Nod Factor Inhibition of Reactive Oxygen Efflux in a Host Legume[1]

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Hydrogen peroxide (H_2O_2) efflux was measured from *Medicago truncatula* root segments exposed to purified Nod factor and to poly-GalUA (PGA) heptamers. Nod factor, at concentrations > 100 pM, reduced H_2O_2 efflux rates to 60% of baseline levels beginning 20 to 30 min after exposure, whereas the PGA elicitor, at > 75 nM, caused a rapid increase in H_2O_2 efflux to >200% of baseline rates. Pretreatment of plants with Nod factor alters the effect of PGA by limiting the maximum H_2O_2 efflux rate to 125% of that observed for untreated plants. Two Nod factor-related compounds showed no ability to modulate peroxide efflux, and tomato (*Lycopersicon esculentum*), a nonlegume, showed no response to 1 nM Nod factor. Seven *M. truncatula* mutants, lacking the ability to make nodules, were tested for Nod factor effects on H_2O_2 efflux. The *nfp* mutant was blocked for suppression of peroxide efflux, whereas the *dmi1* and *dmi2* mutants, previously shown to be blocked for early Nod factor responses, showed a wild-type peroxide efflux modulation. These data demonstrate that exposure to Nod factor suppresses the activity of the reactive oxygen-generating system used for plant defense responses.

The interaction of *Sinorhizobium meliloti* with the host legume, *Medicago truncatula*, results in a permissive bacterial infection and the eventual induction of a plant nodule where symbiotic nitrogen fixation occurs. The interaction is characterized by a reciprocal exchange of small molecule signals. Induction of bacterial gene expression by plant flavonoids results in the production of lipo-chitooligosaccharide signaling molecules, termed Nod factors (for review, see Dénarié et al., 1996; Long, 1996). The perception of small, carbohydrate-based signaling molecules also occurs during non-race-specific pathogen interactions (for review, see Hahn, 1996; Nürnberger, 1999). Contact of the plant and microorganism leads to the liberation of plant- and pathogen-derived oligosaccharide fragments that, in turn, trigger both short-term changes in physiology and longer term effects through plant gene expression (Lamb et al., 1989; Jabs et al., 1997). Here, we report the use of purified Nod factor and poly-GalUA (PGA) fragments to ask if the symbiotic signal elicits an increase in oxidative radical efflux typical for plant-pathogen interactions.

Higher plants dramatically increase the rate of reactive oxygen evolution in response to pathogenic elicitor molecules. This oxidative burst response cross links cell wall molecules (Bradley et al., 1992; Brisson et al., 1994) and results in the induction of both plant and microbial gene expression (Levine et al., 1994; Lamb and Dixon, 1997; Yang et al., 1997; Grant and Loake, 2000; Xu et al., 2001). The response begins with the interaction of the pathogenic elicitor, often a carbohydrate molecule, with a specific receptor (Hahn, 1996). Binding of the receptor and subsequent activation of calcium channels to produce an internal calcium flux leads to the modulation of a kinase/phosphatase system regulating NADPH oxidase activity. The plasma membrane NADPH oxidase complex is composed of at least five subunits, including two intrinsic membrane proteins, two cytoplasmic proteins (that bind to the plasma membrane complex), and a member of the Rac-like small GTPase family (Xing et al., 1997). Dephosphorylation of a cytoplasmic subunit leads to binding of that subunit at the membrane and, in the presence of activated Rac, results in reduction of NADP and the concomitant production of superoxide radicals on the exocytoplasmic face of the membrane.

The bacterially derived Nod factor molecule has a small chitin backbone with a C:16 to C:18 lipid group on the nonreducing end (Spaink et al., 1991). The degree of lipid saturation and other modifications, such as an acetyl moiety or a reducing end sulfate, provide information for host specificity in the symbiosis. Application of purified Nod factor to the host legume at 10^{-11} M concentrations triggers several specific responses, including root hair deformation (Dénarié et al., 1996; Oldroyd et al., 2001) and calcium spiking (Ehrhardt et al., 1996). Higher concentrations induce membrane depolarization (Ehrhardt et al., 1992; Felle et al., 1995) and rapid ionic flux, including a sizeable increase in cytoplasmic calcium ions similar to that observed for pathogenic elicitors (Felle et al., 1999; Shaw and Long, 2003). Existing nodulation mutants that lack the above-listed Nod factor responses suggest a possible hierarchy in Nod factor signal transduction (Catoira et al., 2000).

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Induction of both a calcium flux response and cellular growth defects in root hair cells suggested the possibility that Nod factor might be triggering a modified pathogenesis response. We have devised an assay to measure the hydrogen peroxide (H_2O_2) efflux from excised root segments. Using this assay on wild-type and mutant *M. truncatula* plants, we demonstrate that Nod factor exposure slows the rate of H_2O_2 efflux during the first hours of treatment. We further show that the H_2O_2 retardation response is specific for Nod factor and is independent or upstream of the previously characterized calcium spiking pathway (Catoira et al., 2000; Ben Amor et al., 2003). The ability of Nod factor to interfere with the elicitation of reactive oxygen efflux by a pathogenic carbohydrate elicitor suggests a possible role in modulating the plant defense response.

RESULTS

Modulation of H_2O_2 Efflux in *M. truncatula* Root Tissue

Intact root segments from *M. truncatula* seedlings were measured for changes in H_2O_2 efflux in response to purified Nod factor and a polysaccharide elicitor. The basal 1 cm of root tissue was excised from 36-h seedlings and pre-incubated for 1 h in buffered nodulation medium (BNM). Individual root segments placed in separate wells of a 96-well microtiter plate were assayed at 5-min intervals for 90 min in a fluorescence plate reader. Conversion of nonfluorescent substrate (Amplex Red) to fluorescent product (resorufin) through reaction with plant-derived H_2O_2 and endogenous plant peroxidases was measured using a standardized (sub-saturating) concentration of substrate (3.3 μM) and reported as accumulated fluorescence versus time (Fig. 1) or percentage of control fluorescence at 80 min (Figs. 2 and 3). Untreated root segments produced a nearly linear increase in accumulated (baseline) fluorescence over the 90-min time course of the experiment, eventually slowing as substrate became limiting (Fig. 1A).

The addition of purified *S. meliloti* Nod factor (RmIV C16:2, S), applied over a biologically active concentration range from 10 pM to 10 nM (Shaw and Long, 2003), resulted in a dose- and time-dependent effect on H_2O_2 efflux (Fig. 1, A and B). Root segments showed no discernible response at 10 pM. At 100 pM and 1 nM Nod factor, a small increase was observed over baseline in the first 15 min ($P < 0.05$ at 1 nM Nod factor at 10 min). This was followed by a significant decrease, relative to untreated plants, in the accumulation of fluorescence beginning 30 min after Nod factor addition (Fig. 1B). Increasing the Nod factor concentration to 10 nM resulted in a measurable increase in accumulated fluorescence at 15 min ($P < 0.01$), followed by suppression, again beginning at 30 min.

Oligogalacturonides elicit increases in reactive oxygen evolution from many plant families, including legumes (Levine et al., 1994; Taylor et al., 2001); we used them here as a control for elicitation of peroxide efflux in the excised root segments. PGA heptamers applied at ≥75 nM (0.1 μg mL^{-1}) rapidly increased H_2O_2 efflux in a dose-dependent fashion relative to baseline levels (Fig. 1, A and C). PGA treatment results in significant depletion of (sub-saturating) substrate during the time course of the assay resulting in the tapering of accumulated fluorescence values. These data demonstrate that the root segments are competent to respond to the elicitor with a rapid increase in peroxide generation typical for the initial phase of the pathogenic oxidative burst response.

Assay Controls and Parameters

Our goal in constructing this assay was to examine peroxide evolution from the legume tissue that responds to nodulating bacteria in other assays (e.g. calcium spiking, root hair deformation, and gene expression). To test that the root segments respond to Nod factor, root hair cells on excised root segments were assayed for the calcium spiking response (Ehrhardt et al., 1996; Wais et al., 2000). Root segments from *M. truncatula* were pre-incubated in BNM for 1 h before iontophoretic microinjection with calcium-green dextran and subsequent imaging as previously described (Wais et al., 2000). Calcium spiking in response to 1 nM Nod factor was recorded for six of eight cells (data from three different plants), demonstrating that root hair cells on excised tissue is competent to respond to Nod factor (data not shown).

BNM was used for this assay to facilitate comparisons with other assays (e.g. nodulation tests and calcium spiking response). Several properties of the buffering conditions and the nonfluorescent substrate were tested to evaluate their possible effects on the assay. Conversion of nonfluorescent substrate to fluorescent product in the absence of plant tissue, with or without Nod factor or PGA, was <1% of plant baseline substrate conversion (data not shown). Addition of Nod factor or PGA to plants in the absence of substrate produced no change in fluorescent signal (data not shown). Plants assayed in BNM and 10 nM resorufin (fluorescent product with no Amplex Red substrate) showed a <1% change in fluorescence over 90 min in all three cases: untreated, 1 nM Nod factor, or 75 nM PGA (data not shown).

Titrating buffer pH from 6.5 to 7.0 resulted in a approximately 10% reduction in total fluorescence relative to controls (Fig. 2, A and B). However, the change in fluorescence for Nod factor- or PGA-treated plants for assays at pH 7.0 was not significantly different from results obtained at pH 6.5. Similar to pH change, a comparison of assay results from trials with and without 50 nM $FeSO_4$ in the BNM indicated that the iron in the medium had little effect

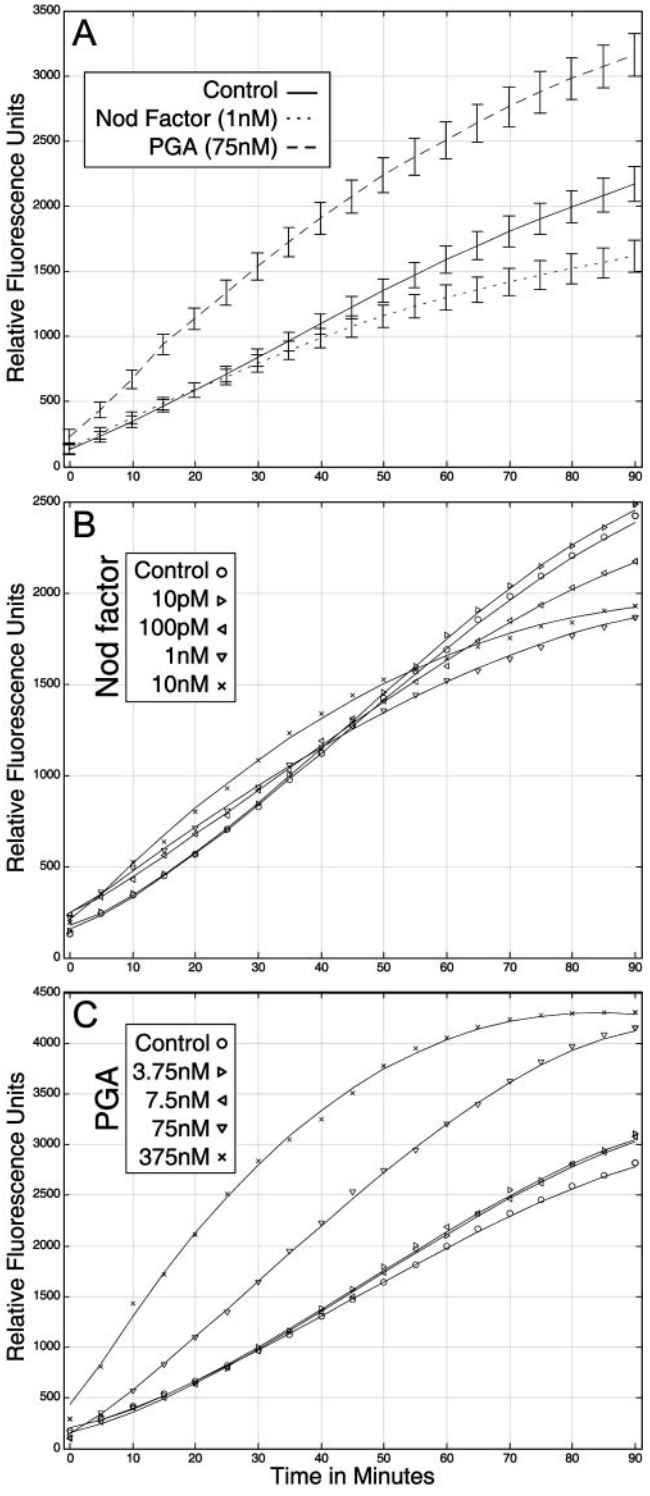

Figure 1. Modulation of peroxide efflux by Nod factor and oligouronide elicitor. Generation of H_2O_2 in plant root segments was measured by the conversion of nonfluorescent substrate to fluorescent product in the presence of endogenous plant peroxidases. A, Accumulated fluorescence measured at 5-min intervals for untreated (n = 101 plants pooled from 16 experiments), 1 nM Nod factor-treated (n = 97), and 75 nM PGA-treated (n = 91) plant segments presented as mean fluorescence. Error bars = three SDs of the mean. B, Nod factor, applied at four different concentrations, shows a retardation response beginning at 20 to 30 min at 100 pM and higher concentrations. Note initial elevation of activity at 10 nM. C, PGA heptamers, applied at four different concentrations, elicit activity at concentrations above 7.5 nM (n > 11 plants from two separate experiments for all experiments in B and C; error bars omitted for clarity).

on the assay results (Fig. 2C). We conclude from these experiments that the conversion of nonfluorescent substrate to fluorescent product occurs only in the presence of plant tissue and that relative changes in fluorescence observed when plants are treated with Nod factor and PGA do not occur because of plant-induced changes to medium pH.

Root segments cut from germinated seedlings are expected to show an initial wounding response that may include increases in H_2O_2 efflux. To verify that the responses to Nod factor and PGA were not caused by or requiring of a wounding response, we tested intact seedlings at a single time point. Whole seedlings were assayed by pre-incubating the root in BNM for 1 h followed by incubation in 400 μL of (3.3 μM) Amplex Red substrate and either Nod factor (1 nM) or PGA (75 nM). After 80 min, 200 μL of assay medium was transferred to a 96-well plate and immediately measured for fluorescence (Fig. 2D). Values for intact seedlings were nearly identical to those obtained using excised root segments. The contribution of injury during assay was tested by brutalizing root segments with forceps for 5 s just before assay (Fig. 2E). Damaging the root tissue immediately before assay led to an increase in peroxide efflux but did not prevent either Nod factor or PGA from achieving respective effects. The intrinsic baseline activity and the divergent responses to Nod factor and PGA in whole (uncut) seedlings argues that wounding can contribute to total assay activity but is not responsible for the baseline output or for the results obtained with either effecter molecule.

Given the 20- to 30-min lag between exposure to Nod factor and slowing of H_2O_2 efflux, we tested the potential role of new protein synthesis using cycloheximide, a protein synthesis inhibitor. Root segments were pre-incubated for 2 h in 5 μM cycloheximide before transfer to plates containing the assay substrate, Nod factor or elicitor treatment, and the same level of cycloheximide. Cycloheximide treatment alone elevated the levels of H_2O_2 efflux for all samples tested relative to solvent controls (Fig. 2F). Nod factor showed no ability to suppress H_2O_2 efflux in root segments treated with cyclohexamide. Moreover, PGA (75 nM) still stimulated activity, indicating that the root segments remained competent to modulate peroxide efflux.

Nod Factor Specificity

The specificity for Nod factor's effect on suppressing H_2O_2 efflux was tested using two compounds

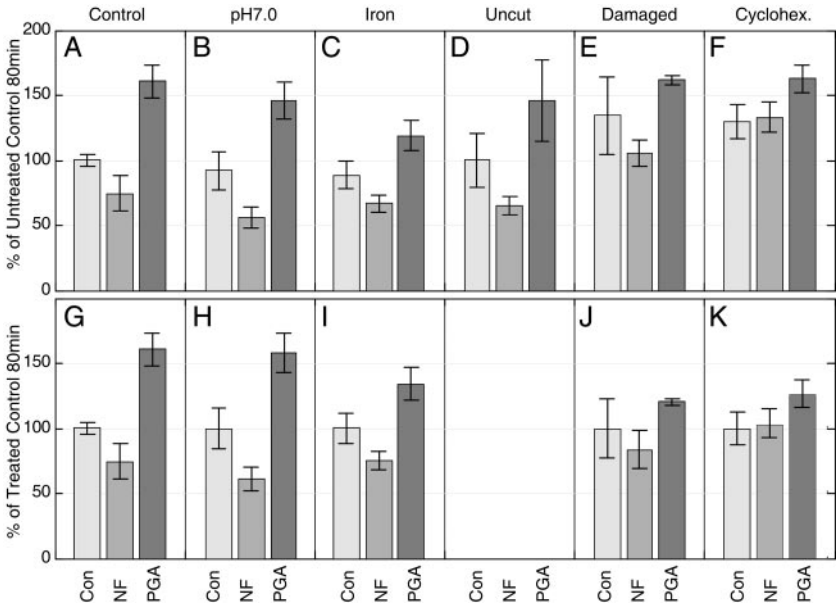

Figure 2. Effect of medium composition and wounding on peroxide response. The effects of buffer conditions and plant damage were evaluated for control (light gray), 1 nM Nod factor-treated (medium gray), and 75 nM PGA-treated (dark gray) plants. Results are presented as the percentage of both untreated control (A–F, upper) and treated control (G–K, lower) at 80 min. Error bars = SD of the mean ($n > 11$ plants for all experiments). Titration of buffer pH from 6.5 to 7.0 decreased total fluorescence relative to controls at pH 6.5 (B) but had little effect on the percent changes relative to the treated control (H). Removal of iron from the medium had little effect on suppression of H_2O_2 generation by Nod factor and only mildly inhibited induction by PGA (C and I). Nod factor and PGA modulate peroxide generation in intact (uncut) seedlings (D) similarly to (excised) root segments (A). Damage to root segments immediately before assay results in an increased total activity (E) that is still modulated by Nod factor and PGA (J). Pre-incubation for 2 h with 5 μM cyclohexamide elicited an increase in total peroxide efflux that Nod factor failed to suppress.

structurally related to Nod factor. Application of either 1 nM sulfated chitotetraose (S-CT4) or 1 nM Nod factor lacking the reducing end sulfate required for host specificity ($NodH^-$ factor) had no measurable effect on efflux when compared with baseline levels of untreated plants (Fig. 3A). Experiments at higher Nod factor analog concentrations were not performed due to limited quantities of the reagents.

Specificity for the host legume was tested by assaying a nonlegume, tomato (Solenaceae), for H_2O_2 efflux in the presence of 1 nM Nod factor or 75 nM PGA (Fig. 3B). Tomato (VF-36) root segments from 4-d-old seedlings were prepared in exactly the same manner as *M. truncatula* root segments. The application of Nod factor resulted in no significant deviation (at $P = 0.05$) from baseline, whereas PGA induced a striking response (Fig. 3B).

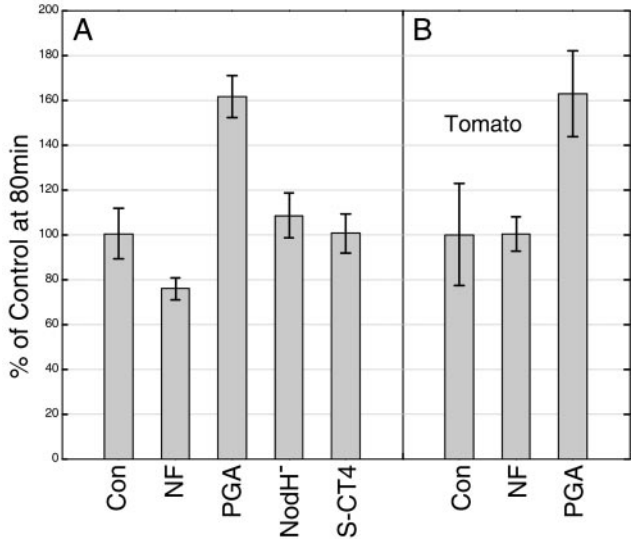

Figure 3. Specificity for host and symbiont. Two compounds structurally related to Nod factor, one lacking the reducing end sulfate ($NodH^-$) and one consisting of the sulfated *n*-acetyl-glucosamine backbone (S-CT4), failed to modulate peroxide efflux when presented at 1 nM final concentration (A). Addition of Nod factor (1 nM) to tomato (*Lycopersicon esculentum*), a nonlegume, had no discernable effect, whereas addition of PGA (75 nM) resulted in a typical increase in peroxide efflux (B). Data are percentage of untreated control plant (fluorescence) at 80 min; error bars = SD of the mean ($n > 11$ plants for all experiments).

Nod Factor Retards Elicitor Induction of Peroxide Efflux

The possibility that Nod factor treatment blocks the action of the carbohydrate elicitor was tested by pretreating plants with Nod factor and subsequently exposing them to PGA. To better approximate the rate of H_2O_2 production, root segments were treated and assayed in a saturating concentration of substrate (66 μM) at 1-min time intervals. Rates were

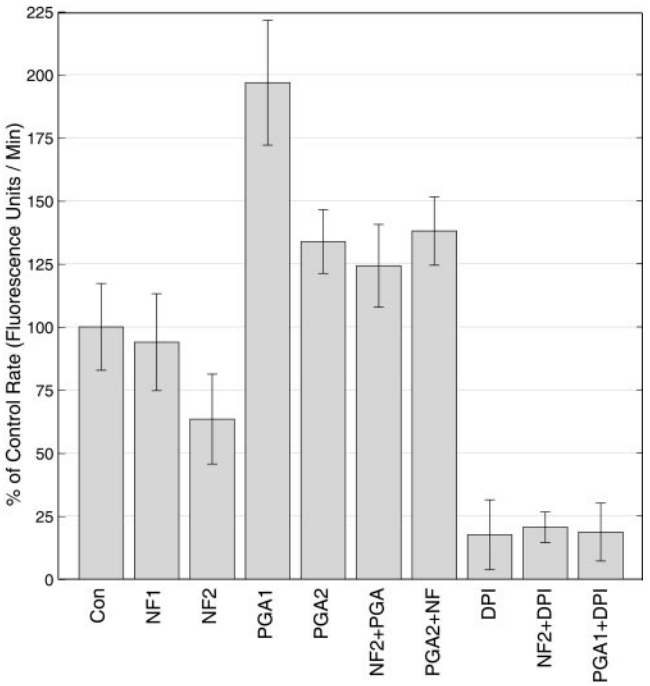

Figure 4. Nod factor interferes with PGA elicitation through a pathway that likely includes the NADPH-oxidase complex. The relative rate of H_2O_2 efflux was assessed using a saturating concentration of nonfluorescent substrate. The percentage of untreated (control) rate was calculated for 1 nM Nod factor-treated and 75 nM PGA-treated plants immediately after treatment (NF1 and PGA1) or after 1 h of pretreatment (NF2 and PGA2). Plants pretreated with either Nod factor or PGA were assayed immediately after the addition of the companion compound (NF2+PGA and PGA2+NF). Rates of H_2O_2 production were also measured for Nod factor- and PGA-treated plants in the presence of the NADPH-oxidase inhibitor, 10 μM diphenylene iodonium (DPI).

calculated as the mean change in fluorescence per minute over a 30-min time period after exposure to substrate. Root segments were either assayed immediately after treatment (NF1 and PGA1 from 5–35 min) or after a 60-min pretreatment and addition of the companion treatment (NF2 and PGA2 from 65–90 min).

Rate approximations were first obtained for untreated, Nod factor-treated, and PGA-treated plants (Fig. 4). Nod factor (1 nM), during the first 30 min of incubation, showed little effect on activity, whereas plants at 60 min produced only 60% of the baseline activity of untreated plants, consistent with experiments using sub-saturating substrate levels (compare Fig. 1A at 15 and 80 min with Fig. 4, NF1 and NF2). PGA (75 nM), assayed in the first 30 min, elicits a 200% increase in peroxide efflux rate compared with untreated plants, dropping to a 140% increase in rate by 60 min after PGA addition.

Pre-incubation with 1 nM Nod factor for 60 min followed by PGA (75 nM) addition resulted in an increased rate of peroxide efflux relative to both untreated and Nod factor-pretreated plants (Fig. 4). Comparing plants treated with Nod factor and then PGA (NF2-PGA1) with untreated plants (Con), the PGA stimulates the absolute level of peroxide production to only 125% of the control value instead of >200% when PGA alone (PGA1) is applied to untreated plants. If the plants treated with Nod factor and then PGA are instead compared with plants that have seen only Nod factor (NF2), the level of PGA stimulation is then about 200%. Hence, Nod factor does not block the carbohydrate elicitor peroxide response but does markedly interfere with the ability of the elicitor to increase the absolute peroxide efflux rate.

Inhibition of NADPH Oxidase Activity with DPI

We hypothesize that the plasma membrane NADPH oxidase complex serves as the primary generator of oxidative radicals being assayed from the root segments. To test this hypothesis, DPI, an inhibitor of NADPH oxidase activity, was used to assess what fraction of assay activity could be attributed to the NADPH oxidase complex (Fig. 4). DPI at 10 μM lowered the baseline rate to 20% of control plants. Addition of DPI to plants either pretreated with Nod factor for 1 h or treated directly with PGA resulted in suppression of peroxide efflux rates to 20% of the untreated control plants. This result suggests that the NADPH oxidase complex is responsible for the increase in efflux observed for PGA addition and for the decrease in efflux, seen with Nod factor exposure. The conversion of substrate to fluorescent product is specific in this assay for H_2O_2 (Mohanty et al., 1997) and requires the presence of endogenous plant peroxidases. We hypothesize that the residual activity in the presence of DPI, 20% of baseline rate, likely comes from the nonspecific conversion of substrate by peroxidases resident in the cell wall.

Analysis of Early Nodulation Mutants

Mutants defective for nodule formation (nod$^-$) have been characterized for gene expression, intracellular calcium responses (i.e. calcium flux and calcium spiking), and the degree of root hair deformation in the model legume, *M. truncatula* (Catoira et al., 2000, 2001; Wais et al., 2000; Ben Amor et al., 2003; Shaw and Long, 2003). Available nod$^-$ mutants were tested for changes in peroxide efflux in response to Nod factor and PGA. The *nfp* mutant, which shows no measurable responses to Nod factor (Ben Amor et al., 2003), showed no response to 1 nM Nod factor in this assay for H_2O_2 production (Fig. 5). In contrast, activity levels for the PGA elicitor in the *nfp* mutant were indistinguishable from wild-type controls. The *dmi1* and *dmi2* mutants, which are defective for calcium spiking (Catoira et al., 2000), exhibit a suppression of peroxide efflux with 1 nM Nod factor and an increase with 75 nM PGA (Fig. 5). These data dem-

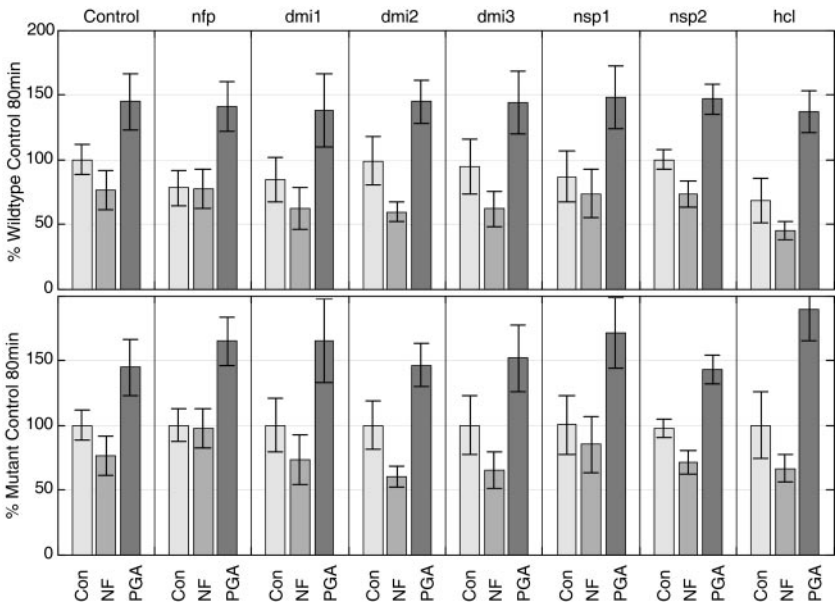

Figure 5. Peroxide retardation response in nod$^-$ mutants. Seven *M. truncatula* mutants that did not make nodules were compared for H_2O_2 efflux in the presence of 1 nM Nod factor or 75 nM PGA. Data are presented as both percentage of (untreated) wild-type control and (untreated) mutant control at 80 min postexposure ($n > 21$ plants from >two experiments for each mutant tested). Note that the *nfp* mutant shows no response to Nod factor, whereas the *dmi1* and *dmi2* mutants show marked suppression of peroxide efflux. The *hcl* mutant appears suppressed for all peroxide efflux, relative to wild-type plants, but does not appear impaired for the peroxide retardation response.

onstrate that the suppression of peroxide efflux is genetically separable from the calcium spiking response. Other mutants, including *dmi3*, *nsp1*, *nsp2* (Oldroyd and Long, 2003), and *hcl* all show suppression of peroxide efflux in the presence of 1 nM Nod factor and an increase when treated with 75 nM PGA. Although experiments with wild-type plants showed very consistent changes in peroxide efflux, both within and between experiments, *hcl* mutants exhibited highly variable responses to Nod factor and a generally suppressed production of H_2O_2.

DISCUSSION

Nod Factor Does Not Elicit an Early Oxidative Burst Response

The original goal of this work was to determine if the chitin-based Nod factor molecule, required for the rhizobia-legume symbiosis, triggers changes in reactive oxygen generation similar to those characterized in plant-pathogen interactions (for review, see Lamb and Dixon, 1997; Grant and Loake, 2000). Reports indicating rhizobia-dependent changes in reactive oxygen levels at the time of infection in *M. truncatula* suggest a possible role for Nod factor in modulating the defense response (Bueno et al., 2001; Santos et al., 2001; Ramu et al., 2002). We found that Nod factor accelerates H_2O_2 efflux only slightly when compared with the action of PGA, a known elicitor of the defense-related oxidative burst. The Nod factor-dependent increase in peroxide efflux was observed only at relatively high agonist concentrations (see below) and is reversed within 20 min. From these data, we conclude that the initial response of *M. truncatula* seedlings to Nod factor does not involve the oxidative burst observed during pathogen response.

Previous studies showing an increase in reactive oxygen species during the *rhizobia*-legume interaction have focused on later time points in the development of the symbiosis. Indications from nitroblue tetrazolium staining of whole plants at 12 to 24 h postinoculation suggest an increase in superoxide concentration in the root (Ramu et al., 2002). These data support the Nod factor-induced up-regulation of the peroxidase gene, RIP1, beginning 12 to 24 h postinoculation (Cook et al., 1995). Most interestingly, the increased peroxide staining was not observed for the nodulation defective mutant, *dmi1*, when exposed to rhizobia. Although the results of Ramu et al. (2002) stand in apparent contradiction to those presented here, we expect that the differences are attributable to the different time points in the development of the symbiosis being investigated. At 12 to 24 h postinoculation, bacteria are physically infecting the root hair cells of the plant, a process that is blocked in the *dmi1* mutant. The first 2 h postinoculation are marked by the induction of cytoplasmic calcium changes and alterations in root hair growth, both of which are observed in the *dmi1* mutant to a limited degree (Catoira et al., 2000; Shaw and Long, 2003). Hence, the modulation of reactive oxygen efflux may play different roles during different developmental stages of the symbiotic interaction.

Nod Factor Suppression of Peroxide Efflux

Application of purified Nod factor to *M. truncatula* root segments had the surprising effect of slowing the rate of H_2O_2 output to 60% of baseline levels. Suppression commenced 20 to 30 min after Nod factor application and occurred at concentrations known to elicit gene expression and calcium spiking (Cook et al., 1995; Wais et al., 2000). Application of

Nod factor structural analogs had no detectable effect on peroxide efflux, and no response was observed in tomato plants exposed to 1 nM Nod factor. We also observed that the nod$^-$ *nfp* mutant showed no suppression of peroxide efflux rates in the presence of Nod factor but responded to PGA. These results indicate that Nod factor is specific for the downregulation of H_2O_2 efflux in root tissue of a host plant.

Using DPI, an inhibitor of NADPH oxidase activity, H_2O_2 efflux rates were reduced to 20% of baseline values, suggesting that the NADPH oxidase complex is responsible for the majority of peroxide efflux measured in this assay. We suspect that the residual assay activity comes mostly from the nonspecific turnover of substrate by cell wall peroxidases, with a lesser contribution coming from cellular metabolism. This supposition is based on turnover of Amplex Red in the presence of exogenous horseradish peroxidase and several attempts to quantify substrate conversion in nonliving plant material using freeze-dried root segments (data not shown).

The NADPH oxidase complex is the likely source for the majority of reactive oxygen species produced in response to pathogenic elicitors (Xing et al., 1997; Sagi and Fluhr, 2001). Consistent with this hypothesis, DPI inhibited the acceleration of H_2O_2 production by PGA, presumably by directly inhibiting NADPH oxidase activity. Creation of superoxide radicals by the NADPH oxidase complex yields an increase in H_2O_2 through the action of hydrogen peroxidases in the cell wall. If Nod factor was exerting its effect by inhibiting these enzymes, then the level of assay activity observed for Nod factor-pretreated plants subsequently exposed to DPI would be less than that for DPI treatment alone. Nod factor pretreatment failed to further suppress assay activity in DPI-treated plants and, further, prevented PGA from fully stimulating H_2O_2 efflux. These results suggest that Nod factor is acting in a pathway that downregulates NADPH oxidase activity or capacity and not through the inhibition of other enzyme activity in the cell wall.

The 20- to 30-min lag between Nod factor exposure and the observation of the peroxide retardation response suggests a possible requirement for new protein synthesis in the peroxide modulation response. Although cycloheximide blocked the ability of Nod factor to retard H_2O_2 production, the drug treatment itself led to an increase in peroxide generation. PGA induced efflux to levels above that seen with cycloheximide alone, indicating that PGA does not require new protein synthesis to modulate efflux rates. Although these results provide limited evidence that new protein synthesis could be required for the retardation effect, the stimulation of peroxide production (i.e. the variable we are testing) by cycloheximide strongly limits the interpretation of these data.

An Early Branch in the Nod Factor Signaling Pathway

Plant mutants lacking the ability to make nodules show a hierarchy of responses to Nod factor. The relationships between existing nod$^-$ mutants have been based on assays for calcium spiking response, cellular growth defects, gene expression, and infection competence (Catoira et al., 2000). The newly discovered *nfp* mutant shows no measurable responses to Nod factor when calcium flux, calcium spiking, or growth defects are examined (Ben Amor et al., 2003). Of the seven nod$^-$ mutants tested, only the *nfp* mutant showed no retardation of peroxide efflux. These results suggest that the wild-type gene encoded by the *nfp* locus is required for linking Nod factor perception to H_2O_2 efflux and that triggering the retardation of peroxide efflux is a very early Nod factor response.

Two results indicate that the peroxide response and the calcium spiking response are independent apart from the requirement for *nfp*. Treatment with 10 pM Nod factor had no effect on peroxide efflux, even though it initiates the calcium spiking response (Ehrhardt et al., 1996; Oldroyd et al., 2001). Further, peroxide retardation was observed for two mutants (*dmi1* and *dmi2*) that are blocked for calcium spiking (Catoira et al., 2000). These data demonstrate that the peroxide retardation response is not in the same pathway as, and is not dependent upon, the calcium spiking response.

Collectively, these data suggest that a branch appears early in the Nod factor signal transduction pathway. Low concentrations of Nod factor ($<$10 pM) trigger calcium spiking but do not elicit a calcium flux response (Shaw and Long, 2003) or the peroxide retardation response. Higher concentrations of Nod factor ($>$100 pM) result in a calcium flux and peroxide efflux retardation, even in the *dmi1* and *dmi2* mutants that do not show calcium spiking. This may represent high- and low-affinity branches of the signal transduction pathway downstream of *nfp* control.

Possible Roles for Peroxide Modulation

The best characterized role for plant peroxide generation comes from studies of the plant defense response (for review, see Lamb and Dixon, 1997). Increases in superoxide and H_2O_2 levels lead to increased cross-linking of proteins in the cell wall and an increase in cell wall strength (Bradley et al., 1992; Brisson et al., 1994). In addition, both superoxides and H_2O_2 can induce defense-related gene expression (Delledonne et al., 2001). Less well understood is the role of basal peroxide production. Basal peroxide production levels were high in the distal root tip of the plant (S.L. Shaw, unpublished data), suggesting that peroxide generation is high in regions of the plant experiencing growth. If peroxides are used to cross-link cell wall materials as a normal facet of cell growth, the Nod factor-elicited retarda-

tion of peroxide efflux could help explain the deformations of growing root hair cells after exposure to Nod factor. A possible link between these two phenomena currently is being investigated (S.L. Shaw, unpublished data).

Pretreatment of plants with Nod factor had a significant effect on the ability of PGA to increase the rate of peroxide efflux: PGA only increased the efflux rate to 125% of baseline, in comparison with 200% for plants not pretreated with Nod factor. It must be pointed out, however, that PGA stimulated the Nod factor pretreated cells from about 60% of baseline levels to 125%, or a roughly 200% change in output rate. Thus, PGA causes a substantial change in the rate of peroxide efflux even in Nod factor-pretreated plants, but the net rate of efflux is only 25% higher than the baseline efflux from untreated plants. Future studies will address the potential effects of modulating peroxide efflux on growth, the symbiotic interaction, and triggering a defense response.

In conclusion, we have demonstrated using purified Nod factor that instead of the oxidative burst, found during pathogenic interactions, reactive oxygen production actually decreases in the presence of the symbiotic signal. Further, Nod factor interferes with the pathogenic elicitor when both signals are present. Given the likelihood that plants in the soil will be attacked by pathogens while undergoing permissive infection by symbiotic bacteria, it is anticipated that some aspects of the defense response will have to be ameliorated, at least locally, for successful nodulation to occur. Slowing the plant peroxide efflux rate in the presence of 100 pM to 1 nM Nod factor could help to permit the changes in cell wall growth required for root hair curling and infection, even under the pressure of pathogenic attack. Further, reducing the amount of peroxide generation may also prevent incidental triggering of defense response genes during permissive infection.

MATERIALS AND METHODS

Plant Growth

Seed from wild type and mutant *Medicago truncatula* (Jemalong) were treated with 70% (v/v) ethanol/water for 40 min, rinsed twice in sterile water, and sterilized with commercial bleach for 40 min. After rinsing in sterile water, seed were imbibed overnight at 4°C in water and germinated overnight in inverted petri dishes. Seedlings were placed on to 1% (w/v) agarose containing BNM (Ehrhardt et al., 1992) and grown overnight at 24°C in the dark. Tomato (*Lycopersicon esculentum*) seed (VF36, Tomato Germplasm Repository, Davis, CA) were imbibed and germinated in an identical manner. Nodulation factor NodRmIV(C16:2, Ac, and S) was purified from *Sinorhizobium meliloti* strain Rm1021.

Assay for Peroxide Efflux

Amplex Red (Molecular Probes, Eugene, OR) at 3.3 or 66 μM in BNM was made fresh from 20 mM dimethyl sulfoxide stocks. A total of 185 μL of reagent was aliquoted into each well of a 96-well microtiter plate (Microfluor2 Black, Thermo Labsystems, Franklin, MA) before addition of treatments, bringing the final volume to 200 μL. Nodulation factor NodRmIV(C16:2, Ac, and S) was diluted from 100 nM stocks in water. PGA heptamers (kind gift of Joss Rose and Mike Hahn, Complex Carbohydrate Research Center, Athens, GA) were diluted from 1 mg mL^{-1} stocks in water. Sulfated chitotetraose and Nod factor lacking the reducing end sulfate group (kind gifts of Dr. Jean Dénarié, Toulouse, France) were diluted from 10 nM stocks in water. Resorufin (Molecular Probes) was diluted from 100 μM stocks in water. Cycloheximide (Calbiochem-Novabiochem, San Diego) was diluted from 5 mg mL^{-1} stocks in dimethyl sulfoxide to final concentrations of 5 μM and 4 mM, respectively.

For assay of plant segments, the basal 1 cm of each of 32 seedling was cut, placed immediately into 5 mL of BNM, and allowed to equilibrate in the dark for >1 h. Plant segments were placed into individual wells of the microtiter plate just before reading. Segments were cut to be wider than the well diameter to prevent movement during assay. Transfer of plant material to plates took approximately 6 min, resulting in a slight broadening of the assay values.

Plates were read at 5- or 1-min intervals for 90 min using a Gemini fluorescence plate reader (Molecular Dynamics, Sunnyvale, CA). The plate was shaken for 2 s before each read. Dye was excited at 544 nm and read through a 570-nm dichroic mirror and a 590-nm high-pass filter. The machine was set to medium sensitivity and took the mean of six individual reads as specified by the manufacturer.

Data Analysis

Data were exported to Excel (Microsoft, Redmond, WA) and Matlab version 7 (The Mathworks, Waltham, MA). Each treatment (e.g. Nod factor or PGA) was given to sets of six or eight plant segments per experiment, and experiments were repeated more than two times. The trials reported refer to the number of plant segments tested and used in the analysis. For instance, two experiments could consist of 16 (two $\times$ eight plant segments) data points for the control and 16 data points for Nod factor. Data were normalized between experiments by conversion to percentage of the mean of the control set for each experiment. Error estimation was calculated from normalized values including the error estimate for the control set. Histograms of values where $n > 30$ appeared to be normally distributed. Student's two-tailed t test for a difference in mean values was applied using functions in Excel or Matlab.

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