RESEARCH ARTICLE

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

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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 NH₄NO₃) 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 Ca²⁺ influx/release (EGTA, La³⁺, 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 Ca²⁺ 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., 1995) in alfalfa root hairs. Ehrhardt et al. (1995) 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^{-1}-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 expressing 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-
ator 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

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**Table 1. Sequences of G Protein Peptide Effectors**

<table>
<thead>
<tr>
<th>Peptide</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<td>Ala</td>
<td>Leu</td>
<td>Ala</td>
<td>Ala</td>
<td>Leu</td>
<td>Ala</td>
<td>Lys</td>
<td>Lys</td>
<td>Ile</td>
<td>Leu-\text{NH}_2^b</td>
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<td>Leu</td>
<td>Ala</td>
<td>Lys</td>
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<td>Leu^c</td>
<td>Leu-\text{NH}_2^b</td>
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</tr>
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<td>Ala</td>
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<td>Leu</td>
<td>Ala</td>
<td>Lys</td>
<td>Leu^c</td>
<td>Leu-\text{NH}_2^b</td>
<td></td>
</tr>
</tbody>
</table>

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^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.

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![Figure 2. Histochemical Localization of GUS Activity in Roots of Transgenic Alfalfa after Treatment with Either the Nod Factor or Mastoparan.](image_url)

(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 μM. However, when the mastoparan concentration was increased to 10 μM, 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 μM (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 Ca²⁺ 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 μM) than for mastoparan. At 2 μM (fivefold lower than for mastoparan), Mas7 ceases to be an agonist for early nodulin gene induction. Again, the presence of 1 mM Ca²⁺ 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 α-helical membrane conformation apparently essential for G protein agonist activity (see Discussion). Significantly, Mas17 (0.2 to 5 μM) totally lacked the capacity to elicit pMtENOD12-GUS expression above background levels (Table 2).

### Table 2. G Protein Peptide Effector Activities

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Concentration (μM)</th>
<th>% GUS-Staining Plants</th>
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</thead>
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<tr>
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<tr>
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<td>93</td>
</tr>
<tr>
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<td>78</td>
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<tr>
<td></td>
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<td>11²</td>
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<td>Minus Ca²⁺(c)</td>
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</tr>
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<td>Mas7</td>
<td>0.2</td>
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<tr>
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<td>60²</td>
</tr>
<tr>
<td>Minus Ca²⁺(c)</td>
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<td>Mas17</td>
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</tr>
<tr>
<td></td>
<td>5</td>
<td>60²</td>
</tr>
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</table>

¹Control in the absence of peptide.  
²Very weak response.  
³Incubation medium lacking calcium.

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**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 Gα subunit, thus preventing GDP 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 Gα cysteine residue, thus interfering with the interaction with the receptor. The results presented in Table 3 show that cholera toxin (up to 100 μg/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 μg/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 μg/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 μg/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 NH₄NO₃ 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
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 NH₄NO₃ 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 NH₄NO₃ 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 (PIP₂) to yield inositol (1,4,5)-trisphosphate (IP₃) 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) 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 aminglycoside, 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>
<thead>
<tr>
<th>Elicitor</th>
<th>Effector</th>
<th>Concentration (μg/mL)</th>
<th>% GUS-Staining Plants</th>
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<td>— b</td>
<td>—</td>
<td>7 c</td>
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<td></td>
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a Control in the absence of elicitor and/or effector.
b Very weak response.
c Clearly reduced response.

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

<table>
<thead>
<tr>
<th>Growth in Presence of 10 mM NH₄NO₃</th>
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</tr>
<tr>
<td>Yes</td>
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<td>10⁻⁶</td>
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a Clearly reduced response.
b Very weak response.
Transient rises in cytoplasmic Ca\(^{2+}\) concentration have been implicated in the responses of higher plants to a wide variety of external signals. We have examined whether the Nod factor–elicited gene expression requires Ca\(^{2+}\) signaling pathway leading to the mobilization of intracellular Ca\(^{2+}\) stores and/or inward flux across the plasma membrane. Ruthenium red is an efficient 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 \(\mu M\) (Table 6), 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) (but not Mg\(^{2+}\)) restored gene expression, showing that this inhibition is specifically due to Ca\(^{2+}\) chelation. A normal epidermal response could also be recovered when 5 mM Ca\(^{2+}\) was added to the medium after a 3-hr incubation with Nod factors in the presence of 2 mM EGTA (Table 7). Significantly, the concomitant addition of increasing concentrations of Ca\(^{2+}\) progressively relieved inhibition of the Nod factor response (Table 7).

The importance of extracellular sources of Ca\(^{2+}\) for mastoparan activity is illustrated by the fact that MtENOD12 expression elicited by the G protein agonist was totally blocked in the presence of 2 mM EGTA. The concomitant addition of increasing concentrations of Ca\(^{2+}\) progressively relieved inhibition of the Nod factor response (Table 7).

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

<table>
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<tr>
<th>Elicitor</th>
<th>Effector</th>
<th>Concentration ((\mu M))</th>
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<td>Neomycin</td>
<td>100</td>
<td>6(^c)</td>
</tr>
<tr>
<td></td>
<td>U73122</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>U73343</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>93</td>
</tr>
</tbody>
</table>

\(^a\) 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.

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

<table>
<thead>
<tr>
<th>Elicitor</th>
<th>Effector</th>
<th>Concentration ((\mu M))</th>
<th>% GUS-Staining Plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>NodRm (10(^{-9}) M)</td>
<td>Ruthenium red</td>
<td>2</td>
<td>93(^b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>12(^c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CA</td>
<td>10</td>
<td>10(^c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+15 min(^a)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+60 min(^a)</td>
<td>10</td>
</tr>
<tr>
<td>Mastoparan (10(^{-6}) M)</td>
<td>Ruthenium red</td>
<td>10</td>
<td>5(^c)</td>
</tr>
</tbody>
</table>

\(^a\) 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.
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\(_4\)NO\(_3\). 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 \(\alpha\)-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 effects.
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; Drebak 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 Gi 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 (Gα, Gβ, Gγ, and Gδ) based on the structural and functional characteristics of the Gα subunits (reviewed in Simon et al., 1991). Mastoparan and Mas7 are believed to interact preferentially with members of the G1 class (including Gαi), with a lower sensitivity for Gαq (Ross and Higashijima, 1994). Pertussis toxin–sensitive Gαi subunits usually belong to the G1 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 Gi or Gαq classes (Simon et al., 1991). Although intact heterotrimeric G proteins have not yet been isolated in plants, a small number of Gα and Gβ subunits genes have been cloned from a variety of plant species (reviewed in Millner and Causier, 1996), and the first putative Gα subunits have been biochemically characterized (e.g., Wise et al., 1997). Sequence analysis shows that these first Gα subunits do not possess the pertussis-sensitive C-terminal ribosylation site, suggesting that other families of heterotrimeric G proteins must probably exist in plants. Sequence comparisons also show that plant Gα 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 G1 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 IP3, 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 IP3 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 IP3 hydrolysis during Nod factor signal transduction in the root epidermis.
Ca\textsuperscript{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\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{cyt}) are implicated in the responses of plants to environmental, hormonal, pathogenic, and developmental signals (reviewed in Bush, 1995). Increases in [Ca\textsuperscript{2+}]\textsubscript{cyt} can potentially arise either from Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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 \textmu 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\textsuperscript{2+} chelator EGTA and the putative calcium channel blocker La\textsuperscript{3+}. Our results show that EGTA totally represses MiENOD12 induction when added to the external medium. This inhibition can be reversed by the subsequent addition of excess Ca\textsuperscript{2+}, thus arguing that ETGA treatment does not prejudice cell viability. The La\textsuperscript{3+} ion, a membrane-impermeable Ca\textsuperscript{2+} channel blocker, inhibits the epidermal Nod factor response when applied in the concentration range 5 to 100 \textmu M. This is in line with typical concentrations used in plant studies (e.g., Huang et al., 1994; Malhó et al., 1994). La\textsuperscript{3+} inhibition can be progressively reversed by the concomitant addition of increasing concentrations of Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} influx/release downstream of the putative G protein–mediated step. In addition, certain components or second messengers (e.g., changes in cytosolic Ca\textsuperscript{2+} concentration) may be invoked more than once in the pathway leading from signal perception to transcription of the epidermal marker gene MiENOD12. 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\textsubscript{3}, which are both generated by PI\textsubscript{3} hydrolysis. Although DAG is known to activate Ca\textsuperscript{2+}-dependent protein kinase C in animal cells, a role for DAG in plant signal transduction has yet to be demonstrated. If IP\textsubscript{3} indeed functions as a secondary messenger, it is likely that the transduction pathway involves intracellular Ca\textsuperscript{2+} release from both IP\textsubscript{3}-responsive receptor channels (IP\textsubscript{3}R) and RYR channels. Finally, although not indicated in the model, it is also conceivable that cytosolic Ca\textsuperscript{2+} fluxes could be mediated directly via G protein regulation of plasma membrane Ca\textsuperscript{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

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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\textsuperscript{2+}\textsubscript{int} and Ca\textsuperscript{2+}\textsubscript{organelle} intracellular (organelle) and extracellular (apoplastic) calcium stores, respectively; G\textsubscript{aGPR}, heterotrimeric G protein; and R, putative Nod factor receptor. \Delta Ca\textsuperscript{2+} 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 alkalization (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 IP3 and Ca2+ itself) coupled to Ca2+ 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 Ca2+ influx/release) are also capable of blocking these different cellular responses.

Ardourel et al. (1994) have proposed, based on studies with R. melioli 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 (Dohman 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 lipochitooleigosaccharide 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 lipochitooleigosaccharides 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 MgSO4, 1 mM KCl, and 1 mM CaCl2, 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 Ca2+ concentration in the buffer was either modified or replaced by additional Mg2+. 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 mellotii NodRm factors (majority of species NodRmIV(C16:2, Ac,S)) were kindly provided by F. Maillet (research group of ) ean Dénarié within the host laboratory). Serial dilutions of Nod factors were performed in H2O. The peptide effectors mastoparan (Sigma), Mas7 (Calbiochem, La Jolla, CA), and Mas17 (Peninsula Laboratories, Belmont, CA) were all dissolved at 1 mg/mL in H2O 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 H2O. Neomycin sulfate and LaCl3 (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 J. e- malong 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-μg 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 μg 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 μL of RT buffer (50 mM Tris–HCl, 75 mM KCl, and 3 mM MgCl2), including 10 mM DTT and 0.5 mM deoxynucleotide triphosphates at 42°C. After heat denaturation of the enzyme, cDNA samples were diluted to ~30 ng of RNA per μL, and 1-μL 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 reaction mixture of 20 μL 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′-CTGCTTTATAGGCCA-CCAC-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′-CTCCATCCACATAATGCCCTCAA-3′ and reverse primer 5′-ATGCATTGCTAGGAGGTCT-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 μM 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 μM was the lowest cycloheximide concentration that totally inhibited epidermal MtENOD12–GUS expression in transgenic M. truncatula plants in the presence of Nod factors.

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