Nod Factor Induction of Reactive Oxygen Species Production Is Correlated with Expression of the Early Nodulin Gene *rip1* in *Medicago truncatula*

Senthil K. Ramu,¹ Hui-Mei Peng,¹ and Douglas R. Cook²

¹Department of Plant Pathology and Microbiology, Texas A&M University, College Station, 77843-2132 U.S.A.;

²Department of Plant Pathology, One Shields Avenue, University of California, Davis, 95616 U.S.A.

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Plant genes that are specifically activated by the rhizobial lipochitooligosaccharide signal molecule (Nod factor) in legume hosts are collectively referred to as nodulins. Although nodulin gene expression is both spatially and temporally correlated with symbiosis, the function of these genes and the molecular events underlying their expression remain unknown. Sequence analysis of *rip1*, an early nodulin gene encoding a putative peroxidase protein, revealed the existence of sequence motifs with homology to reactive oxygen species (ROS) responsive *cis* elements. Here we report that recognition of compatible Nod factor rapidly stimulates a spatially localized production of reactive oxygen species in legume roots. *Sinorhizobium meliloti* mutants that produce an altered Nod factor structure and a nonnodulating plant mutant, *dmi1-1*, that is implicated in Nod factor signal transduction are equally impaired in the ability to elicit ROS production and *rip1* expression. Interestingly, both *rip1* transcription and ROS production exhibit the same tissue-specific pattern of localization. Moreover, exogenous hydrogen peroxide is sufficient to activate *rip1* transcription. Taken together, these results suggest that ROS production is a consequence of specific Nod factor perception and implicate H₂O₂ produced during this response as a mediator of Nod factor-induced *rip1* expression.

The symbiotic interaction between rhizobia and leguminous plants results in the development of a root nodule, a specialized plant organ in which nodule-inducing bacteria and their legume host conduct symbiotic nitrogen fixation (Long 1996). Rhizobial *nod* genes specify the synthesis of a lipochitooligosaccharide signal molecule called Nod factor (Dénarié et al. 1996). Nod factors play a pivotal role in the initial stages of symbiosis and are capable of eliciting symbiotic gene expression and nodule morphogenesis on legume hosts with the same specificity as the rhizobial symbiont (Dénarié et al. 1996). Purified Nod factors induce plant responses that are reminiscent of signaling events in other eukaryotic systems. For example, in root hairs of *Medicago* species, Nod factors produced by compatible *Sinorhizobium meliloti* induce plasma membrane depolariza-

tion (Erhardt et al. 1992), intracellular alkalization (Felle et al. 1996), and calcium spiking (Erhardt et al. 1996). These inducible responses occur within minutes to hours of Nod factor application and may be part of a series of cellular events that lead to symbiotic development (Downie and Walker 1999).

In addition to several electrophysiological and cellular changes, Nod factors specifically trigger the expression of plant genes referred to as nodulin genes in legume roots. Of the known nodulin genes, one of the most rapidly induced is *rip1*, encoding a peroxidase protein. Nod factor is both necessary and sufficient for *rip1* induction, and Nod factor-producing *S. meliloti* induces tissue specific expression of *rip1* that is tightly correlated with early symbiotic processes, first in the differentiating root epidermis and then in nascent nodule primordia (Cook et al. 1995; Peng et al. 1996).

Despite the importance of Nod factor as an elicitor of early symbiotic responses, including plant gene expression, the molecular mechanisms underlying Nod factor perception and signal transduction remain unclear. Genetic analyses of model legume systems, such as *Medicago truncatula* (Cook 1999) and *Lotus japonicus* (Handberg and Stougaard 1992), have recently led to the identification of several host genes that are required for proper symbiotic development (Penmetsa and Cook 1997; Catoira et al. 2001). In particular, a genetic pathway for Nod factor signal transduction has been proposed based on the identification of multiple alleles at four loci that are pleiotropic for a suite of Nod factor-induced responses in *M. truncatula* (Catoira et al. 2000; Wais et al. 2000).

In conjunction with these genetic analyses, we are investigating the mechanism of transcriptional activation of *rip1* by compatible *Sinorhizobium meliloti* and Nod factor. Based on sequence analysis of the *rip1* promoter, we identified three copies of a putative OCS (octopine synthase) element and eight copies of a putative OBF binding protein (OBP) element positioned within 500 bp of the *rip1* transcription start site (Peng et al. 1996) (Fig. 1). OCS and OBP motifs have been identified as *cis* regulatory elements of certain plant genes that are transcriptionally activated by H₂O₂ and, in several cases, the corresponding gene products function as protectants from oxidative damage or from pathogen ingress (Zhang et al. 1995; Chen et al. 1996). The existence of OCS- and OBP-like motifs in the *rip1* promoter prompted us to explore the possible involvement of ROS in *rip1* transcription during symbiosis.

Here, we present evidence that recognition of Nod factor can stimulate the tissue-specific production of superoxide radical, coincident with the temporal and spatial distribution of *rip1*

Corresponding author: Douglas R. Cook; E-mail drcook@ucdavis.edu; Telephone: +530.754.6561; Fax: +530.754.6617

Current address for S. K. Ramu: Department of Genetics, Harvard Medical School.

transcript during early nodulation. Using *S. meliloti* mutants producing an inactive structural variant of Nod factor and a plant mutant defective for early Nod factor-induced responses, a functional link was established between the Nod factor signal and elicitation of this ROS response. We have also determined that the exogenous application of H₂O₂ is sufficient to induce *rip1* expression. These results suggest ROS production is a consequence of specific Nod factor perception and implicate H₂O₂ produced during this response as a mediator of Nod factor-induced *rip1* expression.

RESULTS

Exogenous hydrogen peroxide is sufficient for *rip1* induction.

DNA sequence analysis of the *rip1* promoter region revealed the presence of putative OCS and OBP *cis* elements in close proximity to the transcription initiation site (Fig. 1). OCS and OBP *cis* elements are common in the promoters of plant genes that are induced by pathogens or pathogen elicitors. In such cases, these *cis* elements are implicated in transcriptional induction by reactive oxygen species (ROS) and, in particular, by H₂O₂ (Chen et al. 1996).

To ascertain whether the transcription of *rip1* is responsive to exogenous H₂O₂, we treated uninoculated roots with varying concentrations of H₂O₂ and assayed *rip1* transcript levels by Northern blot analysis. In parallel to the analysis of *rip1* expression, we monitored the induction of a glutathione-S-transferase gene (*gst*). GSTs function as an antioxidant (Daniel

1993) and, by analogy to other plant systems, GST gene expression should provide a reporter for H₂O₂-responsive transcription. As shown in Figure 2A, increasing levels of H₂O₂ were correlated with increased levels of *rip1* transcript. Moreover, in replicate experiments, both *rip1* and *gst* exhibited minimal induction in the range of 200 μ M H₂O₂ and maximum induction at 2 to 5 mM H₂O₂. These results are in agreement with the dose-dependent induction of *gst* transcript by H₂O₂ reported for *Arabidopsis* (Chen et al. 1996) and soybean (Levine et al. 1994).

Because both *S. meliloti* Nod factors (Cook et al. 1995) and H₂O₂ are sufficient for *rip1* induction, it is possible that early responses to Nod factor perception might include increases in ROS. As an indirect test of this hypothesis, we analyzed *gst* expression in roots 12 h after inoculation with wild-type *S. meliloti* or its Nod factor-deficient derivative SL44. Strain SL44 contains a deletion for nod genes *nodD1* to *nodABC* and is, therefore, null for Nod factor biosynthesis. As shown in Figure 2B, inoculation with wild-type *S. meliloti*, but not strain SL44, was correlated with increased *gst* transcript. Thus, in addition to induction of early nodulin genes such as *rip1*, Nod factor is likely to activate an endogenous antioxidant pathway.

S. meliloti Nod factor induces ROS production in the nodulation zone of *M. truncatula* roots.

The rapid induction of *rip1* by exogenous H₂O₂ and the Nod factor-dependent induction of *gst* in roots prompted us to explore the possibility that Nod factor can elicit ROS in legume roots. By analogy to plant responses to pathogen elicitors, the production of ROS may involve initial production of superoxide radical (O₂⁻) and its rapid enzymatic conversion into H₂O₂ by superoxide dismutase (SOD) (Levine et al. 1994). We monitored superoxide production *in situ* by treating roots with nitroblue tetrazolium (NBT), a histochemical reagent that reacts with superoxide to a blue formazan precipitate (Doke 1983), and we assessed the specificity of the assay for O₂⁻ based on its sensitivity to exogenous SOD. As shown in Figure 3A, the uninoculated root tip is a site of constitutive NBT reduction. Within 12 h following inoculation with wild-type *S. meliloti* the region of formazan staining had expanded to include tissue adjacent to the root tip, fully encompassing the zone of root hair differentiation (data not shown); by comparison, inoculation with a Nod factor-deficient derivative of *S. meliloti* (strain SL44) failed to increase the zone of formazan staining (Fig.

A	1	-459	tac ctcttttcg tcctt	-443	
	2	-428	t tc cggttttagtcctg	-412	
	3	-369	t tc ctcttttgatccct	-353	
	4	-296	tcc ca cttttggtccct	-280	
	5	-265	ct cc acttttggtccct	-249	
	6	-187	t tc ca cttttgg tcctt	-203	
	7	-127	t tc cggtttcagtcctt	-143	
	8	-96	tac ctcttttcg tcctt	-112	
Mt consensus			ttcncttttggtccct		
OBP1			tacac <u>ttttt</u> gg		
B	1	-336	gctgacgtga	-345	
	2	-323	aattacgtcc	-314	
	3	-220	gcttacgtgg	-214	
	OCS			gattacgtcc	
	alternate nt			g caa	

Fig. 1. Structure of OCS- (octopine synthase) and OBF binding protein-like sequence motifs 5' of the *rip1* transcription initiation site. **A**, Sequence of individual 17-bp repeats within the *rip1* promoter region that share sequence similarity with the 11-bp OBP1 binding site (OBS) of the *Arabidopsis* *gst6* promoter (Chen et al. 1996). Numbering corresponds to the position of each repeat relative to the transcription initiation site determined by Peng and associates (1996). Bolded nucleotides share identity with OBP1. Mt consensus = the consensus of the *Medicago truncatula* repeats. 'ctttt' corresponds to the footprint of OBP1 (Chen et al. 1996). **B**, Sequence of three 10-bp repeats within the *rip1* promoter region that share sequence similarity with the OBF5 binding site (OCS) of the *Arabidopsis* *gst6* promoter (Chen et al. 1996). Numbering corresponds to the position of each repeat relative to the transcription initiation site of *rip1*. Bolded nucleotides share identity with the OCS consensus. The tg(t)acg motif represents the DNase I footprint of OBF5 (Chen et al. 1996). The OCS consensus sequence is based on comparison of 10 pathogen promoters including CaMV 35S, NOS, and *ocs* promoters (Bouchez et al. 1989).

Fig. 2. Gene expression analysis. **A**, Effect of H₂O₂ on *rip1* and *gst* induction. Northern blots of total root RNA isolated from roots following treatment with various concentrations of H₂O₂ for 1 h are shown. Blots were successively probed with ³²P-labeled *rip1*, *gst*, and histone H3 as indicated. **B**, Analysis of *rip1* and *gst* expression in response to *Sinorhizobium meliloti* 1021 and its Nod factor-deficient derivative SL44. Autoradiographs are of gel blots containing total RNA isolated from roots after inoculation with *S. meliloti* 1021 (Rm 1021) or *S. meliloti* SL44. Rm1021 and SL44 RNAs were analyzed on the same membrane and represent identical exposure times. Blots were successively probed with ³²P-labeled *rip1*, *gst*, and histone H3 as indicated. Time is reported in hours postinoculation.

3A). Similar results were obtained with purified Nod factor, which also induced NBT reduction in the root tip proximal zone (Fig. 3B). Both the *S. meliloti*- and Nod factor-induced responses were effectively blocked by SOD (Fig. 3B), which should rapidly deplete O_2^- . These results suggest that the molecular species under assay is indeed O_2^- . Importantly, this Nod factor-induced response is restricted to the root tip proximal zone, where well-characterized Nod factor-specific responses are also localized (Journet et al. 1994; Pingret et al. 1998; Catoira et al. 2000), including the tissue-specific induction of *rip1* transcript (Cook et al. 1995).

Nodule primordia, which are first macroscopically evident on inoculated roots at 60 h postinoculation with *S. meliloti*, are also a location of *rip1* transcript (Peng et al. 1996). NBT reduction to formazan was readily detected in these nascent nodule primordia (Fig. 3C), and formazan formation was blocked by treatment with SOD. However, in contrast to the 12 h time point (Fig. 3A, 2011 panel) when NBT staining was widely distributed throughout the inoculated symbiotic zone, NBT staining at 60 h postinoculation was confined to nodule primordia and to vascular tissue (Fig. 3C, top panel). Thus, similar to *rip1* transcript (Cook et al. 1995; Peng et al. 1996), NBT staining is first evident throughout the symbiotically competent root zone and subsequently focused at sites of nodule initiation.

Superoxide accumulation is spatially coincident with *rip1* transcript.

The spatial distribution of *rip1* transcript in roots is closely correlated with early symbiotic processes (Cook et al. 1995; Peng et al. 1996). In uninoculated roots, basal levels of *rip1* transcript are associated with the root tip. By 3 h following inoculation with the bacterial symbiont, *rip1* transcript is observed within the differentiating root epidermis, the zone of impending infection by *S. meliloti*, and by 72 h postinoculation, *rip1* transcript is evident in nascent nodule primordia, the site of sustained bacterial infection.

To determine the precise spatial correlation between *rip1* transcript and superoxide production, we performed in situ hybridization and NBT staining on roots immediately before and 12 or 72 h after inoculation with *S. meliloti* and examined the tissue sections to compare the tissue specificity of both responses. As shown in Figure 4, *rip1* transcript and superoxide production were spatially coincident throughout all time points analyzed. In uninoculated roots, *rip1* transcript and superoxide production were localized to cells in the extreme root tip (Fig.

4A and B). At 12 h postinoculation with *S. meliloti*, prior to bacterial infection, *rip1* transcript and superoxide production were detected in the differentiating root epidermis and subtending vascular tissue (Fig. 4C and D). At 72 h postinoculation *rip1* transcript and superoxide were detected in nascent nodule primordia (Fig. 4E and F). Thus, there is a tight spatial correlation between the presence of *rip1* transcript and the detection of superoxide accumulation by means of NBT.

Specific aspects of Nod factor structure are required for elicitation of ROS production and *rip1* induction.

Nodulation of *Medicago* species by *S. meliloti* has been shown to have a requirement for specific aspects of Nod factor structure. In particular, the presence of a sulfate moiety at the reducing end is necessary for the induction of several host nodulin genes, root hair deformation, rhizobial infection, and the induction of nodule primordia on *Medicago* (Long 1996; Dénarié et al. 1996). To determine if the production of ROS and *rip1* induction share specificity for Nod factor structure, we assayed *rip1* transcript and superoxide production in plants inoculated with wild-type bacterium or with *S. meliloti nodH* mutants that produce Nod factors lacking the reducing-end sulfate (Roche et al. 1991). As shown in Figure 5, a Rm 2011 *nodH* mutant was unable to induce either *rip1* expression or superoxide production. Other well-characterized *nodH*-dependent host responses, including induction of the early nodulin gene ENOD12 and the formation of nodule primordia, were absent from similarly treated plants (data not shown). To confirm these observations, we analyzed *rip1* transcript and superoxide production following inoculation with a *nodH* mutant of a second strain of *S. meliloti*, Rm 1021, and we obtained similar results (data not shown). Thus, Nod factor induction of *rip1* transcript and superoxide elicitation have a similar stringent requirement for Nod factor O-sulfation, consistent with these responses being products of the same specific Nod factor perception pathway.

A symbiotic mutant of *M. truncatula* is defective for *rip1* induction and ROS production.

Recent genetic analyses in *M. truncatula* have identified numerous single gene mutations that affect the symbiosis with *S. meliloti*. Among these mutants are four loci that are pleiotropic for the absence of several early Nod factor-induced responses and, on this basis, they are proposed to be defective in Nod factor signal transduction (Catoira et al. 2000; Wais et al.

Fig. 3. Localization of superoxide accumulation in whole roots. **A**, Nitroblue tetrazolium (NBT) staining in the root tip proximal zone. Staining was performed 12 h postinoculation with *Sinorhizobium meliloti* (Rm) 2011, the *nodD1ABC* deletion mutant SL44, or mock-inoculated control. The blue formazan precipitate is indicative of superoxide radical accumulation. **B**, Nod factor induction of NBT staining and its inhibition by superoxide dismutase (SOD) in the root tip proximal zone. NBT staining was performed 12 h postinoculation. **C**, NBT staining in nodule primordia. Staining was performed 60 h postinoculation with *S. meliloti* 2011 with or without superoxide dismutase. np = nodule primordia.

2000). One such mutant, named *dmil-1* for “doesn’t make infections,” was tested for induction of superoxide production and *rip1* expression in response to compatible *S. meliloti*. As shown in Figure 6B, inoculation with *S. meliloti* was unable to elicit either superoxide production or *rip1* expression in the *dmil-1* mutant. In agreement with previous findings (Catoira et al. 2000), we found that *dmil-1* is also impaired for *enod12* and *enod40* expression (Fig. 6B). The lack of *S. meliloti*-induced superoxide production in *dmil-1* plants implies that the mutant is blocked at a stage upstream of ROS generation. To determine if *rip1* expression in *dmil-1* is also insensitive to exogenous H_2O_2 , we analyzed *rip1* transcript levels in noninoculated *dmil-1* plants following treatment with 1 to 5 mM H_2O_2 . Interestingly, exogenous H_2O_2 was still able to induce *rip1* expression in *dmil-1* roots (Fig. 6C).

DISCUSSION

We have shown that *rip1* transcription can be activated by exogenous H_2O_2 in the absence of Nod factor signal, with a dose response similar to that of other characterized ROS-responsive genes (Chen et al. 1996). Evidence for increases in ROS during symbiotic development comes from the observation that expression of an antioxidant gene, *gst*, is also increased during symbiosis in a Nod factor dependent manner. Interestingly, among the bacterial genes that are expressed during *S. meliloti* infection of *Medicago* species are those with potential roles in protection against reactive oxygen species (Oke and Long 1999), consistent with the possibility that the bacterial symbiont encounters oxidative stress in the plant environment. In support of this hypothesis, SOD-deficient *S. meliloti*

mutants are only slightly impaired in free-living growth but severely impaired in the ability to colonize host *Medicago* plants (Santos et al. 2000). We also provide direct evidence that application of Nod factor or inoculation with wild-type *S. meliloti* leads to an increase in ROS in the symbiotically competent root zone. This response is characterized by increases in O_2^- in the differentiating root epidermis and root vasculature, as well as in nodule primordia, coincident with the tissue specificity of *rip1* expression. These spatial and temporal patterns of O_2^- accumulation are tightly correlated with the known morphological and gene-expression changes that characterize early symbiotic development (Long 1996; Mylona et al. 1995).

The observation that *dmil-1*, a plant mutant that is blocked early in symbiotic development, is simultaneously affected for induction of ROS production and *rip1* expression further strengthens the correlation between these responses and symbiotic signaling. The fact that *dmil-1* plants retained the ability to respond to exogenous H_2O_2 by expressing *rip1* and *gst* suggests that the *dmil-1* gene functions at or upstream of ROS generation. Interestingly, the *dmil-1* mutant consistently displays elevated basal levels of *gst* transcript (Fig. 6C) as compared with near-isogenic wild-type plants, suggestive of an altered redox state or response in *dmil-1*. Additional genetic evidence from inoculation experiments with bacterial mutants suggests that *rip1* induction and ROS production may be products of the same specific Nod factor perception pathway. In particular, *S. meliloti nodH* mutants, which produce inactive Nod factors that lack a reducing-end sulfate substitution (Dénarié et al. 1996), were unable to induce either the ROS response or *rip1* expression in *M. truncatula* roots.

One can envision several possible roles for ROS during symbiosis. One possibility, for example, is that activation of ROS production is a lateral consequence of Nod factor perception, and *rip1*, being a peroxidase enzyme, could itself function in the metabolism of otherwise harmful H_2O_2 . Alternatively, ROS could fulfill a critical role in nodulation, for example, by providing oxidant for peroxidase-mediated cell wall modifications during *S. meliloti* infection and nodule organogenesis. Many of the known early nodulin genes, including *enod12* (Scheres et al. 1990), *enod10* (Lobler and Hirsch 1993),

Fig. 4. Spatial distribution of superoxide accumulation in tissues expressing *rip1* transcript. **A**, In situ hybridization of an uninoculated root tip (distal 2 mm) hybridized with *rip1* antisense probe. Note the intense signal uniformly distributed in all cell types. For all time points, negative control hybridizations using a sense-strand *rip1* probe did not produce signal above background (data not shown). **B**, Transverse section comparable to **A**, stained with nitroblue tetrazolium (NBT). The blue staining is taken to be indicative of superoxide radical accumulation. **C**, In situ hybridization of roots 12 h after inoculation with *Sinorhizobium meliloti* ABS7. Tissue sections were taken from the 1 cm root zone immediately adjacent to the root tip. *rip1* Signal is detected in the epidermis (e) and vascular tissue (v). **D**, Transverse section comparable to **C**, stained with NBT. **E**, In situ hybridization of 72-h nodule primordia (np). **F**, Transverse section comparable to **E**, stained with NBT.

Fig. 5. Effect of wild-type and *nodH* *Sinorhizobium meliloti* strains on induction of reactive oxygen species production and *rip1* expression. **A**, Nitroblue tetrazolium (NBT) staining of roots inoculated with *S. meliloti* 2011 (Rm2011) or the corresponding Nod factor mutant *nodH*. All images were taken 12 h postinoculation. **B**, Northern blots of RNA isolated from samples inoculated as in **A**. Northern blot and NBT staining analyses were conducted in parallel. The blot was probed with ^{32}P labeled *rip1*.

enod11 (Cook et al. 1995; Journet et al. 2001) and *MtPRP4* (Wilson et al. 1994), encode proline-rich proteins (PRPs) that are members of the extensin superfamily of cell wall proteins (Kieliszewski and Lampert 1994). With the notable exception of *enod12*, each of these nodule-specific PRPs include a series of tyrosine-containing pentameric motifs that have been implicated in cross-linking of cell wall proteins by a process that involves cell wall-bound peroxidases and H_2O_2 (Bradley et al. 1992). The oxidative cross-linking of cell wall proteins, potentially involving *rip1* peroxidase, could be important at sites of infection thread formation (Salzwedel and Dazzo 1993) or in dividing cells during nodule development. Consistent with this model, Santos and associates (2001) analyzed ROS production during later stages of nodule development (6 to 18 days postinoculation) and observed elevated levels of O_2^- and H_2O_2 at sites of *S. meliloti* infection. Finally, ROS might have a direct role in regulation of plant signaling proteins. Thus, Nod factor-induced changes in redox balance within symbiotically competent root cells could cause alterations in the activity of key signaling proteins, which in turn might activate pathways for *S. meliloti* infection and nodule development. Subtle changes in redox potential can have dramatic effects on protein folding and function (Gamaley and Klyubin 1999), including the activity of transcription factors and small GTP binding proteins, and redox potential may influence the phosphorylation poise that regulates diverse aspects of eukaryotic function. In the analyses presented here, wild-type *S. meliloti* or purified Nod factors consistently induced higher levels of *rip1* transcript than H_2O_2 relative to control untreated tissues. Although this result might imply that H_2O_2 acts indirectly to affect *rip1* transcript levels, interpretation of this result is complicated by the dissimilar nature of the various reactants, i.e., unstable H_2O_2 , ampipathic Nod factor, and living *Sinorhizobium meliloti*.

The rhizobium-legume symbiosis shares features in common with plant-pathogen interactions. These features include host genotype-specificity, induction of host genes by microbial elicitors, and redirection of host metabolism (Vance 1983; McKhann and Hirsch 1994). Hirsch and colleagues, in particular, have advanced the hypothesis that, in addition to symbiotic

responses, rhizobial Nod factors trigger host defense responses in a manner analogous to pathogen elicitors (McKhann and Hirsch 1994). Interestingly, several studies have shown that pathogen elicitors (specific pathogen avirulence products and fungal oligosaccharides) can induce many of the same physiological responses induced by Nod factor (Downie and Walker 2000; Kikuyama et al. 1997; Kuchitsu et al. 1995, 1997; Lamb and Dixon 1997), including membrane depolarization, transient extracellular alkalization, ion fluxes, and the induction of host response genes. The present data extend this parallel and suggest an additional common element, namely the induction of ROS production in response to specific Nod factor signals.

An important goal for future research will be to determine if the similarities between the proposed events of legume Nod factor signal transduction and plant responses to pathogen elicitors share common genetic elements or if separate but related components are operative in the two cases. Activation of early nodulin gene *enod40* by chitin pentamer is suggestive of cross talk between these two phenotypically distinct pathways (Minami et al. 1996). Conversely, nonlegume species can respond to Nod factors by induction of plant gene expression (Reddy et al. 1998; Staehelin et al. 1994). Whether H_2O_2 might also be sufficient for induction of other early nodulin genes, such as *enod12* and *enod40*, has not yet been tested, although the nodulation mutant *dmi1-1* failed to increase the corresponding transcripts in response to Nod factor or *S. meliloti* treatments. An intriguing possibility is that the response of legumes to Nod factor may be derived from an ancient conserved pathway for plant responses to certain classes of pathogen elicitors. It will be important to examine ROS and related phenotypes in other nodulation mutants and, ultimately, the cloning of plant genes operative in the symbiotic signaling pathway, such as *dmi1*, will be crucial to resolving these issues.

MATERIALS AND METHOD

Plant material, bacterial strains, and growth conditions.

M. truncatula ecotype A17 and its near-isogenic, nonnodulating mutant *dmi1-1* were grown aeroponically and inoculated

Fig. 6. Analysis of a nonnodulating plant mutant, *dmi1-1*, for elicitation of reactive oxygen species and early nodulin gene expression. **A**, Nitroblue tetrazolium (NBT) staining in the wild-type parent A17 (WT) and its nonnodulating mutant *dmi1-1*. Roots were stained with NBT 12 h after inoculation with *Sinorhizobium meliloti* 2011. **B**, Northern blot containing total RNA isolated from roots of the wild-type parent A17 (WT) and its nonnodulating mutant *dmi1-1* after inoculation with *S. meliloti* 2011. WT and *dmi1-1* RNAs were analyzed on the same membrane and represent identical exposure times. The same blot was successively probed with ³²P-labeled *rip1*, *ENOD12*, *ENOD40*, and *histone H3*. The time course is reported in hours postinoculation. Note: The increase in histone H3 transcript observed in response to inoculation of wild-type plants with *S. meliloti* is a nodulation-related response that has been documented in other studies (R. V. Penmetta and D. Cook, unpublished data). **C**, Northern blot analysis of *dmi1-1* plants treated with H_2O_2 or mock-treated with nutrient medium. The same blot was successively probed with ³²P labeled *rip1*, *gst*, and *histone H3*.

with *S. meliloti* as previously described (Cook et al. 1995). Bacterial inoculations were performed using *S. meliloti* wild-type strains ABS7, 1021, 2011, or strains mutated for *nodD1ABC* (SL44, a gift from S. Long) or *nodH* (Debelle et al. 1986). For Nod factor treatment, purified Nod factor (a gift from J. Dénarié, CNRS-France) was dissolved in 50% ethanol and added directly to 10 l of inorganic nutrient media (Lullien et al. 1987) in an aeroponic chamber to a final concentration of 5×10^{-9} M. Plant material for all experiments was grown in a growth room with a 12 h photoperiod (18°C night and 22°C day) and a light intensity of 300 $\mu\text{M m}^{-2} \text{s}^{-2}$.

Assay for *rip1* induction by exogenous H_2O_2 .

For studies involving hydrogen peroxide, 5-day-old uninoculated plants were removed from the aeroponic chamber and the roots were immersed in 50 ml inorganic nutrient media (Lullien et al. 1987) containing specified concentrations of H_2O_2 (Chen et al. 1996). After 1 h of incubation, the roots were harvested for Northern blot analysis.

Nucleic acid methods.

RNA isolation, Northern blot analysis, and in situ hybridization experiments were conducted as previously described (Cook et al. 1995). For analysis of *rip1* induction by *Sinorhizobium* strains, shown in Figure 5B, the terminal approximately 3 mm of the root was removed prior to RNA extraction; this manipulation served to reduce background levels of *rip1* transcript that were associated with the extreme root tip. Hybridization probes used were *rip1* (Cook et al. 1995), *enod12* (Pichon et al. 1992), *enod40* (Crespi et al. 1994), *actin*, *gst*, and *histone H3*. The *actin*, *gst*, and *histone H3* probes were generated by degenerate polymerase chain reaction (PCR) amplification of DNA from *M. truncatula* ecotype A17 followed by cloning of individual PCR products into pBlueScript vector (Stratagene, La Jolla, CA, U.S.A.). The identity of individual clones was determined by DNA sequence analysis.

In situ hybridizations were performed essentially as described by Cook and associates (Peng et al. 1996). Briefly, root tissue was embedded in paraffin following fixation in 2% formaldehyde and 0.5% glutaraldehyde. Sections (8 to 10 μm thick) were placed on poly-L-lysine-coated slides for hybridization experiments. Single-stranded riboprobes were prepared by incorporation of ^3S -UTP using either T3 or T7 polymerase according to manufacturer's instructions (Stratagene). Antisense probes were used to detect *rip1* transcript, as shown in Figure 4. Controls consisted of nonhybridized tissue sections and of tissue sections hybridized with sense strand probes of *rip1* (data not shown). Hybridized transcript was detected with Kodak NBT-2 emulsion, and specimens were observed by bright field optics using a Zeiss (Batavia, IV) Axioskop light microscope.

Superoxide detection by nitroblue tetrazolium.

For in situ localization of O_2^- , 5-day-old plants were inoculated with different *Sinorhizobium* strains or purified Nod factor in an aeroponic chamber. At 12 h postinoculation, the plants were removed from the aeroponic chamber, and the roots were excised. For NBT staining, the excised roots were incubated for 1 h at 37°C in 10 ml of 10 mM sodium phosphate buffer (pH 7.8) containing 10mM NaN_3 and 0.1% NBT (Jabs et al. 1997). The stained roots were then cleared in two changes of lactophenol at 65°C (Dietrich et al. 1994) and examined with an Olympus SZH10 stereo microscope using dark field optics. For localization of O_2^- in nodule primordia, whole roots were harvested 60 h after inoculation with *S. meliloti*. Nodulated roots were first vacuum infiltrated with 10 mM phosphate buffer (pH 7.8) and then incubated for 1 h at 37°C in phosphate

buffer containing 0.1% NBT (Jabs et al. 1997). The stained roots were subsequently cleared and analyzed as described above. In treatments involving SOD, the roots were preincubated in 10 ml phosphate buffer containing SOD (2000 U/ml) for 2 h at 37°C. After 2 h of preincubation, 0.1% NBT was added to the phosphate buffer and SOD solution and incubated again at 37°C for 1 h. Plants preincubated for 2 h in phosphate buffer without SOD were used as controls in experiments involving SOD. Sectioning of plant material was performed after staining the roots with NBT. Root segments (terminal 2 cm) or nodule primordia were embedded in agarose (3% in water) and sliced into 50 to 70 μm width with a DSK microslicer DTK 100 (Ted Pella Inc., Redding, CA, U.S.A.). The sections were subsequently examined with a Zeiss Axioplan-2 compound microscope using bright-field or dark-field optics.

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