16:2, Ac,S)) were kindly provided by F. Maillet (research group of Jean Dénarié within the host laboratory). Serial dilutions of Nod factors were performed in $H_2O$. The peptide effectors mastoparan (Sigma), Mas7 (Calbiochem, La Jolla, CA), and Mas17 (Peninsula Laboratories, Belmont, CA) were all dissolved at 1 mg/mL in $H_2O$ and stored at −20°C. Buffered native cholera and pertussis toxins (Calbiochem) were resuspended at stock concentrations of 1

and 0.1 mg/mL, respectively, before use. Solutions (4 mM) of the aminosteroids U73122 and U73343 (Calbiochem) were freshly prepared in ethanol. A ruthenium red (Fluka Chemie AG, Buchs, Switzerland) solution (10 mM) was freshly prepared in $H_2O$. Neomycin sulfate and $LaCl_3$ (Sigma) solutions were prepared as 10 and 100 mM aqueous stocks, respectively.

### Reverse Transcriptase–Polymerase Chain Reaction Assay to Detect *MtENOD12* Transcripts

For each time point (0 to 6 hr), reactive root zones (between 1 and 2 cm above the primary root tip) of 30 10-day-old *M. truncatula* cv Jemalong plants were collected and ground in liquid nitrogen, and total RNA was extracted by a standard phenol–SDS protocol. Before reverse transcriptase–polymerase chain reaction (RT-PCR), genomic DNA was eliminated by treating 1- to 5-$\mu$g aliquots of total RNA with 10 units of DNase I (Pharmacia LKB, Uppsala, Sweden) in RT buffer (see below) at 37°C, followed by phenol–chloroform extraction and ethanol precipitation. First-strand cDNA synthesis using 1 to 3 $\mu$g of total RNA was performed with oligo(dT)$_{12-18}$ (400 ng) and 80 units of Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD) in a total volume of 15 $\mu$L of RT buffer (50 mM Tris–HCl, 75 mM KCl, and 3 mM $MgCl_2$), including 10 mM DTT and 0.5 mM deoxynucleotide triphosphates at 42°C. After heat denaturation of the enzyme, cDNA samples were diluted to $\sim$30 ng of RNA per $\mu$L, and 1-$\mu$L aliquots were used for PCR amplification. Controls without RT were used to confirm the absence of genomic DNA contamination. PCR reactions were conducted using Taq DNA polymerase (Life Technologies), following the standard protocol, in a 20-$\mu$L total reaction volume. Each experiment was performed at least twice.

   *MtENOD12* cDNA was amplified (25 cycles at 94°C for 30 sec, 59°C for 30 sec, and 72°C for 30 sec) between positions 88 and 295 (Pichon et al., 1992) by using forward primer 5′-CCTGCTTATAGGCCACCAC-3′ and reverse primer 5′-CTTCTGCTGGAGGATGCC-3′. To control for equivalent cDNA levels, PCR amplifications were also performed for *MtPR10-1* (GenBank accession No. Y08726), a constitutively and strongly expressed gene in roots (Gamas et al., 1998). *MtPR10-1* cDNA was amplified (25 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec) between positions 149 and 433 by using forward primer 5′-CTCCATCCCACAATATGCCTCCA-3′ and reverse primer 5′-ATCGATGCTAGGTGGAGGCT-3′. PCR products were analyzed by DNA gel blotting using the 500-bp SphI-BamHI fragment of *MtENOD12* (Pichon et al., 1992) and the complete cDNA for *MtPR10-1*. We confirmed that under our experimental conditions, PCR yields were proportional to the initial quantity of cDNA. For protein synthesis inhibition experiments, cycloheximide (5 $\mu$M final) was added to the root incubation medium 30 min before Nod factors. The choice of inhibitor concentration was based on experiments showing that 5 $\mu$M was the lowest cycloheximide concentration that totally inhibited epidermal *pMtENOD12–GUS* expression in transgenic *M. truncatula* plants in the presence of Nod factors.

## REFERENCES

**Allen, G.J., Muir, S.R., and Sanders, D.** (1995). Release of $Ca^{2+}$ from individual plant vacuoles by both $InsP_3$ and cyclic ADP-ribose. Science **268,** 735–737.

**Allison, L.A., Kiss, G.B., Bauer, P., Poiret, M., Pierre, M., Savouré, A., Kondorosi, E., and Kondorosi, A.** (1993). Identification of two alfalfa early nodulin genes with homology to members of the pea *Enod12* gene family. Plant Mol. Biol. **21,** 375–380.

**Ardourel, M., Demont, N., Debellé, F., Maillet, F., de Billy, F., Promé, J.-C., Dénarié, J., and Truchet, G.** (1994). *Rhizobium meliloti* lipooligosaccharide nodulation factors: Different structural requirements for bacterial entry into target root hair cells and induction of plant symbiotic developmental responses. Plant Cell **6,** 1357–1374.

**Armstrong, F., and Blatt, M.R.** (1995). Evidence for $K^+$ channel control in *Vicia* guard cells coupled by G-proteins to a 7TMS receptor mimetic. Plant J. **8,** 187–198.

**Bauer, P., Crespi, M.D., Szécsi, J., Allison, L.A., Schultze, M., Ratet, P., Kondorosi, E., and Kondorosi, A.** (1994). Alfalfa *Enod12* genes are differentially regulated during nodule development by Nod factors and *Rhizobium* invasion. Plant Physiol. **105,** 585–592.

**Berridge, M.J., and Dupont, G.** (1994). Spatial and temporal signaling by calcium. Curr. Opin. Cell Biol. **6,** 267–274.

**Bono, J.J., Riond, J., Nicolaou, K.C., Bockovich, N.J., Estevez, V.A., Cullimore, J.V., and Ranjeva, R.** (1995). Characterisation of a binding site for chemically-synthesized lipooligosaccharidic NodRm factors in particulate fractions prepared from roots. Plant J. **7,** 252–260.

**Bush, D.S.** (1995). Calcium regulation in plant cells and its role in signaling. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46,** 95–122.

**Chabaud, M., Larsonneau, C., Marmouget, C., and Huguet, T.** (1996). Transformation of barrel medic (*Medicago truncatula* Gaertn.) by *Agrobacterium tumefaciens* and regeneration via somatic embryogenesis of transgenic plants with the *MtENOD*12 nodulin promoter fused to the *gus* reporter gene. Plant Cell Rep. **15,** 305–310.

**Chen, Q.Y., and Boss, W.F.** (1991). Neomycin inhibits the phosphotidylinositol monophosphate and phosphatidylinositol bisphosphate stimulation of plasma-membrane ATPase activity. Plant Physiol. **96,** 340–343.

**Conklin, B.R., and Bourne, H.R.** (1993). Structural elements of $G_\alpha$ subunits that interact with $G_{\beta\gamma}$, receptors and effectors. Cell **73**, 631–641.

**Dénarié, J., Debellé, F., and Promé, J.-C.** (1996). *Rhizobium* lipochitooligosaccharide nodulation factors: Signaling molecules mediating recognition and morphogenesis. Annu. Rev. Biochem. **65**, 503–535.

**Dohlman, H.G., Thorner, J., Caron, M.G., and Lefkowitz, R.J.** (1991). Model systems for the study of seven-transmembrane-segment receptors. Annu. Rev. Biochem. **60**, 653–688.

**Drøbak, B.K., and Watkins, P.A.C.** (1994). Inositol (1,4,5)-trisphosphate production in plant cells: Stimulation by the venom peptides, melittin and mastoparan. Biochem. Biophys. Res. Commun. **205**, 739–745.

**Ehrhardt, D.W., Atkinson, E.M., and Long, S.R.** (1992). Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. Science **256**, 998–1000.

**Ehrhardt, D.W., Wais, R., and Long, S.R.** (1996). Calcium spiking in plant root hairs responding to *Rhizobium* nodulation signals. Cell **85**, 673–681.

**Felle, H.H., Kondorosi, E., Kondorosi, A., and Schultze, M.** (1995). Nod signal–induced plasma membrane potential changes in alfalfa root hairs are differentially sensitive to structural modifications of the lipochitooligosaccharide. Plant J. **7**, 939–947.

**Felle, H.H., Kondorosi, E., Kondorosi, A., and Schultze, M.** (1996). Rapid alkalinization in alfalfa root hairs in response to rhizobial lipochitooligosaccharide signals. Plant J. **10**, 295–301.

**Franklin-Tong, V.E., Drøbak, B.K., Allan, A.C., Watkins, P.A.C., and Trewavas, A.J.** (1996). Growth of pollen tubes of *Papaver rhoeas* is regulated by a slow-moving calcium wave propagated by inositol 1,4,5-trisphosphate. Plant Cell **8**, 1305–1321.

**Galione, A.** (1994). Cyclic ADP-ribose, the ADP-ribosyl cyclase pathway and calcium signaling. Mol. Cell. Endocrinol. **98**, 125–131.

**Gamas, P., de Billy, F., and Truchet, G.** (1998). Symbiosis-specific expression of two *Medicago truncatula* nodulin genes, *MtN1* and *MtN13*, encoding products homologous to plant defense proteins. Mol. Plant-Microbe Interact., in press.

**Gehring, C.A., Irving, H.R., Kabbara, A.A., Parish, R.W., Boukli, N.M., and Broughton, W.J.** (1997). Rapid, plateau-like increases in intracellular free calcium are associated with Nod factor–induced root hair deformation. Mol. Plant-Microbe Interact. **10**, 791–802.

**Gotor, C., Lam, E., Cejudo, F.J., and Romero, L.C.** (1996). Isolation and analysis of the soybean *SGA2* gene (cDNA), encoding a new member of the plant G-protein family of signal transducers. Plant Mol. Biol. **32**, 1227–1234.

**Hansen, M., Boitano, S., Dirksen, E.R., and Sanderson, M.J.** (1995). A role for phospholipase C activity but not ryanodine receptors in the initiation and propagation of intercellular calcium waves. J. Cell Sci. **108**, 2583–2590.

**Heidstra, R., and Bisseling, T.** (1996). Nod factor–induced host responses and mechanisms of Nod factor perception. New Phytol. **133**, 25–43.

**Heidstra, R., Geurts, R., Franssen, H., Spaink, H.P., Van Kammen, A., and Bisseling, T.** (1994). Root hair deformation activity of nodulation factors and their fate on *Vicia sativa*. Plant Physiol. **105**, 787–797.

**Heidstra, R., Nilsen, G., Martinez-Abarca, F., Van Kammen, A., and Bisseling, T.** (1997). Nod factor–induced expression of leghemoglobin to study the mechanism of $NH_4NO_3$ inhibition on root hair deformation. Mol. Plant-Microbe Interact. **10**, 215–220.

**Higashijima, T., Uzu, S., Nakajima, T., and Ross, E.M.** (1988). Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G proteins). J. Biol. Chem. **263**, 6491–6494.

**Higashijima, T., Burnier, J., and Ross, E.M.** (1990). Regulation of $G_i$ and $G_o$ by mastoparan, related amphiphilic peptides, and hydrophobic amines. J. Biol. Chem. **265**, 14176–14186.

**Horvath, B., Heidstra, R., Lados, M., Moerman, M., Spaink, H.P., Promé, J.C., Van Kammen, A., and Bisseling, T.** (1993). Lipo-oligosaccharides of *Rhizobium* induce infection-related early nodulin gene expression in pea root hairs. Plant J. **4**, 727–733.

**Huang, J.W., Grunes, D.L., and Kochian, L.V.** (1994). Voltage-dependent $Ca^{2+}$ influx into right-side-out plasma membrane vesicles isolated from wheat roots: Characterization of a putative $Ca^{2+}$ channel. Proc. Natl. Acad. Sci. USA **91**, 3473–3477.

**Jan, L.Y., and Jan, Y.N.** (1997). Receptor-regulated ion channels. Curr. Opin. Cell Biol. **9**, 155–160.

**Journet, E.P., Pichon, M., Dedieu, A., de Billy, F., Truchet, G., and Barker, D.G.** (1994). *Rhizobium meliloti* Nod factors elicit cell-specific transcription of the *ENOD12* gene in transgenic alfalfa. Plant J. **6**, 241–249.

**Kim, H.Y., Coté, G.G., and Crain, R.C.** (1996). Inositol 1,4,5-trisphosphate may mediate closure of $K^+$ channels by light and darkness in *Samanea saman* motor cells. Planta **198**, 279–287.

**Knight, M.R., Smith, S.M., and Trewavas, A.J.** (1992). Wind-induced plant motion immediately increases cytosolic calcium. Proc. Natl. Acad. Sci. USA **89**, 4967–4971.

**Kurkdjian, A.C.** (1995). Role of the differentiation of root epidermal cells in Nod factor (from *Rhizobium meliloti*)–induced root-hair depolarization of *Medicago sativa*. Plant Physiol. **107**, 783–790.

**Legendre, L., Yueh, Y.G., Crain, R., Haddock, N., Heinstein, P.F., and Low, P.S.** (1993). Phospholipase C activation during elicitation of the oxidative burst in cultured plant cells. J. Biol. Chem. **268**, 24559–24563.

**Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J.-C., and Dénarié, J.** (1990). Symbiotic host specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature **344**, 781–784.

**Long, S.R.** (1996). *Rhizobium* symbiosis: Nod factors in perspective. Plant Cell **8**, 1885–1898.

**Malhó, R., Read, N.D., Pais, M.S., and Trewavas, A.J.** (1994). Role of cytosolic free calcium in the reorientation of pollen tube growth. Plant J. **5**, 331–341.

**McDonald, L.J., and Mamrack, M.D.** (1995). Phosphoinositide hydrolysis by phospholipase C modulated by multivalent cations $La^{3+}$, $Al^{3+}$, neomycin, polyamines, and melittin. J. Lipid Mediat. Cell Signal. **11**, 81–91.

**Meldolesi, J.** (1993). Keeping the stores full. Curr. Biol. **3**, 910–912.

**Millner, P.A., and Causier, B.E.** (1996). G-protein coupled receptors in plant cells. J. Exp. Bot. **47**, 983–992.

**Munnik, T., Arisz, S.A., de Vrije, T., and Musgrave, A.** (1995). G protein activation stimulates phospholipase D signaling in plants. Plant Cell **7**, 2197–2210.

**Niebel, A., Bono, J.J., Ranjeva, R., and Cullimore, J.V.** (1997). Identification of a high affinity binding site for lipo-oligosaccharidic NodRm factors in the microsomal fraction of *Medicago* cell suspension cultures. Mol. Plant-Microbe Interact. **10,** 132–134.

**Pichon, M., Journet, E.-P., Dedieu, A., de Billy, F., Truchet, G., and Barker, D.G.** (1992). *Rhizobium meliloti* elicits transient expression of the early nodulin gene *ENOD12* in the differentiating root epidermis of transgenic alfalfa. Plant Cell **4,** 1199–1211.

**Quarmby, L.M., Yueh, Y.G., Cheshire, J.L., Keller, L.R., Snell, W.J., and Crain, R.C.** (1992). Inositol phospholipid metabolism may trigger flagellar excision in *Chlamydomonas reinhardtii.* J. Cell Biol. **116,** 737–744.

**Roche, P., Debellé, F., Maillet, F., Lerouge, P., Faucher, C., Truchet, G., Dénarié, J., and Promé, J.C.** (1991). Molecular basis of symbiotic host specificity in *Rhizobium meliloti: nodH* and *nodPQ* genes encode the sulfation of lipo-oligosaccharide signals. Cell **67,** 1131–1143.

**Rodriguez, A., Rioult, M.G., Ora, A., and Andrews, N.W.** (1995). A trypanosome-soluble factor induces $IP_3$ formation, intracellular $Ca^{2+}$ mobilization and microfilament rearrangement in host cells. J. Cell Biol. **129,** 1263–1273.

**Röhrig, H., Schmidt, J., Walden, R., Czaja, I., Miklasevics, E., Wieneke, U., Schell, J., and John, M.** (1995). Growth of tobacco protoplasts stimulated by synthetic lipochitooligosaccharides. Science **269,** 841–843.

**Ross, E.M., and Higashijima, T.** (1994). Regulation of G-protein activation by mastoparans and other cationic peptides. Methods Enzymol. **237,** 26–37.

**Scheres, B., Van de Wiel, C., Zalensky, A., Horvath, B., Spaink, H., Van Eck, H., Zwartkruis, F., Wolters, A.M., Gloudemans, T., Van Kammen, A., and Bisseling, T.** (1990). The *ENOD12* gene product is involved in the infection process during the pea–*Rhizobium* interaction. Cell **60,** 281–294.

**Simon, M.I., Strathmann, M.P., and Gautam, N.** (1991). Diversity of G proteins in signal transduction. Science **252,** 802–808.

**Smith, R.J., Sam, L.M., Justen, J.M., Bundy, G.L., Bala, G.A., and Bleasdale, J.E.** (1990). Receptor-coupled signal transduction in human polymorphonuclear neutrophils: Effects of a novel inhibitor of phospholipase C dependent processes on cell responsiveness. J. Pharmacol. Exp. Ther. **253,** 688–697.

**Subbaiah, C.C., Bush, D.S., and Sachs, M.M.** (1994). Elevation of cytosolic calcium precedes anoxic gene expression in maize suspension-cultured cells. Plant Cell **6,** 1747–1762.

**Tanimura, A., Matsumoto, Y., and Tojyo, Y.** (1991). Mastoparan increases membrane permeability in rat parotid cells independently of action on G-proteins. Biochem. Biophys. Res. Commun. **177,** 802–808.

**Thompson, A.K., Mostafapour, S.P., Denlinger, L.C., Bleasdale, J.E., and Fisher, S.K.** (1991). The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. A role for $G_p$ in receptor compartmentation. J. Biol. Chem. **266,** 23856–23862.

**Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Promé, J.C., and Dénarié, J.** (1991). Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature **351,** 670–673.

**Vijn, I., Martinez-Abarca, F., Yang, W.C., das Neves, L., Van Brussel, A., Van Kammen, A., and Bisseling, T.** (1995a). Early nodulin gene expression during Nod factor–induced processes in *Vicia sativa.* Plant J. **8,** 111–119.

**Vijn, I., Yang, W.C., Pallisgård, N., Jensen, E.Ø., Van Kammen, A., and Bisseling, T.** (1995b). *VsENOD5, VsENOD12* and *VsENOD40* expression during *Rhizobium*-induced nodule formation on *Vicia sativa* roots. Plant Mol. Biol. **28,** 1111–1119.

**Wakamatsu, K., Okada, A., Miyazawa, T., Ohya, M., and Higashijima, T.** (1992). Membrane-bound conformation of mastoparan-X, a G-protein–activating peptide. Biochemistry **31,** 5654–5660.

**Wallace, M.A., and Carter, H.R.** (1989). Effects of wasp venom peptide, mastoparan, on a phosphoinositide-specific phospholipase C purified from rabbit brain membranes. Biochim. Biophys. Acta **1006,** 311–316.

**Wise, A., Thomas, P.G., Hedley Carr, T., Murphy, G.A., and Millner, P.A.** (1997). Expression of *Arabidopsis* G-protein GPα1: Purification and characterisation of the recombinant protein. Plant Mol. Biol. **33,** 723–728.

**Wong, W.S.F., and Rosoff, P.M.** (1996). Pharmacology of pertussis toxin B-oligomer. Can. J. Physiol. Pharmacol. **74,** 559–564.

This information is current as of April 19, 2019

| References | This article cites 61 articles, 24 of which can be accessed free at: /content/10/5/659.full.html#ref-list-1 |
|---|---|
| Permissions | https://www.copyright.com/ccc/openurl.do?sid=pd_hw1532298X&issn=1532298X&WT.mc_id=pd_hw1532298X |
| eTOCs | Sign up for eTOCs at: http://www.plantcell.org/cgi/alerts/ctmain |
| CiteTrack Alerts | Sign up for CiteTrack Alerts at: http://www.plantcell.org/cgi/alerts/ctmain |
| Subscription Information | Subscription Information for *The Plant Cell* and *Plant Physiology* is available at: http://www.aspb.org/publications/subscriptions.cfm |