pSym *nod* Gene Influence on Elicitation
of Peroxidase Activity from White Clover and Pea Roots
by Rhizobia and Their Cell-Free Supernatants

Janet L. Salzwedel and Frank B. Dazzo

Department of Microbiology, Michigan State University, East Lansing 48824 U.S.A.

The activities of salt-elutable peroxidases from roots of white clover and pea were examined during the early interaction of these legume hosts with strains of *Rhizobium leguminosarum* in homologous and heterologous combination. Peroxidase-specific activity from clover root hairs began to increase 6 hr after inoculation with *R. l.* bv. *viciae* RL300 and was localized over the entire area of their deformations. In contrast, the onset of elicitation of peroxidase activity from root hairs was delayed after inoculation with *R. l.* bv. *trifolii* ANU843 and was localized only at the site of infection thread initiation. Three wild-type strains (*R. l.* bv. *trifolii* ANU843, *R. l.* bv. *viciae* RL300 and 1003) and one hybrid transconjugant strain of *R. leguminosarum* containing pSym from *R. l.* bv. *viciae* 248 (RBL5715) elicited increased specific activity of peroxidases eluted from pea and clover roots in heterologous combination. A comparison of peroxidase activity eluted from pea roots inoculated with ANU843 or its pSym-cured derivative indicated that pSym is required for elicitation of peroxidase on this heterologous host. The level of peroxidase activity elicited by *nodE* mutants (which have extended host range) is decreased on their new host. An extracellular fraction of RL300 contained flavonoid-dependent, heat-stable, and ethanol-soluble elicitor(s) of peroxidase activity. Treatment of clover seedlings with this cell-free fraction decreased the number of root hairs infected by ANU843. We propose that elicitation of root hair peroxidase may contribute to the infection process in this *Rhizobium*-legume symbiosis by altering root hair wall structure at sites of incipient penetration.

Additional keywords: host specificity, plant defense, symbiosis.

Rhizobium leguminosarum bv. *trifolii* forms a mutually beneficial symbiosis with clover species. *R. l.* bv. *viciae* is the symbiont for peas and vetch. Both microsymbionts infect root hairs by infection thread formation and induce

nitrogen-fixing root nodules on their respective "homologous" hosts but are incapable of doing so in "heterologous" combination with these legumes (Li and Hubbell 1969).

The basis of host specificity in this plant-microbe symbiosis is an area of intense investigation. The host-specific nodulation (*hsn*) genes (*nodFERLMN*) govern the specific host range for *R. l.* bv. *trifolii* and *R. l.* bv. *viciae* and are found on the symbiotic plasmid (pSym) (Weinman et al. 1988; Djordjevic and Weinman 1991). Expression of these genes requires the regulatory gene *nodD* plus specific flavonoid compounds exuded by the host root (Innes *et al.*1985). The plant cell wall is an important component influencing host range as illustrated by the report of Al-Mallah et al. (1987), which showed that a brief exposure of white clover roots to a mixture of cellulase and pectolyase allows *R. loti* to nodulate this heterologous host. In addition to the action of bacterial factors that deform host root hairs (Hollingsworth *et al.* 1990; Lerouge *et al.* 1990; Spaink *et al.*1991; Schultze *et al.* 1992) and host lectin that recognizes the bacterial symbiont (Bohlool and Schmidt 1974; Dazzo *et al.* 1978; Diaz *et al.* 1989), it has been suggested that successful infection may also depend on the ability of rhizobia to escape or suppress induction of structural and chemical defenses of homologous hosts that normally serve to prevent infection by microorganisms (Vance 1983; Djordjevic *et al.* 1987).

The effectiveness of inducible defense responses often depends on their rapid initiation and development and involves hypersensitive host cell death, production of antimicrobial phytoalexins, or deposition of wall-strengthening polymers such as lignin, suberin, or the hydroxyproline-rich glycoprotein "extensin" at sites of attempted penetration by microbes (Misaghi 1982). Wall-associated peroxidases are believed to participate not only in the normal growth and maturation of plant cell walls but also in defense (Campa 1991) by catalyzing the cross-linking of isodityrosine residues in extensin and polymerizing the aromatic constituents of lignin and suberin (Gaspar *et al.* 1982; Espelie and Kolattukudy 1985; Everdeen *et al.* 1988; Lewis and Yamamoto 1990). Thus, increased peroxidase activity during plant defense responses is thought to increase the amount of cross-linking in cell wall polymers, which hinders penetration by a pathogen (Campa 1991).

In the present study, we examined the activities of salt-elutable peroxidases during the early interaction between roots of white clover and pea with strains of *R. legu-*

minosarum in homologous and heterologous combination. We hypothesized that rhizobia may influence peroxidase activity, particularly in root hair walls, and that this plant response may contribute to the success or failure of infection. Included among the test strains of *R. leguminosarum* were wild-type *R. l.* bv. *viciae* and *R. l.* bv. *trifolii*, a pair of hybrid transconjugants, and transposon insertion mutant strains to explore the role of bacterial pSym *nod* and *hsn* genes in eliciting (or suppressing) peroxidase activity as a plant defense response. Portions of this work were presented at the 1989 International Congress on Nitrogen Fixation at Knoxville, TN, and the 1991 Workshop on Molecular Biology of the *Rhizobium*-Legume Symbiosis in Anacapri, Italy.

RESULTS

The location of peroxidase activity in white clover root hairs was shown by the deposition of the insoluble brown product of DAB oxidation. A low level of peroxidase activity was evenly distributed over entire root hairs on uninoculated roots (Fig. 1A) and was inactivated by heating roots at 100° C for 2 min before staining (figure not shown). Plasmolyzed root hairs showed that peroxidase activity was present in both the cytoplasm and the cell wall (Fig. 1B). Hairs on seedlings inoculated with *R. l.* bv. *trifolii* ANU843 accumulated the stain for peroxidase activity in the center of markedly deformed shepherd's crooks (Fig. 1C) and where infection thread formation initiated (Fig. 1D). The intracellular infection thread itself did not accumulate any more stain than did the cytoplasm of the root hair (Fig. 1D). In contrast, after inoculation with *R. l.* bv. *viciae* strain RL300 or hybrid transconjugant strain RBL5715, root hairs had dark deposits of peroxidase product over the entire irregular deformation, particularly at hair tips (Fig. 1E,F).

The time course for peroxidase elicitation was examined in clover root hairs during the first 24 hr after being inoculated with wild-type *R. l.* bv. *trifolii* ANU843 and *R. l.* bv. *viciae* RL300 (Fig. 2). The specific activity of root hair peroxidase increased earlier and to a higher level after inoculation with RL300 than with ANU843 or the uninoculated control. Beginning at the first sampling point (<30 min) and continuing over the next 12 hr, the mean level of peroxidase activity associated with root hairs was about 50% lower when inoculated with ANU843 than with the NF control. After 24 hr of incubation with ANU843, the level of peroxidase activity in the root hair preparation rose to a level that was not significantly different from the uninoculated NF control. Similar assays of 0.6 μg of protein from ANU843 or RL300 grown for 24 hr in BIII broth with 2 μM DHF or naringenin showed no detectable guaiacol peroxidase activity.

Native polyacrylamide gel electrophoresis resolved four acidic peroxidase isozymes salt-eluted from whole axenic clover roots (Fig. 3). The most slowly migrating isozyme stained 2.8-fold more intensely in samples derived from roots inoculated with *R. l.* bv. *viciae* RL300 than with *R. l.* bv. *trifolii* ANU843. The other three isozymes from clover roots incubated with either inoculant strain produced equivalent amounts of product. The same gel electrophoresis of proteins eluted from pea roots resolved only one peroxidase isozyme. This isozyme had low mobility and stained 1.5-fold more intensely in samples from roots inoculated with ANU843 than with RL300. Relative to the uninoculated control, the activity stain of this isozyme was increased by 57% upon inoculation with ANU843, in contrast to a modest 7% increase with RL300. No new peroxidase isozymes with different mobilities were detected following inoculation of clover or pea with ANU843 or RL300. Peroxidase samples from uninoculated clover roots (not shown) consistently showed an isozyme pattern identical to samples from clover inoculated with ANU843, including the most slowly migrating peroxidase isozyme.

The specific activities of salt-eluted peroxidases from clover and pea roots were increased following inoculation with wild-type strains of *R. leguminosarum* in heterologous combination (Table 1). The levels of salt-eluted peroxidase activity did not significantly change after inoculation of clover or pea roots with wild-type strains in homologous combination. The level of peroxidase activity eluted from
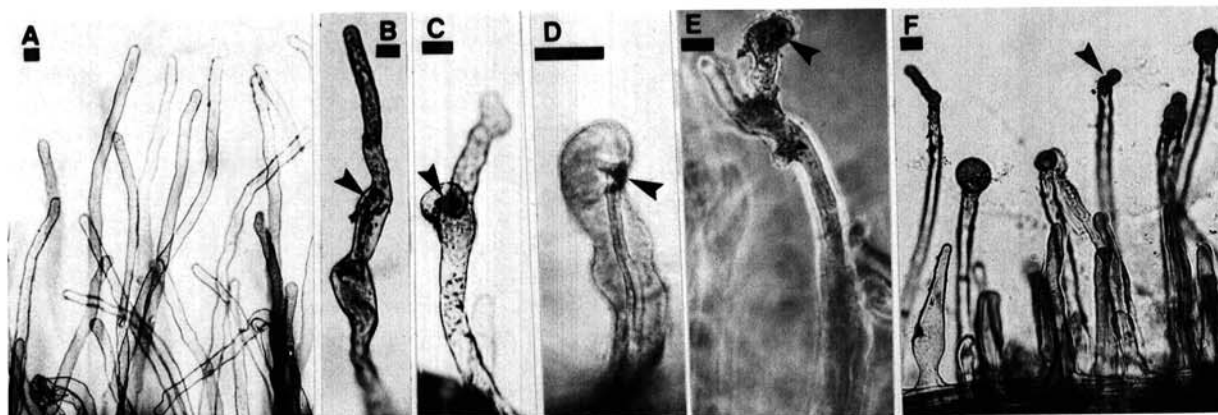

Fig. 1. White clover root hairs stained with diaminobenzidine for *in situ* localization of peroxidase activity. Treatments were **A,** uninoculated control; **B,** plasmolyzed uninoculated control (note activity stain in wall and cytoplasmic strand); **C,** 1 day postinoculation with ANU843; **D,** 5 days postinoculation with ANU843 (note infection thread); **E,** 5 days postinoculation with RL300; **F,** 5 days postinoculation with RBL5715. Arrowheads indicate areas of enhanced staining. Bar scale = 15 μm.

pea roots inoculated with the pSym-cured derivative of ANU843 (strain ANU845) was similar to the levels eluted from roots inoculated with the homologous strains (*R. l.* bv. *viciae* RL300 and 1003) but significantly lower than the levels elicited by wild-type *R. l.* bv. *trifolii* strain ANU843.

Mutant derivatives of *R. l.* bv. *trifolii* ANU843 or hybrid strain RBL5602 with a Tn5 insertion in a single *hsn* gene, *nodE*, elicited significantly less peroxidase activity on its respective heterologous host than did the *nodE*+ isogenic strain (ANU843 and RBL5715, respectively; Table 1). The differential responses on homologous and heterologous hosts inoculated with *R. l.* bv. *trifolii* ANU251 (*nodL*::Tn5)

followed the same trend as with the *nodE*::Tn5 mutant strain ANU297. The specific activity of salt-eluted peroxidases from clover roots following inoculation with ANU297 was much higher than with its parent ANU843 and similar to the levels elicited by the heterologous *R. l.* bv. *viciae* strains RL300 and 1003 (Table 1).

The effect of cell-free *Rhizobium* factors on elicitation of peroxidase activity from uninoculated clover roots was examined. The specific activity was increased upon incubation with a cell-free bacterial washing from *R. l.* bv. *viciae* RL300, but only if the bacterial culture was grown with *nod*-activating flavonoids (Table 2). The peroxidase-eliciting activity in this extracellular fraction from RL300 was resistant to autoclaving and in some degree ethanol-soluble (Table 2).

The number of infected root hairs and nodule primordia on clover seedlings grown for 4 days with *R. l.* bv. *trifolii*
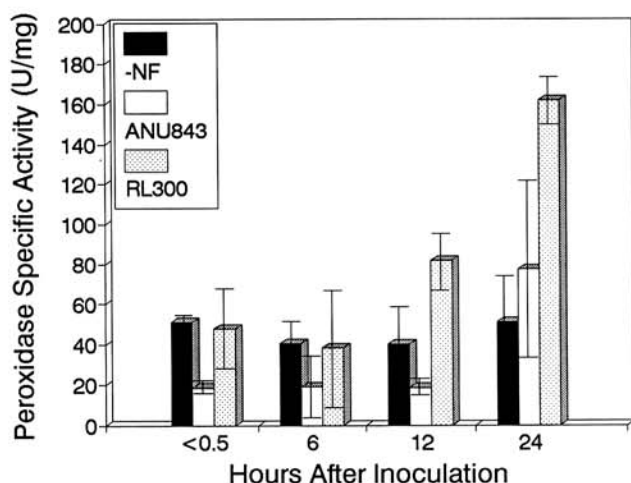

Fig. 2. Time course of changes in peroxidase-specific activity from white clover root hairs after inoculation with wild-type *Rhizobium leguminosarum* bv. *trifolii* ANU843 or *R. l.* bv. *viciae* RL300. One unit of activity equals ΔOD_{470nm} per minute. Specific activity equals units per milligram of protein. Data points represent the mean of two experiments ± SE.

Table 1. Specific activity of salt-elutable guaiacol peroxidases from pea and white clover roots after 24 hr incubation with *Rhizobium leguminosarum* strains

Inoculum (Biovar, strain)	Peroxidase specific activity[a] ($\Delta OD_{470 nm}$/min/mg protein)	
	Pea	Clover
Uninoculated control	891 ± 234	546 ± 194
viciae RL300	643 ± 270	900 ± 194
viciae 1003	595 ± 225	1,044 ± 224
trifolii ANU843	1,759 ± 234	765 ± 173
trifolii ANU845 (pSYM⁻)	356 ± 92	778 ± 294
trifolii ANU297 (*nodE*::Tn5)	648 ± 235	1,150 ± 172
trifolii ANU251 (*nodL*::Tn5)	646 ± 308	860 ± 144
viciae RBL5715	399 ± 225	1,149 ± 224
viciae RBL5602 (*nodE*::Tn5)	651 ± 288	713 ± 151

[a] Values are the mean of at least three experiments ± SE.

Table 2. Specific activity of peroxidases eluted from clover roots after 24 hr exposure to cell-free wash fluid from *Rhizobium leguminosarum*

Strain and sample treatment	Peroxidase specific activity[a] ($\Delta OD_{470 nm}$/min/mg protein)
NF medium (untreated control)	453*
NF medium + 4 μM NAR	472+
RL300 grown without flavone	605*
RL300 grown with 4 μM NAR	1,223*
RL300 grown with 4 μM DHF	1,311+
RL300 grown with 4 μM NAR, autoclaved	1,280+
RL300 grown with 4 μM NAR, EtOH-soluble	961+
ANU843 grown with 4 μM DHF	726*

[a] All values followed by * were derived from triplicate experiments, whereas those followed by + were derived from a composite sample of multiple plants in one replicate.
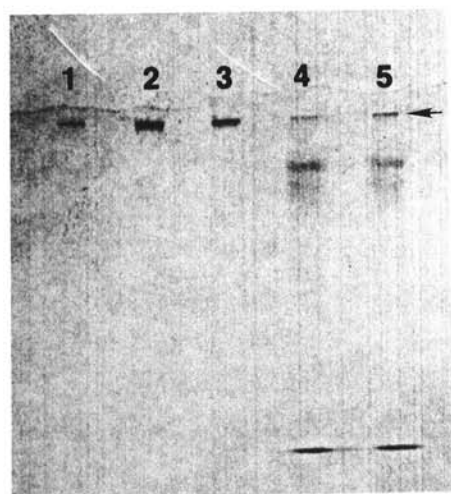

Fig. 3. Native polyacrylamide gel stained for peroxidase activity with 3-amino-9-ethyl carbazole. Peroxidases were salt-eluted from pea and white clover roots 24 hr after inoculation with rhizobia. Each lane was loaded with 2 μg of protein. Treatments included: lane 1, pea/NF uninoculated control; lane 2, pea/ANU843; lane 3, pea/RL300; lane 4, clover/ANU843; lane 5, clover/RL300. The isozyme that exhibited the major change upon inoculation is indicated by the arrow.

Table 3. Effect of culture supernatant from *Rhizobium leguminosarum* strains on the number of infected root hairs and nodule primordia formed by ANU843 on cv. Dutch white clover[a]

Culture supernatant of	Infected root hairs per plant	Nodule primordia per plant
Uninoculated control (BIII medium)	22.2 ± 4.8	0.7 ± 0.3
ANU843 grown with DHF	30.4 ± 3.0	4.1 ± 0.7
RL300 grown with naringenin	1.8 ± 0.1	1.4 ± 0.4

[a] Values are the mean ± SE for nine plants.

ANU843 was increased by preincubating the roots with culture supernatant of DHF-grown ANU843 before bacterial inoculation (Table 3). Culture supernatant from *R. l.* bv. *viciae* RL300 grown with naringenin reduced the number of root hairs infected by ANU843. Neither clover root growth (about 3 mm/day) nor *in vitro* growth of ANU843 in BIII medium (4.5 hr per generation) was affected by the *R. l.* bv. *viciae* RL300 culture supernatant.

DISCUSSION

In this study, we have examined the activity of root-associated peroxidases in relation to the infection process in the *Rhizobium*-legume symbiosis. Activity staining of roots on axenic clover seedlings showed a low level of peroxidase activity associated with root hairs. In contrast, localized areas of elevated activity were found on root hairs inoculated with wild-type *R. l.* bv. *trifolii* ANU843, *R. l.* bv. *viciae* RL300, and hybrid strain RBL5715 of *R. leguminosarum*. In the homologous combination with ANU843, peroxidase activity on root hairs increased specifically at two sites where host specificity in the infection process is expressed: The center of the markedly curled shepherd's crook where an aggregate of bacteria has been sandwiched between overlapping folds of the root hair cell wall, and the initiation of infection threads where the bacteria have penetrated the host wall. These are the only places on the root epidermis where increased staining of peroxidase activity is evident, even 5 days after inoculation. Intracellular infection threads induced by ANU843 did not show enhanced peroxidase activity within white clover root hairs. A localized increase in peroxidase activity has also been found at infection thread initiation in alfalfa root hairs inoculated with *R. meliloti* (C. Vance, personal communication). Thus, the elevated peroxidase response in the above homologous combinations is associated with the root hair cell wall at the localized site of penetration, rather than continued, successful development of the infection thread itself or other wall deformations. We predict that this localized peroxidase activity is important to successful infection in these homologous combinations by being involved in repair and occlusion of the eroded root hair wall where the bacteria have penetrated. In contrast, it is the entire root hair deformation of white clover that accumulated peroxidase activity stain in the heterologous combinations used in this study. We predict that this increase in enzyme activity could possibly modify the structure of the deformed root hair wall and, as a result, hinder penetration by these heterologous strains of rhizobia. There are numerous reports that correlate plant resistance to microbial infection and increased peroxidase activity. In particular, Stermer and Hammerschmidt (1987) induced resistance in cucumber seedlings and found that the cell walls were more resistant to degradation by fungal enzymes and that the level of insoluble extensin (cross-linked by peroxidase) was increased.

Time-course studies showed distinct differences in peroxidase activity associated with clover root hairs following inoculation with wild-type homologous *R. l.* bv. *trifolii* ANU843 and heterologous *R. l.* bv. *viciae* RL300 strains of rhizobia, particularly during the first 6–12 hr

after inoculation. Evidence was found suggesting a more rapid elicitation by RL300 than by ANU843. Although localized elicitation of peroxidase by ANU843 is evident in cytochemical tests (>24 hr), the time-course study indicates an immediate and transient reduction during the first 12 hr after inoculation with this homologous strain. This early period is likely to be a critical time for root hair/rhizobial interactions which determine the success or failure of initial penetration. By analogy, the effectiveness of a defense response often depends on its rapid initiation and development (Misaghi 1982). The rapid, transient reduction in peroxidase activity in clover root hairs suggests that preformed factor(s) in the ANU843 inoculum may affect peroxidase activity. We predict that rhizobial EPS may be such a preformed factor, since this polymer binds rapidly to root hair tips (Dazzo and Brill 1977), increases the frequency of root hair infections in white clover (Abe *et al.*1984) and the efficiency of alfalfa root infection (Olivares *et al.*1984), and inhibits *in vitro* β-1,3-glucan (callose) synthase from soybean and pea (Ahlborn and Werner 1991). Other studies have implicated a role of rhizobial EPS in avoiding elicitation of plant defensive responses during invasion of legume roots or nodules (Djordjevic *et al.* 1988; Puhler *et al.*1991; Rolfe *et al.*1992).

Elution of wall proteins from isolated clover root hairs unavoidably includes cytoplasmic proteins as well. Therefore, we determined whether the *Rhizobium*-induced changes in peroxidase activity eluted from the surface of intact roots (which includes root hairs) and from isolated root hairs were similar. The results showed that the specific activity of peroxidase eluted from both whole roots and isolated root hairs was greater with the heterologous combinations of rhizobia tested. Unlike the results for root hairs, however, peroxidase activity from clover roots was not reduced by inoculation with homologous wild-type strain ANU843. This may reflect the constitutive background of peroxidase present in the walls of all the epidermal cells of the root which obscures the localized suppression in root hair peroxidase, and/or the 24 hr of incubation of inoculated roots was too late to detect a possible suppression. Nevertheless, heterologous elicitation of peroxidase activity is an early, dominant response in these *Rhizobium*-legume interactions.

For comparison, resistance responses to incompatible pathogens occur more rapidly than pathogenic responses induced by compatible pathogens. For example, washings from the surface of bean roots incubated for 4 days with *Pseudomonas putida* contained twofold higher peroxidase activity than washings from uninoculated roots (Albert and Anderson 1987). The guaiacol peroxidases from intercellular washings of resistant barley leaves increased rapidly 8–24 hr after inoculation with the pathogenic fungus, *Erysiphe graminis* f. sp. *hordei*, and were fivefold greater than uninoculated leaves after 72 hr (Kerby and Sommerville 1989).

Analysis of pea and clover root peroxidase activity by native gel electrophoresis suggests that this increase in specific activity following inoculation with wild-type strains ANU843 and RL300 was due to elevated activity of one constitutively produced peroxidase isozyme. The low mobility of the responding isozyme suggests that it

was weakly acidic at pH 9 and/or had a high molecular weight (possibly complexed with other root surface components). Spectrophotometric assays indicate that pea and clover roots respond to inoculation with these heterologous strains of rhizobia by increasing the specific activity of their salt-elutable peroxidases; however, the response is substantially greater in pea. This difference is likely due to a higher background of constitutive peroxidase isozymes in the root extract from clover than from pea.

We reasoned that if the elicitation of peroxidase is important to the specificity of legume infection, then it should be influenced by functions of the host-specific nodulation (*hsn*) genes present on pSym in *R. leguminosarum*. Consistent with this hypothesis are the findings that 1) the pSym-cured derivative of ANU843 (strain ANU845) has completely lost the ability to elicit peroxidase activity on pea; and 2) the pea-nodulating hybrid transconjugant RBL5715 elicits peroxidase activity on the heterologous clover host. These results clearly indicated that elicitation of peroxidase activity by these strains is pSym-dependent, and warranted further investigation of the possible involvement of pSym *hsn nod* genes.

Of the *hsn* genes, *nodE* is pivotal (Djordjevic *et al.*1985; Spaink *et al.*1989) and is believed to function as an avirulence gene in which the loss of function leads to increased host range (Djordjevic *et al.* 1987). ANU843*nodE*::Tn*5* mutants can nodulate peas (Djordjevic *et al.*1985) and the *nodE* mutant strain RBL5602 induces small white, presumably Fix⁻ nodules on clover (J. Salzwedel, unpublished). Consistent with their nodulation phenotypes, these *nodE*::Tn*5* mutants with extended host range elicited less peroxidase activity on their new host. Interestingly, the ANU843*nodE*::Tn*5* mutant elicited higher peroxidase specific activity on clover roots than did ANU843 and displays a delayed nodulation phenotype on this host. The ANU843*nodL*::Tn*5* mutant also elicited a lower peroxidase-specific activity than did ANU843 on pea roots, indicating that either the Tn*5* insertion in *nodE* has a polar effect on the expression of *nodL* which then is a key gene controlling the elicitation of pea root peroxidase activity by ANU843, or both *nodE* and *nodL* of ANU843 are needed to elicit this plant response. Based on recent studies with *R. leguminosarum* (Spaink *et al.* 1991), we predict that both *nodE* and *nodL* gene products contribute to the production of some bacterial factor(s), which elicits peroxidase activity and affects the efficiency of successful infection.

Bioassays using axenic clover plants showed that cell-free washings from plate-grown RL300 could elicit peroxidase activity within 24 hr just as did the live bacteria, suggesting that an extracellular elicitor(s) was present. This activity was flavonoid-dependent, heat-stable, and largely ethanol-soluble. Because this eliciting activity was increased when the bacteria were cultured with the flavone DHF, it is anticipated that RL300 can produce elicitor(s) in the external clover root environment. The correlation between peroxidase elicitation by the extracellular fraction of RL300, and the negative effect of this fraction on the number of clover root hairs infected by ANU843 is consistent with our hypothesis. The few infections that

did occur after this treatment continued to initiate nodules at a frequency equivalent to the untreated control. Thus, the negative effect of the extracellular fraction of RL300 in this bioassay of the *R. trifolii*-white clover symbiosis is exhibited primarily during infection of root hairs rather than during nodule initiation.

In summary, our model for successful infection of white clover by *R. l.* bv. *trifolii* ANU843 includes the transient suppression of peroxidase activity in root hairs, which we predict leads to fewer cross-links of wall polymers during extension growth and to an increase in susceptibility to bacterial penetration. After penetration, highly localized peroxidase activity could facilitate repair of the wall at the site of bacterial entry and infection thread initiation. Elicitation of a rapid increase in peroxidase activity in root hairs by the *R. l.* bv. *viciae* strains used in this study might result in increased cross-linking of wall polymers and make the wall more resistant to bacterial penetration. We further propose that the production, modification, and/or export of the bacterial factor(s) responsible for this elicitation require flavonoid-dependent expression of *nod* gene(s), including *nodE* and/or *nodL*. We interpret the increased peroxidase activity elicited by these *R. leguminosarum* strains on heterologous clover and pea hosts as a plant defense response that contributes to expression of host specificity during the infection process in the *Rhizobium*-legume symbiosis.

MATERIALS AND METHODS

Bacteria and plant cultures.

Bacterial strains used in this study are listed in Table 4. Cells were grown at 30° C on plates of BIII defined medium (Dazzo 1982) solidified with 1% purified agar (U. S. Biochemical, used throughout) and amended with appropriate antibiotic to retain recombinant plasmids. Inocula were prepared by suspending cells to a density of 5×10^7 cells per milliliter in nitrogen-free (NF) plant growth medium (Fahraeus 1957). Seeds of pea (*Pisum sativum* L.) cv. Little Marvel and white clover (*Trifolium repens* L.) cv. Dutch were surface-sterilized by shaking in 70% ethanol for 4 min followed by 3 × 10 min washes with 1/10 strength commercial bleach, and then washed several times with sterile water. Plants were grown in NF medium in a growth chamber with a 14-hr photoperiod of mixed incandescent/fluorescent lighting (about 400 $\mu E/$ m²/sec), 23° C day/20° C night cycle, and 70% relative humidity.

Peroxidase activity associated with clover root hairs.

Seeds were germinated in the dark for 2 days on NF agar plates. Seedlings were inoculated by gently swirling in the bacterial suspension for 30 min. Sterile NF medium was substituted throughout as the uninoculated control. To localize peroxidase activity *in situ* on root hairs, inoculated seedlings were incubated vertically on NF agar plates in the growth chamber and then transferred intact to glass microscope slides. Roots were covered with coverslips and incubated for 5 min at room temperature with the substrate mixture of Zaar (1979) containing 12 mg of 3,3'-diaminobenzidine (DAB) and 50 μl of 30% H_2O_2

in 3 ml of 60 Na-K-phosphate buffer, pH 5.5. After rinsing the roots with NF medium, the root hairs were examined by brightfield light microscopy and photographed.

To obtain peroxidases from root hairs, seedlings were inoculated as described above, rinsed with sterile water, separated on sterile moist filter paper, covered, and incubated in the growth chamber. Seedlings were then placed in glass vials and immersed in liquid nitrogen. The frozen vials were shaken vigorously to selectively fracture the root hairs (Gerhold et al.1985). Fragments of roots were poured out, leaving root hairs adhering to the vial walls. The vials were thawed and rinsed with 1.5 ml of 1 M NaCl. The root-hair suspensions were pooled and submerged in a sonic bath (Cole-Parmer Ultrasonic Cleaner, Model 8846-50) for 20 min at 4° C. The protein extract was centrifuged at 5,000 × g for 20 min and the supernatant was dialyzed (12,000–14,000 mol. wt. cutoff) against water for 2 days at 4° C.

In vitro assay for peroxidase activity.

Fresh substrate mixture was made daily and contained 125 μl of guaiacol (Sigma) and 350 μl of H_2O_2 in 50 ml of 10 mM Na-phosphate buffer (pH 6.0) (Hammerschmidt et al.1982). The reaction mixture was prepared by mixing 0.6 μg of salt-eluted protein in 100 μl with 1 ml of substrate mixture in a cuvette. Product formation was measured for 3 min (20 measurements per minute) at OD_{470nm} on a Gilford Response UV/Vis scanning spectrophotometer. The Gilford kinetics software package was used to calculate the initial rate of the reaction from the linear region of the product formation curve. The protein content of samples was measured by the Bradford dye binding assay using bovine serum albumin as standard.

Salt-eluted peroxidases from clover and pea roots.

Clover seeds were embedded in blocks of NF agar and suspended on stainless steel wire mesh supports over 50 ml of NF medium in 15-cm-diameter covered glass dishes (Dazzo 1982). Seeds were germinated for 3 days, and then the original growth medium was replaced with 50 ml of fresh NF medium containing the inoculum or sample. Pea seeds were germinated for 2 days in the dark in sterile water, then placed on NF agar (three seedlings per plate) and incubated vertically in the growth chamber for 2 days. Each pea root was inoculated with 8.5 × 10^6 bacteria, incubated horizontally at room temperature for 1 hr, and then incubated vertically in the growth chamber for 24 hr. After treatments, the wire mesh containing clover roots was immersed in liquid nitrogen and the frozen roots broken off into a chilled beaker. Pea roots were cut from cotyledons with a razor blade. Isolated roots were stored at −20° C until peroxidases were eluted by 1 M NaCl combined with agitation in a sonic bath as was used for root hair peroxidase. Microscopic examination of clover roots after this treatment showed that the damage to root hairs was minimal and that they retained their cytoplasm. Following dialysis against water, root peroxidases were assayed in vitro as previously described.

Separation of acidic peroxidase isozymes by alkaline native gel electrophoresis.

The stacking gels were made with 2.5% acrylamide in 20 mM Tris-phosphoric acid buffer (pH 6.7). Running gels were made 1 mm thick with 7.5% acrylamide in 150 mM Tris-HCl buffer (pH 9.1). The upper electrode buffer was 40 mM Tris-glycine (pH 8.9), and the lower electrode buffer was 100 mM Tris-HCl (pH 8.1). Lanes were loaded with samples containing 2 μg of protein eluted from clover or pea roots and mixed with 5× sample buffer (0.05% bromophenol blue and 10%, v/v, glycerol in 0.5 M Tris-HCl, pH 6.8), run through the stacking gel at 10 mA, then at 24 mA until the tracking dye had migrated three-quarters of the length of the running gel. The electrophoresis unit was cooled by running tap water. Peroxidase activity was visualized by soaking the gel in a solution containing 40 mg of 3-amino-9-ethylcarbazole (Sigma) dissolved in 10 ml of N,N-dimethylformamide and mixed with 66 μl of H_2O_2 in 190 ml of 50 mM Na-acetate buffer

Table 4. Rhizobium leguminosarum strains used in this study

| Biovar and strain | Relevant characteristics | Nod phenotype | | Source |
		Clover	Pea	
bv. trifolii				
ANU843	Wild type	+	−	Djordjevic et al. 1983
ANU845	pSym⁻ deriv. of ANU843	−	−	Djordjevic et al. 1983
ANU297	843nodE::Tn5, Km^r	delayed +	+	Djordjevic et al. 1985
ANU251	843nodL::Tn5, Km^r	+	−	Weinman et al. 1988
bv. viciae				
RL300	Wild type	−	+	Brewin et al. 1980
1003	Rif^r deriv. of wt 1001	−	+	A. Squartini[a]
Hybrid transconjugants				
RBL5715	pSym-cured derivative of R. l. bv. trifolii RCR5 (RBL5039) and containing pSym pRL1JI from wild-type R. l. bv. viciae 248 with a Tn5 in a nonsymbiotic locus	−	+	C. Wijffelman[b]
RBL5602	5039pRL1JInodE1::Tn5	+	delayed + on vetch	C. Wijffelman et al. 1985 and R. Okker[c]

[a] University of Padova, Italy.
[b] University of Leiden, The Netherlands.
[c] University of Leiden, The Netherlands.

(pH 5.5) (Graham *et al.* 1965). In preliminary tests, this substrate would detect the same activity bands of peroxidase as guaiacol but had the advantage of producing an insoluble product and therefore a more permanent stain. After activity staining of bands, the gels were rinsed with water for 20 min to stop the reaction and stored in 50% methanol, 5% acetic acid (v/v) in water. Band intensity was measured with an Ambis densitometer.

Cell-free bacterial wash fluid.

Cells were grown for 3 days on BIII plates with or without 4 μM 4',7-dihydroxyflavone (DHF, Spectrum Chemical Corp.) or naringenin (NAR, Sigma) to express *nod* genes, then suspended and gently shaken in NF medium for 1 hr, and centrifuged at 10,780 $\times$ *g* for 30 min. The supernatant was sterilized by sequential passage through 0.8-, 0.45-, and 0.2-μm Millipore filters and adjusted to an OD_{245nm} between 0.08 and 0.18. An aliquot of this bacterial-wash fluid from *R. l.* bv. *viciae* strain RL300 was autoclaved for 20 min at 120° C and then clarified by centrifugation. Two volumes of cold 95% ethanol were mixed with another aliquot and centrifuged at 10,000 $\times$ *g* for 40 min at 4° C. The supernatant was evaporated to dryness under vacuum, and the ethanol-soluble residue was redissolved in sterile NF medium to the original sample volume. Fifty milliliters of each of these solutions (original, autoclaved, ethanol-soluble fraction) was added to cover the roots of axenic clover seedlings grown in wire mesh assemblies. After 24 hr of incubation, the roots were removed for salt-elution of peroxidases as described above.

Influence of the extracellular fraction from broth-grown bacteria on infection thread and nodule initiation.

Cells were grown in shaken flasks (175 rpm) containing BIII medium supplemented with 2 μM DHF or NAR for strains ANU843 and RL300, respectively. After 2–3 days, the cultures were centrifuged at 16,000 $\times$ *g* for 30 min. The supernatants were filter-sterilized and diluted with fresh BIII medium to an OD_{245nm} of 0.625–0.633. One-day-old clover seedlings were applied to the surface of NF agar plates and grown vertically for 1 day. Then, 40 μl of diluted bacterial supernatant was applied to the roots and covered with a sterile 18-mm^2 coverslip positioned with the lower edge just below the root tip. After 4 hr of vertical incubation, 10 μl of ANU843 inoculum (10^5 cells/seedling) was introduced under the coverslip without rinsing, and then seedlings were incubated vertically. After 4 days, roots were transferred to microscope slides, stained with 0.01% methylene blue (in NF medium), rinsed with NF medium, and examined by phase contrast microscopy to count the root hairs containing infection threads. Later, the roots were cleared in 33% bleach under a vacuum, rinsed in water, restained with methylene blue, and examined by brightfield light microscopy to count the nodule primordia in the root cortex (Truchet *et al.*1989). Root growth was measured as the distance between the root tip marks made at the time of inoculation and staining.

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