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THE ROLE OF PLANT-DEFENSE RESPONSES IN THE DETERMINATION OF HOST SPECIFICITY FOR THE *RHIZOBIUM*-LEGUME SYMBIOSIS

By

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ABSTRACT

THE ROLE OF PLANT DEFENSE RESPONSES
IN THE DETERMINATION OF HOST SPECFICITY
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Our objective was to test whether rhizobia elicited a plant defense response which could affect the determination of host specificity in the *Rhizobium*-legume symbiosis. Heterologous *Rhizobium leguminosarum* biovars elicited increased specific activity of salt-elutable peroxidase from the surface of pea and clover roots. The cell-free supernatant of *R. leguminosarum* bv. *viciae* also elicited increased peroxidase activity from clover roots. The excreted elicitor of peroxidase activity was flavone-dependent, heat-stable, and ethanol-soluble. Peroxidase activity was localized to the site of attempted penetration in clover root hairs. Peroxidase activity in clover root hairs began to increase 6 hrs after heterologous inoculation. Inoculation with homologous bv. *trifolii* suppressed peroxidase activity for 12 hrs. The transient suppression of activity could be mimicked by treatment with purified EPS from bv. *trifolii*. The Sym plasmid-cured strain, *nodE*::Tn*5* mutant, *nodL*::Tn*5* mutant, and hybrid recombinant bv. *trifolii* strain containing the host specific nodulation (*hsn*) genes from bv. *viciae* each elicited less peroxidase activity from pea roots than did the wild type bv. *trifolii*. We conclude that *hsn* genes interact with others present on the Sym plasmid to contribute to host-specificity through the modification of an elicitor which increases root hair peroxidase activity which in turn may alter the structure of the root hair wall at

the site of incipient penetration.

Micro pH electrodes were used to measure the response of single white clover root hairs to inoculation with rhizobia or treatment with purified LPS. Cells of homologous bv. trifolii or their LPS induced a rapid (30 min) increase in pH at the surface of individual root hairs from pH 6.5 to 6.7. Heterologous rhizobia did not elicit this response. The nodD::Tn5 and nodL::Tn5 mutants of bv. trifolii also failed to elicit this response. We conclude that since homologous (compatible) rhizobia elicit this increase in pH, this response is fundamentally different than the H^+ uptake by host cells undergoing a hypersensitive response elicited by incompatible pathogens. This Rhizobium-induced neutralization at the root hair surface may enhance successful infection by overcoming the acid-inhibition of early infection events.

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LITERATURE REVIEW

Introduction

The mutually beneficial symbiosis between soil bacteria of the genus Rhizobium and plants in the legume family is agriculturally important world wide. The fixation of atmospheric N_2 into NH_3 by the bacteria residing inside root nodules eliminates the need for costly nitrogenous fertilizers. The symbiosis is characterized by highly evolved and restricted host specificity with only Rhizobium leguminosarum bv. viciae able to infect peas and vetch, R. leguminosarum bv. trifolii able to infect clovers, and R. meliloti able to infect alfalfa.

The sequence of events leading to successful nodulation for the above Rhizobium-legume combinations are well known. Microscopy has revealed that rhizobia are attracted to host roots, attach to the root hair tip, induce marked curling of the tip, and penetrate the root hair within the confines of the advancing infection thread (Nutman, 1959; Vincent, 1980). Eventually, the bacteria are released from the infection thread into the proliferating cortical cells and are surrounded by a plant derived peribacteroid membrane. The bacteria then differentiate into bacteroids and a nitrogen fixing nodule emerges.

Rhizobium-legume host specificity.

The genes governing host specificity in the fast-growing rhizobia have been well characterized (Horvath et al., 1986; Debellé and Sharma,

1986; Djordjevic et al., 1986; Martinez et al., 1990). For R. leguminosarum bv. trifolii, bv. viciae, and R. meliloti, successful symbiosis culminating in a nitrogen-fixing nodule filled with bacteroids depends on the presence of one to several large bacterial plasmids, appropriately known as symbiotic plasmids (pSym). In bv. trifolii strain ANU843, there is one Sym plasmid on which genes essential for white clover nodulation are found (Djordjevic et al., 1983). To date, the nod genes in ANU843 that have been named are the common nod genes nodDABC; the host-specific nodulation (hsn) nodFERLMNT genes; and two genes that affect infection thread development, nodLJ. "Common nod genes" are defined as genes having homologous DNA sequences among the rhizobia, whose loss by mutation or deletion renders the bacteria unable to infect their own or any host, and which when mutated or deleted, can be replaced by the equivalent gene from another Rhizobium species to restore nodulation ability on the original host. To be designated an hsn gene, either (1) the gene must alter the bacterial host range when that gene function is lost (e.g. Tn5 insertion in nodE extends bv. trifolii host range to nodulate peas), or (2) the gene must be transferred to a recipient strain of a different Rhizobium species in order to allow that recipient to efficiently nodulate the homologous host of the donor Rhizobium (e.g. bv. trifolii nodFERLMN genes transferred to bv. viciae enable the latter to efficiently nodulate white clover, Djordjevic et al., 1986). Thus, manipulation of the bacterial hsn genes affects the determination of host specificity. The precise function of the hsn genes is still unknown. Faucher et al. (1988), however, have reported that the bacterial production of a host specific extracellular factor which deforms root hairs is dependent on

nodH in R. meliloti. The authors believe that the common nod genes encode a non-specific hair deformation factor and it is the nodH gene product which then modifies the factor to produce the host-specific form of the molecule known as nodRm1 (Lerouge et al., 1990).

Root exudates contain various carbohydrates, carboxylic acids, phenolic compounds, and amino acids (Klein et al., 1990). Within this milieu, the class of compounds called flavones are a pivotal plant signal which stimulates expression of the fast-growing Rhizobium nodulation genes. Flavones are synthesized from one branch of the phenylpropanoid pathway with other branches giving rise to lignin precursors, anthocyanins, and pterocarpan phytoalexins. The greatest stimulation of nod gene expression depends upon the specific flavone and the particular Rhizobium biovars or species being examined. The most stimulatory flavones are 7,4'-dihydroxyflavone (DHF) for bv. trifolii, naringenin for bv. viciae, and luteolin for R. meliloti (Redmond et al., 1986; Peters et al., 1986). Very low concentrations (10^{-9}-10^{-6} M) of these compounds will induce Rhizobium nod genes. The pattern of hydroxylation specifies whether the flavone will be stimulatory, inhibitory, or have no effect on the particular strain being examined. Some host specificity is dictated by the specific flavone to which a particular Rhizobium species responds, and the basis of this flavone specificity lies in specific domains of the protein product of the pSym regulatory gene, nodD (Spaink et al., 1987). Those genes shown by transcriptional lacZ fusions to be expressed after exposure to flavones have a conserved upstream region of DNA known as a "nod box". The nodD protein binds to this nod box region of DNA (Fisher et al., 1988, Kondorosi et al., 1988).

In addition to release of nod gene-inducing flavones, the plant contribution to host-specificity is thought to involve specific recognition and attachment of bacteria to the host root hairs. At the root hair surface, an initial reversible attachment occurs that is not symbiont-specific (Dazzo et al., 1984b) and may involve a Ca^{++}-dependent bacterial adhesin (Smit et al., 1986). Within an hour, this step is followed by a symbiont-specific, lectin-mediated step of bacterial aggregation (Dazzo et al., 1984b). In soybean, pea, and clover, lectins which specifically bind the EPS or LPS of the homologous symbiont have been observed (Bohlool and Schmidt, 1974; Dazzo and Hubbell, 1975; vander Schaal et al., 1983). In white clover, immunofluorescence localization has shown that the lectin, trifoliin A, is present at root hair tips (Dazzo and Brill, 1977). The most compelling evidence that a lectin is a host-encoded determinant of symbiont specificity has come through genetic manipulation of the host plant. Diaz et al. (1989) showed that transgenic white clover containing the gene for pea lectin could be infected with R. leguminosarum bv. viciae.

The barrier of host specificity has also been breached through external manipulation of the host plant. Al-Mallah et al. (1987) succeeded in infecting white clover with Rhizobium loti at a low frequency after the roots had been treated with a mixture of cellulase and pectolyase in the presence of polyethylene glycol. Similar treatment allowed nodulation of the non-legume Brassica napus by rhizobia (Al-Mallah et al., 1990).

It is the early interaction between Rhizobium and the root hair which must determine host-specificity (Li and Hubbell, 1969). Both homologous and heterologous rhizobia attach to root hairs, but they

differ in their pattern of attachment. Homologous rhizobia are heavily concentrated at the root hair tip with additional bacteria polarly attached along the length of the root hair. This has been termed the symbiont specific pattern of attachment. The heterologous rhizobia also bind to root hairs, though not in the symbiont specific pattern of attachment (Dazzo et al., 1984). Both homologous and heterologous rhizobia induce root hair deformations, but it is only the homologous which induces the symbiont specific deformation called a shepherd's crook which leads to infection thread initiation. Heterologous rhizobia have never been observed to form an infection thread. Thus, the first structural barrier encountered by rhizobia is the root hair wall.

Mechanisms of plant defense against microorganisms.

During the life cycle of a plant, its roots are exposed to a full spectrum of rhizosphere-colonizing organisms including saprophytic, beneficial, and pathogenic fungi and bacteria. The first line of defense against colonization by microbes is preformed structures including the cell wall. The wall is a matrix composed of cellulose fibrils, hemicelluloses, pectins, pectic acid, interwoven with a network of hydroxyproline-rich glycoprotein (extensin) (Lamport, 1986; Varner and Lin, 1989). A number of enzymes are also covalently or ionically bound to the wall matrix. In legume roots, additional proteins such as lectins and adhesins are found on root hair tips. Further protection of roots is thought to be provided by a hydrophobic suberin layer. Suberin is a heterogeneous polymer composed of both long chain (C16-C26) fatty acids and alcohols, and aromatic compounds as suggested by the appearance of p-hydroxybenzoic acid, vanillin, and syringaldehyde after nitrobenzene oxidation (Kolattukudy, 1977). While cutin is the

protective polymer covering the aerial parts of plants, suberin is thought to be the protective covering for roots and tubers. Thus far, analysis of the skin of root vegetables such as carrot, parsnip, and turnip, and the epidermis of young bean roots has shown that the characteristic components of suberin are present (Kolattukudy et al., 1975; Sijmons et al., 1985).

Plants have also evolved inducible structural and chemical defense mechanisms to resist attack by pathogens. The hypersensitive reaction (HR) is often cited as a host-specific defense mechanism. The HR is induced in incompatible (non-host) interactions with pathogenic bacteria. It is characterized by a rapid localized host cell death and necrosis of tissue which is thought to limit further pathogen ingress. The earliest event observed in the HR is the uptake of H^+ and concomitant efflux of K^+ within 30 min after inoculation of tobacco suspension cell cultures (Atkinson et al., 1985). After several hours there is decompartmentalization of the cells, loss of membrane integrity, a general efflux of ions, and finally cell death. The HR cell death can be delayed by high external pH (Salzwedel et al., 1989) and HR induced peroxidation of the host cell plasmamembrane can be inhibited by addition of the enzyme superoxide dismutase (Keppler and Novacky, 1987).

Another inducible defense response which is prevalent among the legumes is the production of antimicrobial metabolites known as phytoalexins. In legumes, pterocarpan derivatives of the phenylpropanoid pathway are primarily induced in the tissues being attacked by pathogens (Ingham, 1982). These compounds are all toxic to fungi while a few are also bactericidal; e.g. soybean glyceollin is

toxic to Pseudomonas syringae pv. glycinea (Wyman and Van Etten, 1978). It is thought that tolerance of host phytoalexins contributes to the virulence of a pathogen, although some non-pathogens are also tolerant (Smith and Banks, 1986). Virulent isolates of the fungus Nectria haematococca detoxify pisatin via pisatin demethylase while avirulent isolates are unable to do so (Van Etten et al., 1980). This system provides the best evidence for the role of tolerance to phytoalexins in the virulence of a pathogen. Many phenolic compounds also have antimicrobial properties. In the resistance response of tobacco and tomato, increased concentrations of phenolics such as scopoletin have been measured (Nadolny and Sequeira, 1980; Bashan et al., 1987).

A number of induced structural modifications of plant tissues are thought to hinder colonization by pathogens. In the epidermal cells of barley coleoptiles, resistance to Erisyphe graminis has been correlated with the rapid formation of oversized cell wall appositions known as papillae at the sites of attempted fungal penetration (Aist and Israel, 1986). Papillae are composed of callose and phenolic residues as indicated by histochemical staining and autofluorescence (Aist and Israel, 1986). In cucumber, non-host resistance and induced resistance to fungi were correlated with deposition of a lignin halo at the site of penetration in epidermal cells of hypocotyls (Hammerschmidt et al., 1985; Hammerschmidt and Kuć, 1982). Deposition of suberin is important in the potato tuber wound response (Kollattukudy and Dean, 1974) and in coating the infected vascular cells of resistant tomato plants inoculated with Verticillium albo-atrum (Street and Ellis, 1986). In melon and cucumber, fungal pathogens induce an increase in the hydroxyproline-rich cell wall glycoprotein known as extensin (Esquerré-

Tugayé and Lamport, 1979; Hammerschmidt et al., 1984). This increase in extensin in melon has been correlated with ethylene-inducible resistance to pathogens (Esquerré-Tugayé et al., 1979). Extensin also accumulates in the cell walls from cucumber seedlings subjected to heat shock. Such extensin-enriched walls are more resistant to pectolytic enzymes than walls from control plants (Stermer and Hammerschmidt, 1987).

Another type of response to infection is the production of pathogenesis related (PR) proteins. In tobacco, resistance to Peronospora tabacina involves systemic induction of both chitinase and β-1,3-glucanase (Ye et al., 1990). Other PR proteins have been identified as proteinase-inhibitors (Bowles, 1990). Numerous reports link an increase in the activity of certain isozymes of peroxidase with resistance responses (Campa, 1991). This enzyme is capable of catalyzing the cross-linking of the various wall strengthening polymers including lignin, suberin, and extensin (Campa, 1991). A detailed review of peroxidase literature appears later in this section.

The above inducible resistance mechanisms are rarely used alone but rather in concert. Since plants are constantly exposed to a legion of potential pathogens, the question remains as to how rhizobia avoid such defense mechanisms in the natural environment.

Plant responses to Rhizobium.

We already know the phenotype of homologous interactions--a nitrogen fixing nodule. Yet, to fully understand the spectrum of events which contribute to the determination of host specificity, it is also necessary to study the heterologous interactions. Vance (1983) suggested that the symbiotic interaction was a beneficial plant disease. A plant's molecular recognition of both pathogens and Rhizobium depends

upon the bacterial polysaccharides residing on the cell surface--EPS, CPS, and LPS (Keen and Holliday, 1982). In his review, Vance (1983) addressed the heterologous Rhizobium-legume interaction in asking: 1. Does pretreatment with heterologous polysaccharide affect infection by homologous bacteria? 2. Are there rhizobial (EPS?) suppressors of plant mechanisms that limit infection? In another review (Djordjevic et al., 1987a) Rhizobium is described as a refined parasite and the authors suggest that the role of lectins in successful infection is to titrate out EPS which would stimulate a defense response.

There are no outward signs of gross defensive responses or injury to the legume host after inoculation with wild type heterologous rhizobia. Still, this metabolically competent bacterium with a full complement of genes and enzymes necessary to infect a host, is unable to penetrate the heterologous root hair. Rhizobia presumably encounter phytoalexins in competition with other rhizosphere organisms which elicit plant defense responses. Rhizobia differ in degree of tolerance to various legume phytoalexins. The slow-growing Bradyrhizobium was sensitive to the major phytoalexins in clover, medicarpin and maakiain, with an Effective Dose (ED_{50}) = 10-60 ug/ml. R. leguminosarum bv. trifolii, bv. viciae, and R. phaseoli were much less sensitive to medicarpin and maakiain with an ED_{50} > 100 ug/ml (Pankhurst and Biggs, 1980). Pisatin purified from peas increases the generation time of R. leguminosarum bv. viciae in vitro, however pisatin was not detectable in pea nodules until bacteroids began to senesce at 30 d after inoculation (Van Iren et al., 1983). Chakraborty and Chakraborty (1989) report that homologous rhizobia did not elicit either of two phytoalexins in pea epicotyls after 5 d. Glyceollin was detected (175 pmole/mg dry weight)

but did not accumulate in either the effective nodules of Glycine max or the ineffective nodules of G. soja PI342434 after inoculation with B. japonicum USDA123 (Parniske et al., 1990). Parniske et al. (1988) found that soybean root hairs isolated after infection with B. japonicum contain 4 new flavonoid compounds, one identified as glyceollin I, although environmental factors can also lead to differences in the flavonoid pattern. Both B. japonicum and R. fredii show geneistein-inducible tolerance to glyceollin (Parniske et al., 1991). The authors conclude that other rhizosphere organisms probably stimulate phytoalexin production and it is the isoflavone-inducible tolerance to the phytoalexin which gives these rhizobia a competitive advantage in the rhizosphere.

Even in interactions between wild type R. leguminosarum bv. trifolii and clover, the majority of the infection threads that are initiated eventually abort (Nutman, 1959). One possible interpretation of such data is that infection threads abort because the plant limits infection by an invading organism with a defense-like response.

Recently, a number of workers claimed to have observed plant-defense responses in legumes when treated with certain mutants of Rhizobium. Inoculation of Macroptillium with an EPS over-producing mutant of NGR234 resulted in rapid accumulation of osmiophillic droplets in the epidermal cells of the host (Djordjevic et al., 1988). The authors suggest this is a hypersensitive response. Pühler et al. (1991) reported that autofluorescent (polyphenolic) materials accumulated in thickened cortical cell walls of pseudonodules on alfalfa induced by an EPS^- mutant of R. meliloti. They propose that EPS is essential to suppress a defense response which would prevent penetration by the

homologous rhizobia. Both of these reports show that plants are capable of responding to Rhizobium mutants defensively. It is not necessarily indicative of the phenomena which contribute to the host specificity of wild type rhizobia in the rhizosphere. What, then, is the plant response to a heterologous wild type Rhizobium and could such a response include a defense which would exclude the bacteria?

The following are possible models to explain why rhizobia are unable to infect the root hairs of a heterologous legume host.

1. Heterologous rhizobia do not bind to host root lectin. Lectins may have multiple functions in interactions with rhizobia, including attachment. Even though Diaz et al. (1989) overcame the host-specificity barrier by introducing the pea lectin gene into white clover, Al-Mallah et al. (1987) overcame the host specificity barrier by removing the tips of clover root hairs with exogenous cell wall degrading enzymes. Regardless of the bacterial mode of entry in this case, the data hint that attachment to the root hair wall is not the sole factor for the determination of host specificity. The data do not preclude the possibility that lectin was present on root hair plasmamembranes, however the non-legume Brassica napus could also be infected by Rhizobium with removal of the root hair wall (Al-Mallah et al., 1990).

2. The rhizobia do not have the enzymes to erode the wall of the heterologous host nor do the rhizobia specifically elicit the host's own wall degrading enzymes. Martinez-Molina et al. (1979) studied cellulase and hemicellulase activity in rhizobia and suggested that hydrolytic enzymes may be an additional factor in host specificity. Such enzymes may be important in distinguishing non-legume species such as in the

Gramineae where wall composition differs markedly from the Leguminosae. Mort's analysis of the root hair walls from a number of plant species showed that within the legume family, cell wall polysaccharide compositions appeared very similar (Mort and Grover, 1988). Even with similar compositions, a thorough analysis of the particular linkages within the wall matrix may yet reveal differences among legume genera. Theoretically, rhizobia that produce cellulases and pectinases should be able to penetrate any legume root hair given that wall substrates are similar. In addition to bacterial enzymes, homologous rhizobia and their isolated EPS stimulate the activity of clover root polygalacturonase (Ljundggren and Fåhraeus, 1961). It was believed that induction of host polygalacturonase led to softening of the root hair wall allowing penetration by the bacteria.

3. The flavones of the host do not effectively induce expression of the heterologous Rhizobium nod genes. Spaink et al. (1987) constructed hybrid strains containing nodABCIJ from R. leguminosarum pRL1JI and the nodD gene from R. leguminosarum, R. trifolii, or R. meliloti. The exudates from Melilotus alba, Pisum sativum, Vicia hirsuta, and Trifolium repens each stimulated the expression of all cloned nodD constructs to at least 50 % of the induction level of luteolin. Both wild type R. leguminosarum bv. viciae and R. leguminosarum bv. trifolii are stimulated equally by apigenin. There is a difference in stimulation by 7-hydroxyflavone (bv. trifolii is stimulated, bv. viciae is not). There may be some host-specificity dictated by the sensitivity of a Rhizobium strain to a particular flavone (Spaink et al., 1987), however clover exudates contain a mixture of stimulatory and inhibitory phenolic compounds (Djordjevic et al., 1987b). In addition, a number of

legume plants produce 7,4'-dihydroxy flavone (DHF) (Venkataraman, 1981) and therefore a more careful analysis of flavones in the rhizosphere is required to understand the contribution of flavones to host-specificity.

4. The heterologous Rhizobium is not recognized as a symbiont and therefore the plant's constitutive level of defense is not reduced sufficiently to allow penetration by the bacteria. All plants possess preformed deterrents to microbial colonization. Recognition of a beneficial microorganism probably involves biochemical cell-cell communication. Such recognition could occur at the level of attachment, during initial cell wall degradation by bacterial or host enzymes, in the release of biologically active bacterial polysaccharides which affect root hairs, other root hair curling factors excreted by the bacteria, or some as yet unknown product of bacterial hsn genes necessary to establish the symbiosis.

5. The heterologous Rhizobium is recognized as an invader and plant defenses are induced which exclude the bacteria from penetration. Plant-pathogen interactions which involve gene-for-gene recognition require a resistance gene in the host and an avirulence gene in the bacteria for expression of resistance (Ellingboe, 1981). Rolfe (pers. comm.) suggests that Rhizobium hsn genes act as avirulence genes since Tn5 mutation in ANU843 nodE (presumed loss of function) results in expanded host range to include peas. Rolfe et al.(1988) claim that increased flavone exudation stimulated by heterologous rhizobia represents a defense response, although the direct consequence of this flavone exudation is not discussed. It is thought that homologous EPS somehow masks elicitors of plant defense which are present in both homologous and heterologous rhizobia (Verma and Nadler, 1984; Djordjevic

et al., 1987a).

6. The heterologous rhizobia do not produce the appropriate extracellular "nod" signal. Lerouge et al. (1990) have isolated a compound called nodRm1 from R. meliloti. This compound is a sulfated-lipooligosaccharide whose synthesis is dependent upon host specificity genes and which is active in root hair deformation and initiation of cortical cell divisions at nanomolar concentrations. The biological activity is restricted to the host plants of R. meliloti.

Although the sixth hypothesis is currently the most popular, no one has yet demonstrated how nod signals alone might be affecting the root hair to allow homologous infections to occur. There must be some mechanism for the bacteria to penetrate the root hair wall. No one hypothesis has ever been shown to be definitive. Most likely, a combination of the above hypotheses for Rhizobium-host interactions will ultimately be needed to explain the determination of host specificity.

Thus, the work in this dissertation addresses one possible determinant contributing to host specificity and has been guided by the fifth hypothesis: namely that Rhizobium biovars are unable to infect a heterologous host because they are recognized as invaders and elicit plant defense reactions which then exclude the bacteria. Many plant resistance reactions to pathogens have been characterized which develop to detectable levels over the course of days. The work presented here examines only the very early phenomena in the interaction of rhizobia and root hairs which must play a role in the short window of opportunity when host specificity is determined. Several different plant reactions could contribute to host specificity, however this dissertation research focuses on the first structural barrier that rhizobia encounter--the

root hair cell wall and induced wall modifications. Numerous reports have correlated increased activity in plant cell wall peroxidase with resistance to microorganisms. Therefore, experiments were performed to test how homologous and heterologous rhizobia affect clover and pea root and root hair peroxidase. In addition, the influence of bacterial host-specificity genes on possible plant responses was examined using a collection of nod gene mutants and recombinants.

Peroxidase Review

The paradigm of plant peroxidases is horseradish peroxidase (Meunier, 1991). This enzyme has a MW of 42,100 and contains 308 amino acids, 1 Fe-protoporhyrin prosthetic group, 2 Ca^{++}, 17% carbohydrate from 8 neutral side chains each with a glycosyl composition of N-acetylglucosamine$_2$, mannose$_3$, fucose, and xylose (Dunford, 1991; Campa, 1991). The reaction catalyzed by peroxidase follows modified ping-pong kinetics with two 1-electron oxidations of two substrate molecules via activated enzyme intermediates formed by interaction with H_2O_2 as follows:

$$E + H_2O_2 \rightarrow E\text{-}I + H_2O$$
$$E\text{-}I + AH_2 \rightarrow E\text{-}II + {}^{\cdot}AH$$
$$E\text{-}II + AH_2 \rightarrow E + {}^{\cdot}AH + H_2O$$

where E is enzyme, A is substrate.

Peroxidase is irreversibly inactivated at pH 11 or greater due to the loss of heme, but is typically stable at room temperature and pH 5-10 (Dunford, 1991). Horseradish peroxidase has a pH optimum of 7.0 (Maehly, 1955); however the pH optimum for many plant peroxidases is slightly acidic (Fielding and Hall, 1978; Everdeen et al., 1988). Because of the strong redox properties of the oxidized forms of peroxidase and the observed long distance electron transfer processes of

proteins, it is possible for peroxidases to oxidize substrates which are not in the direct vicinity of the active site (Meunier, 1991). This explains why peroxidases can oxidize a large range of natural and artificial substrates including O-methoxyphenol (guaiacol), O-dianisidine, 3,3'-diaminobenzidine, 3-amino-9-ethylcarbazole, 4-methoxy naphthol, 2,2'-azino-di[3-ethyl-benzothiazoline-(6) sulfonic acid] (ABTS), pyrogallol, gallic acid, acetosyringone, 3-hydroxy-flavone, ferulic acid, vanillic acid, syringaldehyde, and indole-3-acetic acid (IAA) (Dunford, 1991; Gaspar et al., 1982; Grison and Pilet, 1985). It is thought that the true function of certain peroxidases is dictated by some substrate specificity in vivo.

Peroxidase has been detected in homogenates of leaves, stems, and roots from numerous plant species (Gaspar et al., 1982). Both cytoplasmic and wall-bound peroxidases have been isolated (Gaspar et al., 1982). Peroxidase may be covalently or ionically bound to cell walls. Treatment with cell wall degrading enzymes was necessary to release covalently bound peroxidase in maize roots and pea epicotyls (Grison and Pilet, 1985; Ridge and Osborne, 1970). Ionically bound wall peroxidases have been recovered from the intercellular fluid from vacuum infiltrated cucumber and barley leaves, lupin hypocotyls, the medium from suspension-cultured peanut cells, the stele, cortex, and tip of pea roots, and the surface of bean roots (Hammerschmidt et al., 1982; Srivastava and VanHuystee, 1977; Albert et al., 1986; Ros Barceló and Muñoz, 1989; Fielding and Hall, 1978).

Tissue prints of cross-sections from untreated pea epicotyls showed peroxidase activity was localized in vascular bundles. Plants treated with ethylene underwent cessation of elongation, increase in radial

growth, and appearance of peroxidase activity in the epidermal and cortical cells (Cassab et al., 1988). Pea roots, however, showed histochemical staining for peroxidase in epidermal cells, stele, and cortical cells (Fielding and Hall, 1978).

Density gradient centrifugation and electron microscopy have shown that peroxidase activity was most often associated with the central vacuole, rough endoplasmic reticulum, golgi bodies, the plasmalemma, and the cell wall (Gaspar et al., 1982). Cress root hair walls stained for peroxidase activity with the strongest reaction at the tips (Zaar, 1979). Golgi bodies within the root hairs also stained for peroxidase activity, thus suggesting the subcellular site of post-translational glycosylation (Zaar, 1979).

Plant peroxidases exist both as anionic (acidic) and cationic (basic) isozymes. Certain peroxidase isozymes differ in their substrate specificity (Endo, 1968; Grison and Pilet, 1985; Gibson and Liu, 1978). In pea tissue homogenates, some isozymes were unique to root, epicotyl, and leaf (Gibson and Liu, 1978). Van Huystee (Srivastava and Van Huystee, 1977), however, has shown that peanut isozymes are artifacts of interaction with phenols. Filtrate from peanut suspension cell cultures contained five different isozymes of peroxidase based on mobility in a native gel. Treatment of the filtrate with Dowex 1-X1 to remove phenolics resulted in the loss of 4 out of 5 isozyme bands. On the other hand, Ros Barceló and Muñoz (1989) suggest that the interaction of peroxidase isozymes with phenolic compounds exerts epigenetic control of peroxidase activity. Their data show that incubation of lupisisoflavone with 2 isozymes purified from lupin cell walls specifically converts the 2 isozymes to a third isozyme which gains the ability to oxidize

scopoletin. The authors propose that the flavone may act as an intermediate in the transfer of a hydrogen atom from scopoletin to peroxidase.

In other plant systems, peroxidase isozymes are selectively stimulated in response to particular stimuli. Kay and Basile (1987) showed that the stimulation or supression of certain peroxidase isozymes was correlated with different stages of organogenesis in tobacco. Wounding of cucumber hypocotyls and of potato tubers results in the increase in certain peroxidase isozymes (Svalheim and Robertsen, 1990; Espelie et al., 1986). In cucurbits challenged with fungi, induced resistance responses are associated with systemic stimulation of particular anionic isozymes (Hammerschmidt et al., 1982; Smith and Hammerschmidt, 1988). In a resistant barley cultivar, resistance to Erysiphe graminis was correlated with an increase in 2 isozymes which did not appear in wounded tissue and did not increase in the near isogenic susceptible cultivar (Kerby and Somerville, 1989).

There are reports that increased peroxidase activity in tobacco and tomato is not correlated with resistance to bacterial pathogens, but rather that increased concentration of phenolic compounds is important (Nadolny and Sequeira, 1980; Bashan et al., 1987).

The presence of peroxidase in cell walls and its potential catalytic activity suggests a possible function in cell wall synthesis. Extensin monomers are cross-linked via isodityrosine ether linkages and peroxidase catalyzes the formation of these cross-links in vitro (Everdeen et al., 1988). In xylem elements and woody tissues, cell walls are impregnated with lignin which is composed of derivatives of p-coumaryl, coniferyl, and sinapyl alcohols covalently bound in a

heterogeneous matrix (Lewis and Yamamoto, 1990). Again, the polymerization of known components of lignin can be achieved *in vitro* by peroxidase (Campa, 1991). Thus, by virtue of its ability to cross-link substrates *in vitro*, wall bound peroxidases are thought to participate in wall synthesis with wall-bound malate dehydrogenase supplying the necessary H_2O_2 from NADPH (Campa, 1991).

Cross-linking of suberin may also be catalyzed by peroxidase. A suberin-associated peroxidase has been immunochemically localized to wounded potato tuber tissues undergoing suberization (Espelie and Kolattukudy, 1985; Espelie *et al.*, 1986). In tomato, resistance to the wilt pathogen *Verticillium albo-atrum* is correlated with the deposition of a suberin coating in the xylem (Street *et al.*, 1986). The cDNA clone for the suberin-associated peroxidase from potato was used to probe Northern blots of total RNA from near isogenic lines of tomato. In tomato suspension cell cultures treated with fungal elicitors, the resistant line showed peroxidase message within 15 min while the susceptible line showed barely detectable hybridization in 3 hr (Mohan and Kolattukudy, 1990).

In addition to cross-linking activity, some peroxidase isozymes can degrade indole acetic acid (IAA) *in vitro*. A number of studies have shown a correlation between IAA catabolism by peroxidase and cessation of exponential plant growth (Gaspar et al., 1982). Cationic peroxidase isozymes have a higher oxido-reduction potential against IAA than do anionic isozymes (Gaspar *et al.*, 1982; Campa, 1991). Such cationic peroxidases have been found in tobacco roots and callus culture, and in isolated vacuoles from tobacco (Campa, 1991). In contrast, peroxidases isolated from cell walls are either strongly or weakly anionic isozymes

(Campa, 1991).

Another possible role for peroxidase in plant-microbe interactions is in the production of molecules with antimicrobial activity. Peroxidase and polyphenol oxidase (PPO) catalyze the oxidation of phenols to quinones which have greater antimicrobial activity than the substrates (Gaspar, 1982). Peroxidase also participates in the generation of active oxygen species, such as superoxide and peroxide, which are toxic to microbes (Elstner, 1982; Katsuwon and Anderson, 1989).

In summary, plant peroxidases have been studied extensively. Yet because of the diversity of substrates which it can accomodate, direct evidence for the role(s) of peroxidase has eluded researchers. None the less, the strong correlations between plant resistance to microorganisms and peroxidase activity make peroxidase an important target for study in the Rhizobium-legume symbiosis and therefore is the main focus of this dissertation.

CHAPTER ONE

NOD GENE DEPENDENCE FOR THE ELICITATION OF PEROXIDASE ACTIVITY FROM CLOVER ROOT HAIRS AND PEA ROOTS BY RHIZOBIUM AND THEIR CELL-FREE SUPERNATANTS

Abstract

Our objective was to test whether rhizobia elicited a peroxidase-dependent plant defense response which could affect the determination of host specificity in the Rhizobium-legume symbiosis. Heterologous Rhizobium leguminosarum biovars elicited increased specific activity of salt-elutable peroxidase from the surface of pea and clover roots. Likewise, the cell-free supernatant of R. leguminosarum bv. viciae also elicited increased peroxidase activity from clover roots. The excreted elicitor of peroxidase activity was flavone-dependent, heat stable, and ethanol soluble. Treatment of clover seedlings with the heterologous cell-free supernatant decreased the number of infected root hairs but not the number of nodule initiations formed by bv. trifolii. In infected root hairs with shepherd's crook deformations, the stain for peroxidase activity only accumulated at the site of infection thread initiation. Heterologous bv. viciae caused irregular root hair deformations with stain for peroxidase activity accumulating over the entire deformation where the bacteria were attached. In isolated clover root hairs, peroxidase activity began to increase 6 hrs after heterologous inoculation. In contrast, inoculation with homologous bv. trifolii suppressed peroxidase activity below the level of the

uninoculated control. The suppression was mimicked by treatment with purified EPS from bv. trifolii. After 12 hrs, the specific activity of root hair peroxidase increased to the level of the control. Thus during the period from 6-12 hrs after inoculation, suppression or elicitation of peroxidase activity may affect the structure of the root hair walls to either facilitate or prevent penetration by the bacteria.

The salt-elutable peroxidase from pea roots migrated as one weakly acidic isozyme in alkaline native gel electrophoresis which had greater activity after inoculation with the wild type biovar trifolii. Neither the bv. trifolii strain cured of its Sym plasmid nor the strain containing the cloned 8 kb fragment of pSym which carries the common nod genes elicited an increase in the specific activity of pea root peroxidase. The cloned 14 kb region containing the common nod genes and the hsn genes restored some elicitation of peroxidase but not to the level of the wild type. The 14 kb region is not sufficient to elicit the wild type level of peroxidase and therefore additional regions of pSym must play a role in the elicitation of peroxidase. Single Tn5 mutations in nodE or nodL effectively reduced the level of peroxidase activity elicited by these mutants which correlates with the extended host range phenotype for a nodE mutant of bv. trifolii. The wild type elicitation of peroxidase can be overcome by presence of homologous hsn genes in hybrid recombinants. Thus, hsn genes may interact with others present on pSym to control host-specificity through the modification of an elicitor which increases root hair peroxidase activity which in turn may alter the structure of the root hair wall at the site of incipient penetration.

Introduction

Rhizobium leguminosarum bv. *trifolii* forms a mutually beneficial symbiosis with *Trifolium repens* (white clover) as well as with other species of the genus *Trifolium*. Microscopy has revealed that the bacteria attach to root hairs, induce marked curling of root hairs, and then penetrate the root hair wall within the confines of an advancing infection thread (Nutman, 1959; Vincent, 1980). Ultimately, the bacteria are released from the infection thread into the cortical cells which have divided and formed a root nodule. The root nodule then becomes the site of nitrogen fixation.

Each biovar or species of *Rhizobium* has a specific host range. For example, *R. leguminosarum* bv. *trifolii* infects and nodulates clovers, *R. leguminosarum* bv. *viciae* nodulates peas and vetch, and *R. meliloti* nodulates alfalfa. The bacterial genes controlling host-specificity have been well characterized in a number of rhizobia (Horvath *et al.*, 1986; Debellé and Sharma, 1986; Djordjevic *et al.*, 1986; Martinez *et al.*, 1990). Among the *R. leguminosarum* biovars, the host specific nodulation (*hsn*) genes are *nodFERLMN* or *nodFELMN* for bv. *trifolii* and bv. *viciae*, respectively (Martinez *et al.*, 1990; B. Rolfe, pers. comm.). These genes are found on the symbiotic plasmid (pSym). The phenotype of bv. *trifolii* ANU843 with a *Tn5* insertion in *nodE* is Nod^+ Fix^- on peas, thus extending the host range of the strain (Djordjevic *et al.*, 1985). In order to extend the host range of bv. *viciae*, however, a vector containing bv. *trifolii* *nodFERLMN* (as in strain Rl300pRt290) must be introduced before efficient nodulation of white clover will occur (Djordjevic *et al.*, 1986).

The biochemical basis for host-specificity is currently under investigation in a number of laboratories. Among various hypotheses, it has been suggested that successful infection depends on the ability of homologous rhizobia to avoid eliciting a plant defense response (Vance, 1983; Djordjevic et al., 1987a; Pühler et al., 1991). Plant defense responses may be structural or chemical deterrants to infection by a microorganism. Inducible defense responses include hypersensitive host cell death (HR), production of antimicrobial phytoalexins, and the deposition of wall-strengthening polymers such as lignin, suberin, or extensin at sites of attempted penetration by a pathogen (Misaghi, 1982).

Some investigators have reported that certain Rhizobium mutants altered in EPS production are capable of eliciting an HR-like necrotic response in plant roots (Djordjevic et al., 1988; Pühler et al., 1991). They suggest that the EPS of homologous rhizobia allows successful infection to occur by masking cell surface determinants of plant defense. This interpretation carries the tacit assumption that heterologous rhizobia, lacking the proper EPS, induce plant defense responses which contribute to its inability to infect. It is likely that the determination of host specifcity involves a combination of mechanisms and plant defense may be one of them.

Host-specificity is determined during the interaction of Rhizobium with the host root hairs but prior to infection thread formation (Li and Hubbell, 1969). Al-Mallah et al. (1987) overcame the barrier to host specificity by treating clover roots with a mixture of cellulase and pectolyase. These enzymes degraded the wall at the tip of root hairs and allowed the heterologous R. loti to nodulate white clover. Thus,

the wall of clover root hairs appears to be an important component in the determination of host-specificity.

The primary plant cell wall is a matrix of cellulose fibrils, hemicelluloses, and pectins interconnected with a network of hydroxyproline-rich glycoprotein called extensin (Lamport, 1986; Varner and Lin, 1989). In addition, a number of enzymes are associated with the cell wall including ionically bound peroxidase (Gaspar et al., 1982). Peroxidase is capable of forming isodityrosine links in extensin and polymerizing the aromatic constituents of lignin and suberin in vitro (Everdeen et al., 1988; Lewis and Yamamoto, 1990; Espelie and Kolattukudy, 1985). Thus, increased peroxidase activity during plant defense responses is thought to increase the amount of cross-links in cell wall polymers which then prevents penetration by a pathogen.

Albert et al. (1986) isolated peroxidase from the surface of bean roots grown in both sterile and non-sterile soil. The activity of peroxidase isolated from non-sterile plants was greater than from sterile plants, suggesting that root colonizing organisms in the non-sterile soil stimulate greater peroxidase activity. A systemic increase in peroxidase activity is a part of the induced resistance response in cucumber inoculated with a fungal pathogen (Hammerschmidt et al., 1982). The activity of two specific peroxidase isozymes increased in a resistant barley cultivar inoculated with the pathogen Erysiphe graminis. The activity of the two isozymes neither increased in wounded plants, nor in the near isogenic susceptible cultivar after inoculation (Kerby and Sommerville, 1989).

In the present study, peroxidase activity was used as a measure of a plant defense response during the early interaction between rhizobia

and the roots of pea and clover. We hypothesized that rhizobia influence peroxidase activity, particularly in root hair walls, which then contributes to the success or failure of infection. We compared peroxidase activity from roots and root hairs after inoculation with homologous and heterologous rhizobia, as well as strains with various genetic constructions to explore the role of bacterial hsn genes in eliciting a defense response.

Materials and methods

Bacteria and cell-free bacterial washings.

Bacteria were grown at 30 C on agar plates containing Bergersen's modified medium (BIII, Dazzo, 1982), amended with the appropriate antibiotics when necessary. The strains used in this study, their source, and relevant characteristics are listed in Table 1. Bacterial inoculum was prepared by suspending 5 d-old bacteria in nitrogen-free Fahraeus medium (-NF, Dazzo, 1982) and adjusted to a density of Klett 5 (5×10^7 cells/ml) as measured with a Klett-Summerson colorimeter using the no. 66 red filter. To obtain cell-free bacterial washings, bacteria were grown for 3 d with and without 4 uM 4',7-dihydroxyflavone (DHF) or naringenin (Nar) on BIII agar plates. The bacteria were scraped off the plates and suspended in liquid -NF medium, gently shaken for 1 hr, and then centrifuged to pellet cells at 10,780 x g for 30 min. This bacterial wash fluid was sterilized by sequentially passing the supernatant through 0.8 um, 0.45 um, and 0.2 um Millipore filters. The filtrates were diluted with sterile -NF medium to an OD_{245} nm which was standardized among treatments for each experiment.

Isolation of EPS.

Strain ANU843 was grown at 30 C for 5 d on BIII agar plates without flavone. Cells were suspended in phosphate buffered saline (PBS), stirred for 30 min at 4 C, and then centrifuged at 16,270 x g for 1 hr. The supernatant was removed and concentrated to ca. 20 ml under vacuum on a rotary evaporator (40 C). Two volumes of cold 99 % ethanol were added and the mixture allowed to precipitate while gently stirring for 24 hr at 4 C. The mixture was then centrifuged at 20,190 x g for 1 hr at 4 C. The supernatant was removed and the EPS pellet dried under vacuum at room temperature for 30 min. The pellet was redissolved in ca. 100 ml of water, rapidly stirred at 4 C until the solution was homogeneous, and then placed in dialysis tubing (12,000-14,000 MWC). The EPS solution was dialyzed against water for 2 d at 4 C. Finally, the solution was concentrated to ca. 10 ml on the rotary evaportator, and then lyophilized for storage.

Seed sterilization.

Seeds of both pea cv. Little Marvel and clover cv. Dutch White were used unless otherwise listed. Seeds were surface sterilized by shaking seeds in 70 % ethanol for 4 min followed by 3 x 10 min washes with 1/10 strength commercial bleach, and finally several washes with sterile water.

Plant growth and inoculation.

For studies on peroxidase from clover roots, surface sterilized clover seeds were embedded in blocks of -NF medium solidified with 1 % purified agar and suspended on stainless steel wire mesh supports over 50 ml of liquid -NF medium in 9 cm covered glass dishes (Dazzo, 1982). Clover seeds were germinated and seedlings were grown for 3 d in a

growth chamber with a day/night regime of 14 hr light/10 hr dark, 23 C day/20 C night, and 70 % relative humidity. For inoculation or treatment of roots in these wire mesh assemblies, the original plant growth medium in each dish was removed and replaced with either 50 ml of bacterial inoculum suspended in fresh -NF or 50 ml of sterile bacterial wash fluid for 24 hr.

For studies on pea roots, surface sterilized seeds were germinated in the dark in sterile water for 2 d, then placed on -NF agar plates (3 seedlings per plate) and incubated vertically in the growth chamber for 2 d. Pea roots were then inoculated by applying 5 drops of a Klett 5 bacterial suspension to each root. The plates were then covered, incubated flat at room temperature for 1 hr, and then incubated vertically in the growth chamber for 24 hr.

Treatment of clover roots with isolated EPS.

The EPS from strain ANU843 was dissolved in sterile -NF medium at concentrations of 5, 50, and 500 ug/ml. EPS solutions were added to clover roots in wire mesh assemblies and incubated for 24 hr.

Removal of roots.

Clover and pea roots were removed after 24 hrs of incubation with bacteria, bacterial wash fluid, or purified EPS. Clover roots were obtained by immersing the wire mesh in a dish of liquid nitrogen. The frozen roots were then broken off into a chilled beaker. Pea roots were cut from cotyledons with a razor blade. Isolated roots were stored at -20 C.

Clover growth and inoculation for studies with root hairs.

Seeds were germinated for 2 d on inverted -NF agar plates. Seedlings were inoculated by gently shaking plants in a suspension of

bacteria (Klett 5) for 30 min. For microscopy of root hairs, seedlings were then incubated vertically on -NF agar plates for 1-5 d. For isolation of root hairs, inoculated seedlings were rinsed with sterile water, separated on moist filter paper, covered, and then incubated for 0, 6, 12, or 24 hr in the growth chamber. Seedlings were then placed in glass vials and immersed in liquid nitrogen. The frozen vials were shaken vigorously according to the method of Gerhold et al. (1985) to fracture the the root hairs. Fragments of roots were poured out, the vials were thawed, and 1.5 ml of 1 M NaCl in water was added to each vial. The inside walls of the vials were rinsed with the NaCl solution to suspend the root hairs, and then root hair suspensions were pooled.

Peroxidase localization in clover root hairs.

Intact seedlings were placed on glass microscope slides, a coverslip added, and the substrate mixture (12 mg 3,3'-diaminobenzidine in 3 ml 60 mM Na-K-phosphate buffer, pH 5.5, with 50 ul H_2O_2, Zaar 1979) injected under the coverslip. The seedlings were rinsed with -NF medium after 5 min of incubation at room temperature. Root hairs were then observed on a Zeiss photomicroscope using the quartz-halogen lamp for brightfield illumination. Photos were taken with Kodak Plus-X black and white film and Kodak Ektachrome color slide film.

Isolation of peroxidase.

To isolate peroxidases from root surfaces or root hairs, plant tissue was placed in 1 M NaCl and subjected to a sonic bath (Cole-Parmer Ultrasonic Cleaner) for 20 min at 4 C. This procedure eluted proteins from surfaces with minimal damage to cells. The debris was pelleted by centrifugation at 5000 x g and the supernatant transferred to dialysis tubing (12,000-14,000 MWC) and desalted in water for 2 d at 4 C. The

protein content of samples was measured by the Bradford dye binding assay using BSA as a standard.

Peroxidase assay.

For kinetic studies of peroxidase activity, the total salt-eluted protein solution was assayed for peroxidase activity by adding 1-100 ul of sample to 1 ml of reaction mixture in a cuvette. The reaction mixture was freshly made each day with 50 ml of 10 mM Na-phosphate buffer, pH 6.0, 125 ul guaiacol (Sigma), and 350 ul H_2O_2 (Hammerschmidt et al., 1982). The increase in OD_{470} due to the formation of tetra-guaiacol was measured for 3 min on a Gilford Response^{tm} UV/VIS scanning spectrophotometer. The Gilford kinetics software package was used to calculate the initial rate of the reaction as OD_{470}/min.

Separation of isozymes by electrophoresis.

Native polyacrylamide gel electrophoresis was performed to separate acidic peroxidase isozymes. Alkaline running gels were made 1 mm thick with 7.5 % acrylamide in 0.15 M Tris-HCl buffer at pH 9.3. The stacking gels were 2.5 % acrylamide in 0.02 M Tris-phosphoric acid buffer at pH 6.7. The lower electrode buffer was 0.1 M Tris-HCl, pH 8.8, and the upper electrode buffer was 0.04 M Tris-glycine, pH 9.6 (Biochemical Handbook). Samples containing 2 ug of protein were mixed with 5X sample buffer (0.5 M Tris-HCl, pH 6.8, 0.05 % w/v bromphenol blue, and 10 % glycerol v/v). Samples were run through the stacking gel at 10 mA, and then run at a constant current of 24 mA until the bromphenol blue had migrated 3/4 of the length of the gel. The electrophoresis apparatus was cooled by running tap water.

Staining gels for peroxidase activity.

Peroxidase isozymes were visualized by soaking the gel in a solution containing 190 ml of 50 mM Na-acetate buffer, pH 5.5, 40 mg of 3-amino-9-ethyl carbazole (Sigma) dissolved in 10 ml of N,N-dimethyl formamide, and 66 ul of H_2O_2. The reaction was stopped after 20 min by rinsing gels with water. Gels were stored in a solution of 50 % methanol, 5 % acetic acid v/v in water.

Heat stability and ethanol fractionation of cell-free bacterial washings.

The bacterial wash fluid from bv. viciae strain 300 which elicited clover peroxidase (OD_{245} 0.18) was divided into two aliquots. One aliquot was autoclaved for 20 min and then centrifuged to remove any precipitates. The original and the autoclaved aliquot were each added to clover roots in wire mesh assemblies (50 ml per assembly) and incubated for 24 hrs. Roots were isolated and peroxidase activity was assayed as described above.

To fractionate the bacterial wash fluid, 2 volumes of cold 95 % ethanol were added to precipitate ethanol-insoluble components. The mixture was centrifuged at 10,000 x g for 40 min at 4 C. The ethanol supernatant was removed, evaporated to dryness under vacuum, and the residue redissolved in a volume of sterile -NF medium equal to the original sample volume. The ethanol-insoluble pellet (primarily polysaccharides) was also redissolved in the same volume of -NF medium. Fifty ml each of the ethanol-soluble and the ethanol-insoluble fractions were incubated with clover roots for 24 hr. The roots were then isolated and the peroxidase activity was assayed.

Infection thread and nodule initiation bioassays.

The cell-free supernatants from broth-grown bacteria were used in infection thread and nodule initiation bioassays. Bacteria were grown at 30 C in shaken flasks (175 RPM) containing BIII medium for 2-3 d. The medium was supplemented with 2 uM DHF or naringenin for ANU843 and R1300, respectively. The bacteria were pelleted by centrifugation at 16,000 x g for 30 min and the supernatants were filter-sterilized as previously described. Supernatants were diluted with BIII medium to an OD_{245} of 0.625-0.633. Seeds of the clover cultivars Dutch White, Ladino, Louisiana S-1, and Nolin & LaBorde '83 were germinated in the dark on inverted -NF agar plates for 1 d, and then seedlings were transferred to fresh -NF agar plates and incubated vertically for 1 d in the growth chamber. Seedling roots were then treated by adding 40 ul of bacterial supernatant and covering with a sterile 18 mm^2 coverslip positioned with the lower edge just below the root tip. After incubating for 4 hr at room temperature, the coverslip was lifted and 10 ul of ANU843 inoculum was applied without rinsing and the coverslip replaced. The final inoculum density was 10^5 cells/seedling. Seedlings were incubated vertically for 4 d, then mounted on microscope slides and stained with 0.01 % methylene blue dissolved in -NF medium. Excess stain was rinsed away with -NF medium and the number of infection threads counted using phase contrast microscopy. Later, the seedlings were submerged in 33 % bleach and cleared under vacuum for 15 min. The seedlings were rinsed with water, restained with methylene blue, and the number of nodule initiations in the root cortex were counted. Root length was measured from the root tip mark made at the time of inoculation to the root tip at the time of staining.

Table 1. *Rhizobium leguminosarum* strains used in this study.

strain no.	description	nod phenotype clover	nod phenotype pea	source/ref.
bv. trifolii				
ANU843	wild type (wt)	+	-	Rolfe/Djordjevic 1983
ANU845	pSym- deriv. of 843	-	-	Rolfe/Djordjevic 1983
ANU297	843, pSym *nodE*::Tn5, Km^r	delayed +	+	Rolfe/Djordjevic 1985
ANU251	843, pSym *nodL*::Tn5, Km^r	+	-	Rolfe/Weinman 1988
ANU032	845pRT032 (14kb HindIII fragment *nodJICBADFERLMN* from 843 pSym, cloned into pKt240), Cbr	+	-	Rolfe/Schofield 1984 and Djordjevic 1985
ANU910	845pRT032Δ910 (8 kb fragment *nodJICBAD* in pKt230), Cbr, Km^r	-	-	Rolfe/Innes 1985
bv. viciae				
RI300	wild type	-	+	Rolfe/Brewin 1980
5039	Rif^r deriv. of wt 248 with pRL1::Tn5 in nonsymbiotic gene, Km^r	-	+vetch	Wijffelman/unpub.
RBL601	5039, pRL1JI *nodE1*:Tn5 (=pRL601), Km^r	+	delayed +vetch	Wijffelman/Wijffelman 1985 and Salzwedel unpub.
1003	Rif^r deriv. of wt 1001	-	+	Squartini /Ph.D. Dissertation
hybrids				
ANU290	RI300pRt290 (9 kb BamH1 frag. *nodFERLMN* from pSym of ANU843 in pKt230), Km^r	+	+	Rolfe/Djordjevic 1986
843-85	Rt843pKTK85 (8.4 kb Kpn1 fragment from RI1001 in pKt230), Sm^r	(Hac+ vetch)		Squartini and Salzwedel /unpub.

[a]Abbreviations and concentrations for antibiotics: Km, kanamycin 30 ug/ml, Cb, carbenicillin 75 ug/ml, Sm, streptomycin 250 ug/ml, Rif, rifampicin 20 ug/ml.

[b]Address of Sources: Barry Rolfe, Plant Microbe Interactions Groups, Research School of Biological Sciences, Australian, National University, Canberra, A.C.T. 2601, Australia.

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Results

Heterologous rhizobia elicited an increase in the specific activity of peroxidase eluted from the roots of pea and clover (Fig. 1). The activity from pea roots inoculated with bv. trifolii was 2-fold greater than from roots those incubated with homologous bv. viciae or the -NF control for 24 hrs. Similarly, the activity from clover roots incubated with bv. viciae strains 5039 and 1003 was significantly greater than from the -NF control, but not significantly greater than the homologous combination after 24 hrs. The peroxidase activity from clover roots inoculated with strain R1300 was not significantly greater than either the -NF control or the homologous ANU843.

Native polyacrylamide gel electrophoresis shown in Fig. 2 revealed 4 acidic peroxidase isozymes from clover roots. One sharp band of activity was extremely mobile, 2 broader bands had moderate mobility, and the 4th band was sharp and appeared just at the interface of the stacking and the 7.5 % running gel. This 4th band stained more intensely in the lane containing protein from clover roots inoculated with heterologous R1300 compared to the other 3 bands which appeared equivalent to the intensity for samples from the homologous combination. In contrast, pea roots yielded only one sharp band of activity with low mobility just past the interface between the stacking and running gels. The intensity of this isozyme was increased after inoculation with the heterologous ANU843.

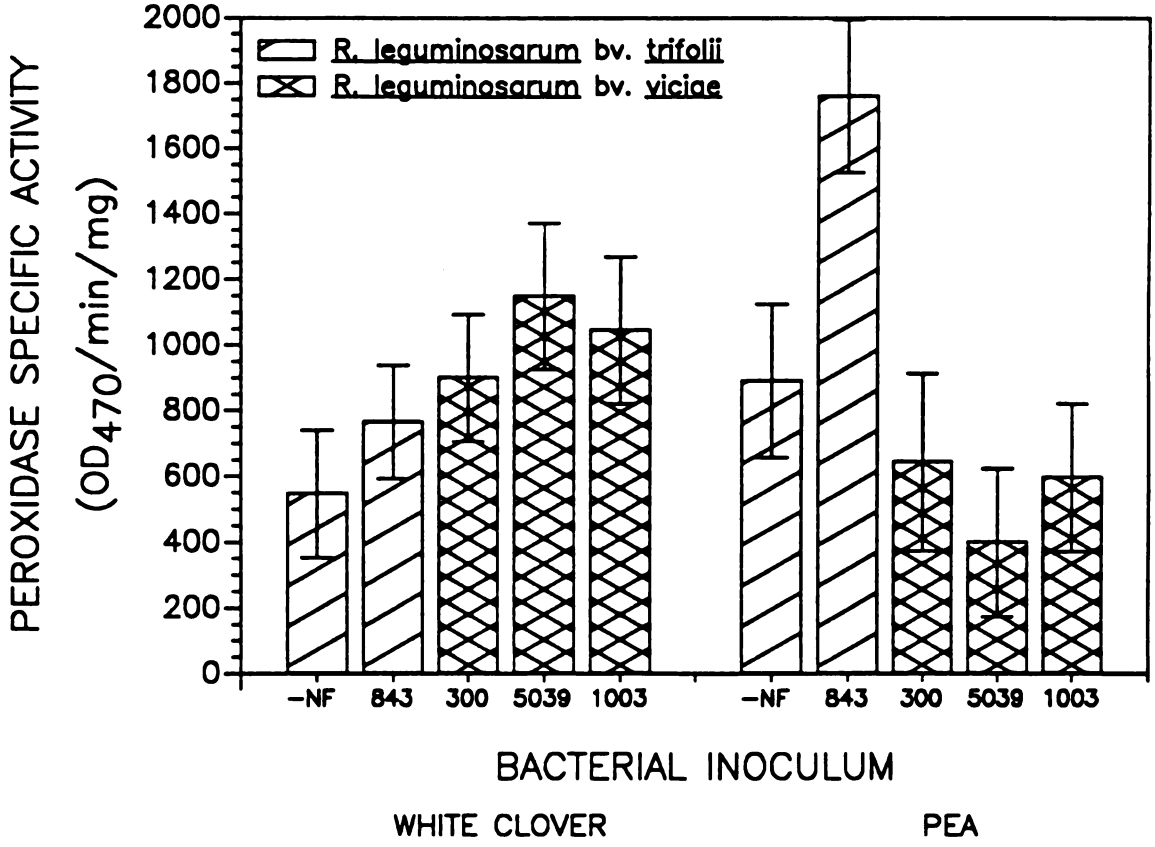


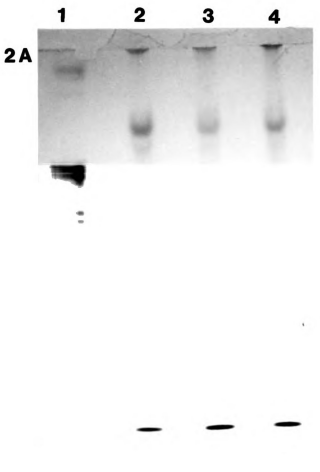
Fig. 1. Specific activity of peroxidase eluted from clover and pea roots 24 hr after inoculation with 5 x 10^7 cells/ml suspension of wild type rhizobia. Each bar represents the mean of at least 3 experiments +/- SE. Nitrogen-free Fahraeus medium (-NF) was used as the control.

Fig. 2.

A. Native polyacrylamide gel stained for peroxidase activity with 3-amino-9-ethyl carbazole. Peroxidase was isolated from clover roots 24 hr after inoculation with rhizobia. Each lane was loaded with 2 ug of protein. Treatments included: lane 1 horseradish peroxidase, lane 2 - NF control, lane 3 ANU843, lane 4 R1300.

B. Native polyacrylamide gel stained for peroxidase activity with 3-amino-9-ethyl carbazole. Peroxidase was isolated from pea roots 24 hr after inoculation. Treatments included: Lane 1 -NF control, lane 2 ANU843, lane 3 R1300.

C. Native gel made with a 3-10 % gradient of acrylamide to illustrate the homogeneity of the band at the interface of 7.5 % native gels.


1
2
3
4
2A


C

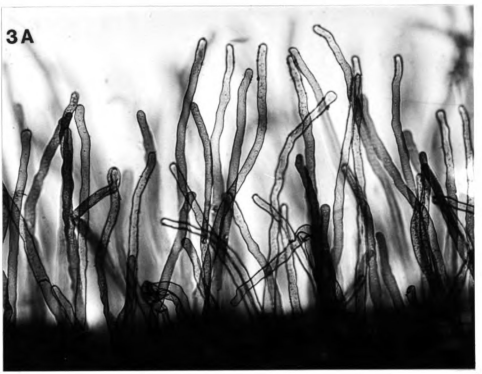

1
2
3
B

The location of peroxidase activity in clover root hairs was shown by the deposition of the insoluble brown product of DAB oxidation (Fig. 3). Uninoculated clover root hairs had an even distribution of golden-brown stain over the entire root hair (Fig. 3A). Clover root hairs formed characteristic shepherd's crooks after inoculation with homologous ANU843 (Fig. 3B,C). In these root hairs, stain for peroxidase activity only accumulated at the point of infection thread initiation both 1 d and 5 d after inoculation. The infection thread itself did not accumulate any more stain than the background. In contrast, after inoculation with heterologous Rl300, clover root hairs show dark deposits over the entire irregular deformation (Fig. 3D).

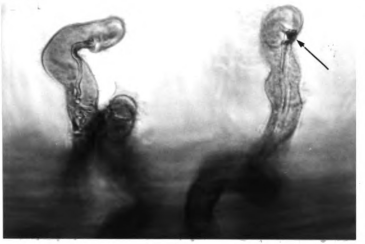
Fig. 3. White clover root hairs stained for *in situ* peroxidase activity with DAB + H_2O_2, pH 5.5.

A. uninoculated
B. 1 d post-inoculation with ANU843
C. 5 d post-inoculation with ANU843
D. 5 d post-inoculation with Rl300

Arrows indicate areas of enhanced staining.



3B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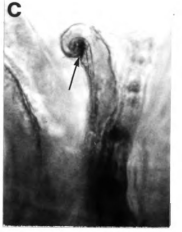
C


3D


D

Clover root hairs were isolated at 0, 6, 12, and 24 hrs after inoculation to determine when peroxidase activity began to increase. The specific activity of peroxidase elicited by R1300 began to increase 6 hrs after inoculation and was greater than either the -NF control or the homologous ANU843 at 12 and 24 hrs (Fig. 4). Surprisingly, the homologous ANU843 suppressed the specific activity of peroxidase from root hairs compared to the -NF control at 0 and 12 hr after inoculation. (Those samples designated time 0 were harvested immediately after addition of inoculum, however preparation of the tissue took aproximately 15-30 min). At 6 hr after inoculation, specific activities for the -NF control and the ANU843 treatment were not significantly different. The specific activity began to increase 12 hrs after inoculation with ANU843, although not significantly greater than the -NF control.

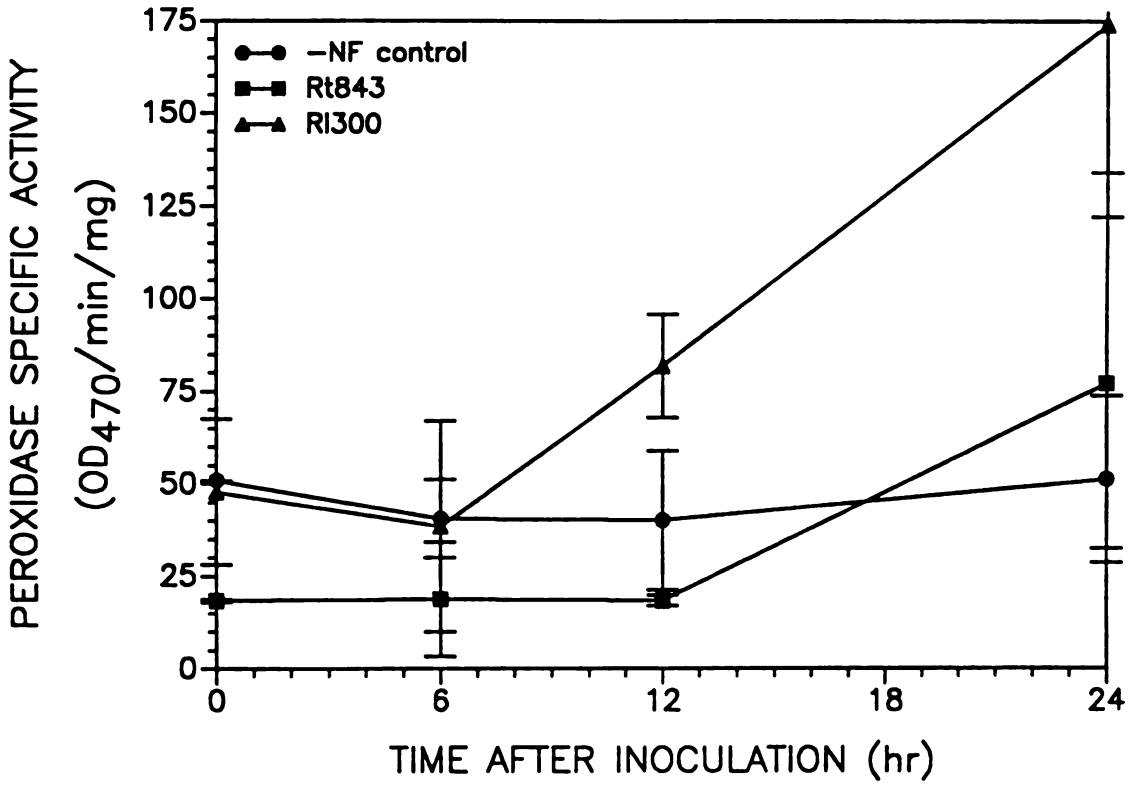

Fig. 4. Specific activity of peroxidase from isolated clover root hairs at 0, 6, 12, or 24 hr after inoculation with wild type rhizobia. Data points represent the mean of 2 experiments +/-SE.

The purified EPS from homologous ANU843 also suppressed the specific activity of peroxidase from clover roots (with root hairs intact) after 24 hrs of incubation (Table 2). Treatment with EPS at 500 ug/ml yielded aproximately 2-fold lower specific activity of root peroxidase compared to the -NF control. Treatment with EPS at 5 or 50 ug/ml resulted in peroxidase activity similar to the -NF control.

Table 2. Effect of purified EPS from bv. trifolii ANU843 on specific activity of peroxidase eluted from clover roots.

treatment	specific activity OD_{470}/min/mg protein
-NF control	794
EPS 5 ug/ml	857
EPS 50 ug/ml	767
EPS 500 ug/ml	416

[a]Seedlings were incubated with EPS solutions for 24 hr.

[b]Data are from one experiment.

The heterologous elicitation of pea root peroxidase was dependent upon the presence of pSym in bv. trifolii. The pSym-cured strain (ANU845) did not elicit an increase in peroxidase activity from pea roots, but rather suppressed the activity below the level from the homologous combination (Fig. 5). Strain 845pRT032 (containing the entire 14 kb nod region from ANU843) elicited slightly more peroxidase activity than strain ANU845, although not as much as did the heterologous wild type ANU843 (Fig. 5). Strain 845pRt032Δ910 (8kb fragment containing common nod genes but not the hsn genes) did not elicit an increase in specific activity from pea roots with the level similar to that found with strain ANU845. For clover roots, ANU845, 845pRT032, 845pRT032Δ910, and ANU843, all elicited specific activities

similar to the -NF control.

The specific activity of peroxidase from pea roots inoculated with the bv. trifolii nodE::Tn5 mutant (strain ANU297) was aproximately 2-fold less than the activity elicited by the heterologous wild type ANU843 (Fig. 6). Similarly, the peroxidase activity from clover roots inoculated with the bv. viciae nodE::Tn5 mutant (strain RBL601) was less than the activity elicited by the heterologous wild type 5039. On pea, the bv. viciae nodE::Tn5 mutant elicited peroxidase activity similar to that of the homologous wild type 5039. On clover, however, the bv. trifolii nodE::Tn5 mutant elicited slightly greater specific activity of peroxidase than did ANU843.

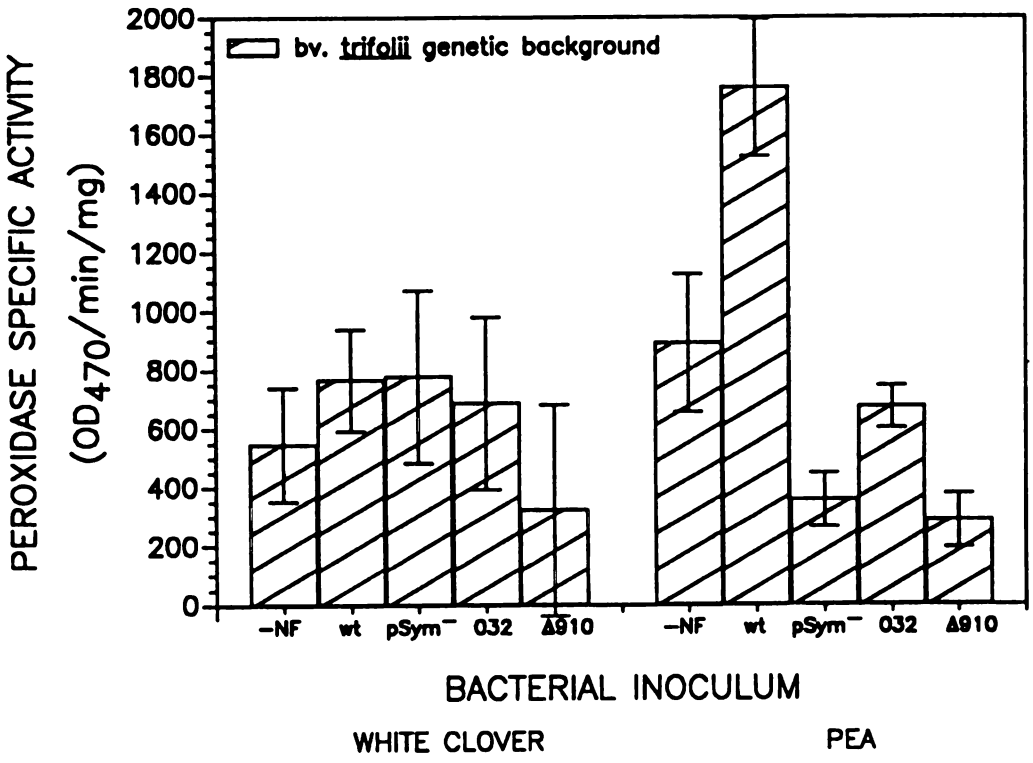

Fig. 5. Specific activity of peroxidase eluted from clover and pea roots 24 hr after inoculation with the pSym-cured bv. trifolii strain or recombinants containing cloned fragments from pSym in the pSym-cured background. -NF = control, wt = wild type, pSym- = ANU845, 032 = 845pRT032 containing 14 kb nod gene region, Δ910 = 845pRT032Δ910 containing 8 kb nodJICBAD. Bars represent the mean of 3 experiments +/- SE.

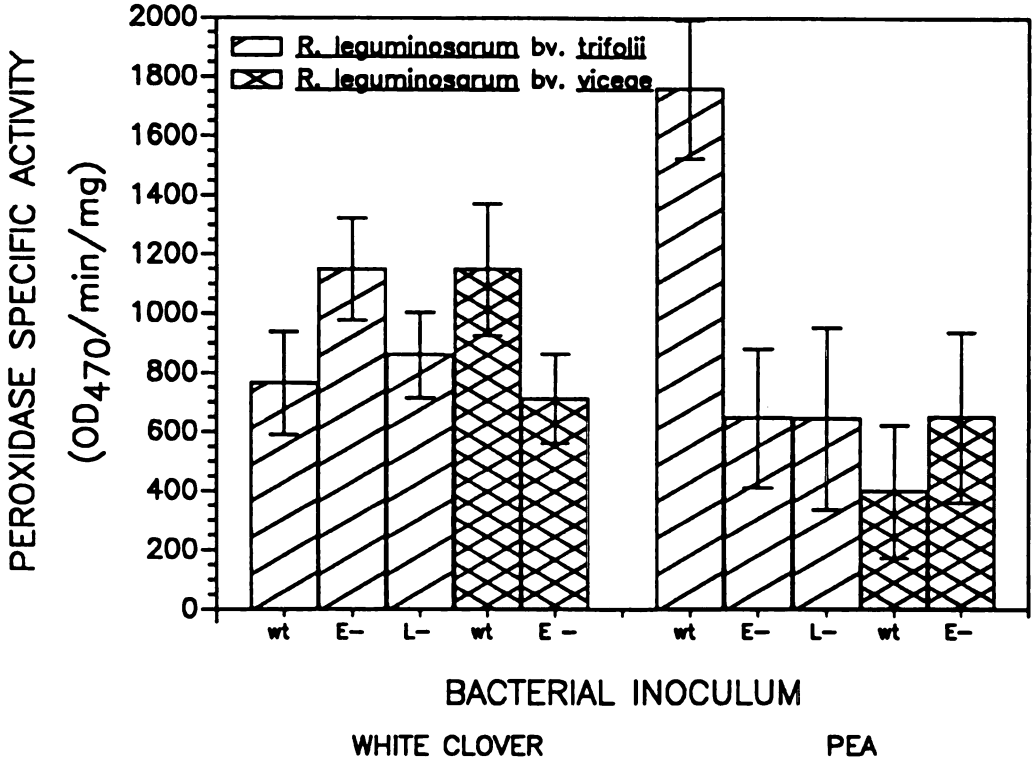

Fig. 6. Specific activity of peroxidase eluted from clover and pea roots 24 hr after inoculation with suspensions of wild type (wt) rhizobia or their respective nodE::Tn5 and nodL::Tn5 mutants. Bars represent the mean of 3 experiments +/- SE.

Further effects of the Rhizobium hsn genes were tested using hybrid recombinants. Specific activity of peroxidase from pea roots after inoculation with 843pKTK85 (pKTK85 contains nodDFELMN genes from R11003) was significantly less than that elicited by the heterologous wild type ANU843 (Fig. 7). This specific activity was also lower than that elicited by the homologous R1300. The specific activity elicited by R1300pRt290 (containing the nodFERLMN genes from ANU843) on peas was not significantly different than the activity elicited by R1300. For clover roots, 843pKTK85, R1300pRt290, ANU843, and R1300 all elicited similar peroxidase activity.

The cell-free bacterial washing from heterologous R1300 grown with naringenin elicited increased peroxidase activity in clover roots while the bacterial washing from R1300 grown without the flavone did not (Fig. 8). The specific activity after treatment with the bacterial washing from ANU843 grown with DHF was similar to treatment with washing from ANU843 without flavone, R1300 without flavone, and the -NF control.

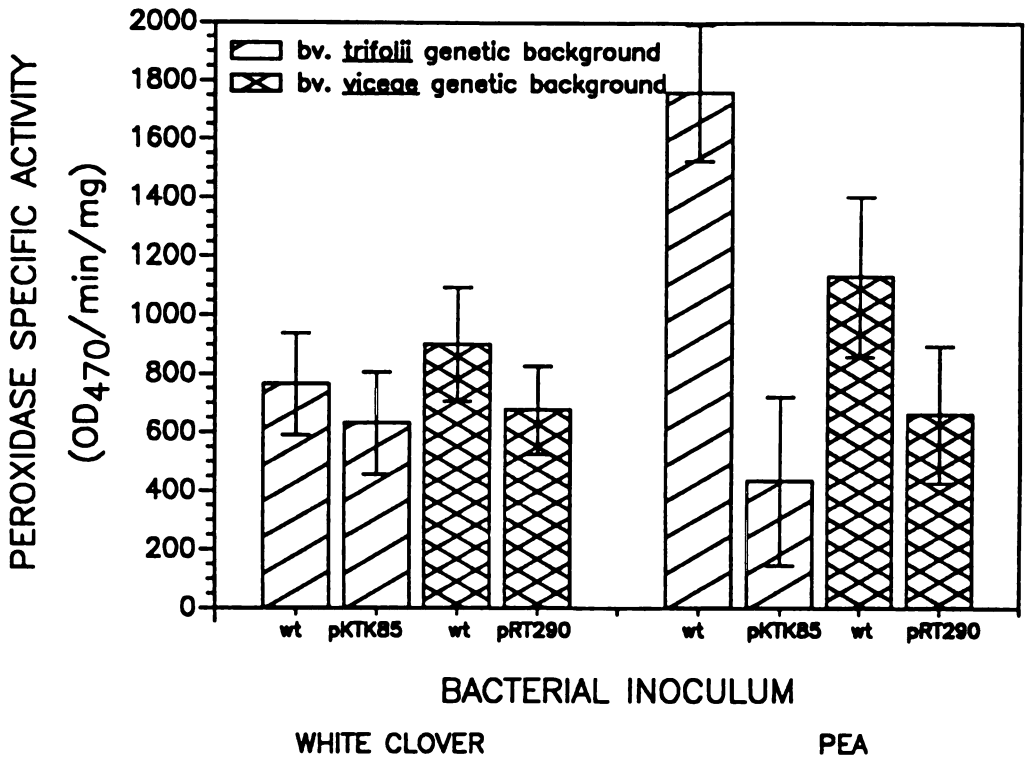

Fig. 7. Specific activity of peroxidase eluted from clover and pea roots 24 hr after inoculation with wild type or hybrid recombinant strains carrying heterologous hsn genes. Wt = wild type, pKTK85 = plasmid carrying bv. viciae nodDFELMN genes, pRT290 = plasmid carrying bv. trifolii nodFERLMN genes. Bars represent the mean of 3 experiments +/- SE.

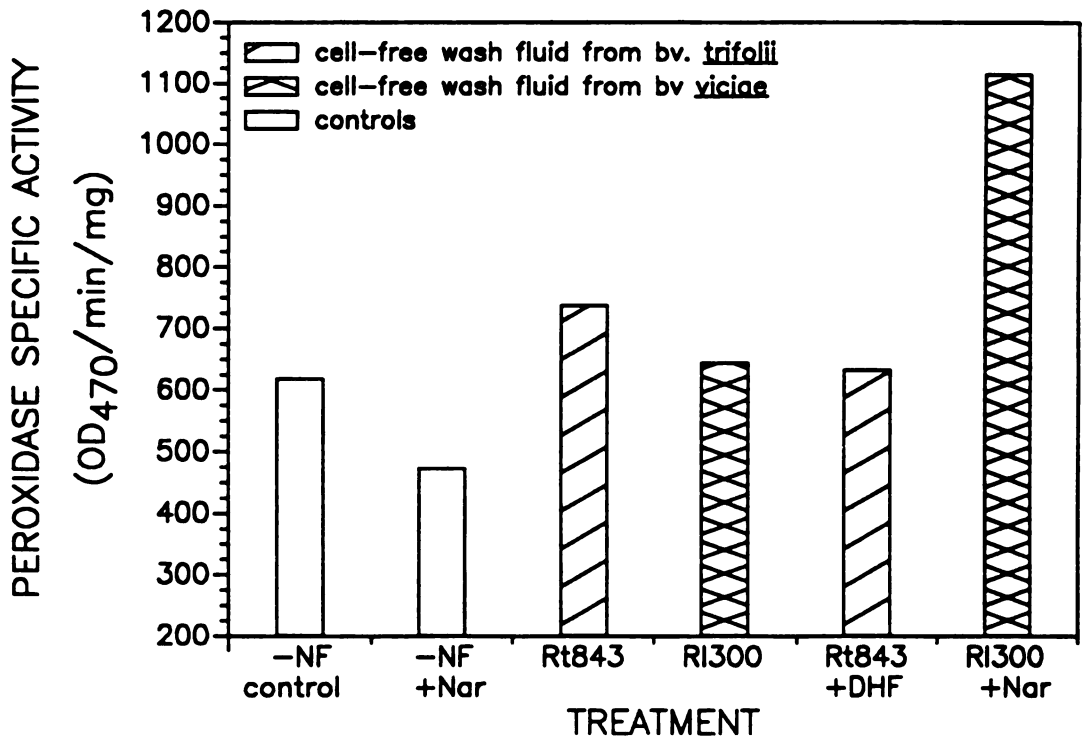

Fig. 8. Specific activity of peroxidase eluted from clover roots 24 hr after treatment with standardized cell-free bacterial washings (OD_{245} = 0.08-0.12) from bacteria grown on plates with and without 4 uM flavone. Data are from one experiment.

The cell-free wash fluid from R1300 grown with DHF also elicited an increase in peroxidase activity from clover roots (Fig. 9). Autoclaved wash fluid retained its ability to elicit peroxidase activity. After fractionation of the wash fluid, the ethanol fraction elicited greater peroxidase activity than the -NF control (Fig. 10). The fraction containing the ethanol-insoluble pellet also elicited some clover root peroxidase activity compared to -NF, although it was less than the ethanol-soluble fraction.

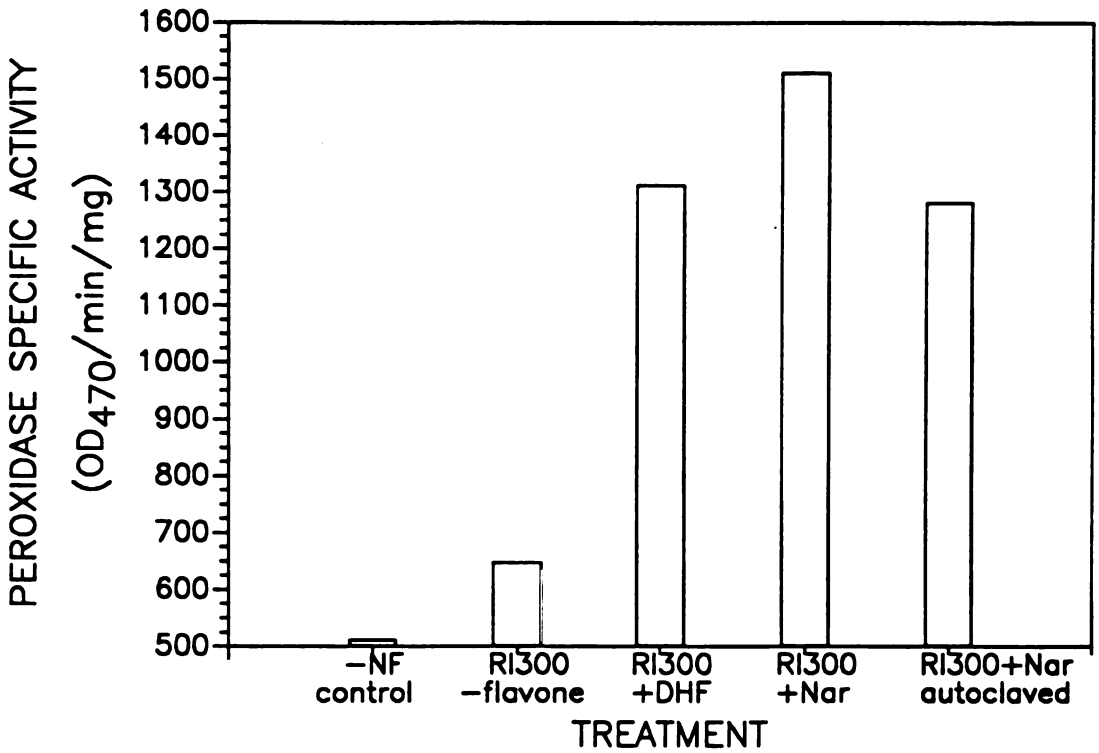

Fig. 9. Specific activity of peroxidase eluted from clover roots 24 hr after addition of autoclaved bacterial washing (OD_{245} = 0.18) from RI300 grown with 4 uM flavone. Data are from one experiment.

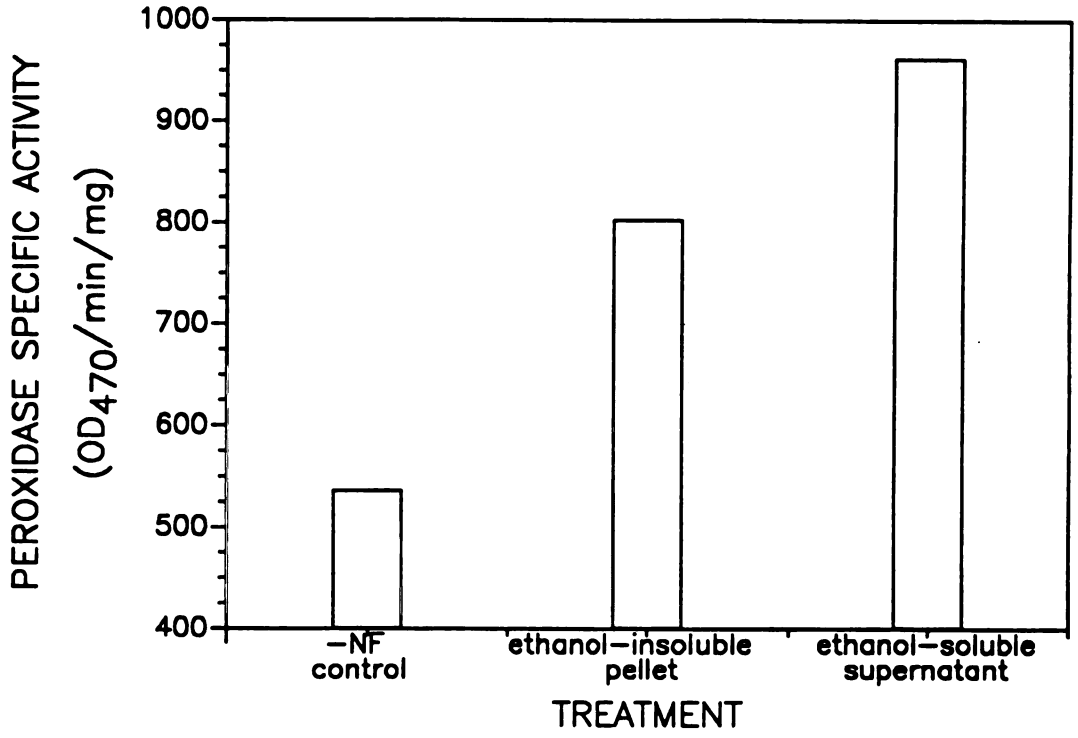

Fig. 10. Specific activity of peroxidase eluted from clover roots 24 hr after treatment with the ethanol-insoluble pellet and ethanol-soluble supernatant fractions of bacterial washings from R1300 grown with 4 uM naringenin. Data are from one experiment.

Culture supernatant from broth-grown bv. viciae reduced the number of infected root hairs but not the number of nodule initiations formed by bv. trifolii on white clover cultivars (Table 3). Root length was not affected. Supernatant from ANU843 grown with DHF increased the number of infected root hairs and nodule initiations compared to the BIII control for cultivar Dutch White.

Table 3. Effect of culture supernatant from broth-grown R. leguminosarum bv. viciae on number of infected root hairs and nodule initiations formed by ANU843 on white clover cultivars.

	Inf/pl		Noi/pl		Root Len./pl (mm)	
CV	BIII	Rl300 sup	BIII	Rl300 sup	BIII	Rl300 sup
1	22.2 ±4.8	1.8 ±0.1	0.7 ±0.3	1.4 ±0.4	10.7 ±1.2	13.7 ±1.7
2	6.7 ±2.1	0.9 ±0.4	0.9 ±0.4	1.4 ±0.4	8.7 ±1.2	9.6 ±0.8
3	8.0 ±4.2	2.4 ±0.9	0.3 ±0.2	0.1 ±0.1	7.8 ±1.4	7.2 ±0.6
4	4.4 ±1.7	1.3 ±0.4	0.0 ±0.0	0.3 ±0.2	5.3 ±0.8	4.8 ±0.6

[a]Cultivar 1 Dutch White, 2 Regal Ladino, 3 Louisiana S-1, 4 Nolin & LaBorde '83.

[b]Forty ul of filter-sterilized culture supernatant from Rl300 grown with 2 uM naringenin (OD_{245} = 0.625) was incubated for 4 hr followed by inoculation with 10^5 cells/seedling of ANU843 without rinsing. Plants were incubated for 4 d. Data are the means per seedling +/-SE, n = 9.

[c]Treatment of cultivar Dutch White with supernatant from ANU843 grown with 2 uM DHF having an OD_{245} = 0.633 followed by inoculation with ANU843 resulted in 30.4 +/-3.0 infected root hairs per plant and 4.1 +/-0.7 Noi per plant.

Discussion

A number of factors are likely to contribute to the determination of host-specificity in the Rhizobium-legume symbiosis. Although peroxidase activity is required for the normal growth and maturation of plant cell walls, localized increases in peroxidase activity contribute to defense against invading microorganisms. We hypothesized that such a plant defense response hindered penetration of root hairs by

heterologous rhizobia. Cytochemical localization of peroxidase activity in clover root hairs provided the initial support for this hypothesis.

The extensive staining of irregular clover root hair deformations in heterologous combination indicates increased peroxidase activity in response to attempted penetration by the bacteria. Such enzyme activity could modify the structure of the root hair wall and thus make it more difficult for the heterologous rhizobia to penetrate. In contrast, the localization of peroxidase activity in clover root hairs infected by homologous bv. trifolii was limited to the site of infection thread initiation both 1 and 5 days after inoculation. It is possible that this peroxidase activity represents repair activity of the root hair wall after the bacteria have successfully penetrated. The light golden stain evenly distributed over the uninoculated root hairs represents the peroxidase present for normal growth.

The DAB substrate for peroxidase localization crosses the root hair wall since both the wall and the cytoplasm were stained in plasmolyzed root hairs. Infected root hairs did not show enhanced peroxidase activity associated with the infection thread. Therefore, the infection thread is compatible with the root hair cytoplasm and once beyond the initial barrier at the cell wall, does not elicit a defense response. An assessment of aborted infection threads was not included in this study.

Peroxidase from isolated clover root hairs was assayed to quantitate the activity elicited by rhizobia. The immediate suppression of peroxidase activity from isolated clover root hairs after addition of homologous inoculum suggests that some preformed factor is present in the inoculum which suppresses the normal level of peroxidase activity in

root hairs. The difference between the -NF and ANU843 treatments was not significant at 6 hrs and may be due to statistical variation for the two replicates. The specific activity began to increase up to control levels between 12 and 24 hr after inoculation with ANU843. This correlates with the enhanced deposition of stain at the site of infection thread initiation since bacterial attachment, curling of the root hair, and initiation of infection threads can take place during the first 24 hr.

To test the possibility that homologous EPS was the preformed factor suppressing peroxidase activity, clover seedlings were incubated with three concentrations of EPS from ANU843. Incubation with 500 ug/ml EPS suppressed the activity of peroxidase from clover roots (root hairs still intact), suggesting that homologous EPS may block peroxidase activity. The homologous rhizobia aggregate at root hair tips and may provide a localized concentration of EPS that is effective in supressing root hair peroxidase activity. To mimic that effect, a large exogenous concentration of EPS is required because the EPS is spread out over the entire root epidermis rather than concentrated at the root hair tips. In addition, enzymes released from clover roots are capable of degrading bacterial polysaccharides and may have reduced the actual concentration of EPS within the 24 hr incubation (Dazzo et al., 1982). A lower concentration of EPS applied to clover roots may be effective earlier than 24 hrs after treatment, although we did not directly test this possibility. By pretreating clover seedlings with oligosaccharides from homologous EPS and CPS, the number of infection threads formed by bv. trifolii strain 0403 on white clover is increased and has been termed infection-related biological activity (Abe et al., 1984). The

suppression of root hair peroxidase by EPS may be a part of this biological activity.

In contrast, bv. viciae cells elicited an increase in the peroxidase activity in heterologous combination with isolated clover root hairs. The increase began between 6 and 12 hrs after inoculation while the rise in activity elicited by homologous ANU843 began 12 hrs after inoculation. This period from 6 to 12 hrs after inoculation may be the critical time when success or failure of initial penetration is determined. The effectiveness of a defense response often depends on its rapid initiation and development (Misaghi, 1982). The peroxidase response for isolated clover root hairs is more rapid and more intense for the heterologous than for the homologous combination.

Thus, our model for the determination of host specificity includes the transient suppression of peroxidase activity in root hairs by homologous rhizobia and their EPS. During this suppression, the root hair wall may continue to grow. Without the normal level of peroxidase activity, however, fewer cross-links among polymers would be formed during extension of the wall. Such a wall would be more susceptible to penetration by the homologous strain. After penetration, highly localized peroxidase activity could facilitate repair of the wall at the site of bacterial penetration and infection thread initiation. At the site of attempted penetration by heterologous rhizobia, a rapid increase in peroxidase activity would contribute to increased cross-linking of wall polymers such as extensin and suberin. The root hair walls would then be more resistant to penetration by the bacteria and may actually exclude the heterologous rhizobia. The localization of activity and the time course for elicitation in clover root hairs provide evidence to

support our plant-defense hypothesis.

Elution of wall proteins from isolated clover root hairs unavoidably includes cytoplasmic proteins as well. Therefore we determined whether the activity of peroxidase from the surface of intact roots (which includes root hairs) was affected by rhizobia similar to alterations observed in root hairs. The heterologous combination of ANU843 on pea significantly increases the specific activity of peroxidase within 24 hr in a manner similar to an incompatible pathogen. Similarly, the heterologous combination of Rl300 on clover resulted in a slight increase in peroxidase specific activity from clover roots, although the increase was not as great as for pea. For peroxidase eluted from the surface of both clover and pea roots, homologous rhizobia failed to suppress the specific activity. This may reflect the constitutive background of peroxidase present in the walls of all the epidermal cells of the root in which a slight suppression in root hair peroxidase is not detected. Heterologous elicitation of peroxidase activity, however, is a dominant effect and thus is detected in peroxidase eluted from roots and root hairs.

The specific activity of an enzyme could be increased due to 1. an increase in the activity of existing enzyme (e.g. increase supply of necessary cofactor), 2. <u>de novo</u> synthesis of a new isozyme, or 3. a decrease in the total protein concentration (with total activity of peroxidase remaining constant). Total activity for peroxidase eluted from clover roots increased after heterologous inoculation (data not shown). Therefore, peroxidase isozymes from the surface of roots were separated by electrophoresis to distinguish between the first two possibilities. Initially, peroxidase isozymes from uninoculated clover

roots were separated by isoelectric focusing followed by staining with 3-amino-9-ethyl carbazole. Five well-resolved bands of peroxidase activity were apparent (data not shown). All but one isozyme migrated to a pI < 7.0 and therefore routine separation of isozymes was performed using alkaline native gels to detect the acidic peroxidases. Four acidic isozymes from clover roots were separated in alkaline native gels and detected with the activity stain. No new isozymes were observed after inoculation with heterologous rhizobia compared to the -NF control. Inoculation with heterologous bv. viciae did enhance the activity of only 1 of the 4 isozymes. Its low mobility suggests that the native enzyme was either very weakly acidic, had a high MW (possibly complexed with other root surface components), or both. In pea roots, only one isozyme was apparent in native gels. It also had low mobility and migrated just slightly farther than the low mobility clover isozyme. It would be interesting to analyze the root isozymes of other legumes to determine whether the peroxidase isozyme increased by heterologous rhizobia was conserved among the legumes.

The magnitude of peroxidase activity elicited by bv. viciae on clover was substantially lower than that elicited by bv. trifolii on pea. This may be due to the method of determining specific activity since the spectrophotometric assay detects peroxidase activity in a mixture of proteins from the root surface. Only 1 of the 4 isozymes from clover roots was increased after heterologous inoculation while a single isozyme was eluted from pea roots. Thus, we believe that the activity of the constitutive peroxidase isozymes from clover roots obscures the increase in the activity of 1 isozyme after heterologous inoculation. In comparison, the changes in the single isozyme from pea

roots are easily detected by the spectrophotometric assay of peroxidase activity.

We reasoned that if the elicitation of peroxidase is an important determinant of host specificity, then elicitation should be controlled by the host-specific nodulation (hsn) genes present on the Sym plasmid. This hypothesis was tested by inoculating roots with strains containing cloned fragments of pSym in a pSym-cured background. We find that deletion of the entire Sym plasmid from ANU843 (strain ANU845) eliminates the heterologous elicitation of peroxidase on pea roots. Thus, some bacterial factor governed by genes on the Sym plasmid must be responsible for the heterologous elicitation of peroxidase activity. Surprisingly, the specific activity after inoculation with ANU845 is even less than the -NF control. We speculate that the lack of pSym affects both the bacterial factors which elicit greater peroxidase activity and which lead to suppression of peroxidase activity.

The 14 kb nod gene region of pSym from ANU843 had been cloned (Schofield et al., 1984) and we determined its influence on the elicitation of pea root peroxidase using the strain 845pRT032. The ability to elicit peroxidase activity was partially restored by the presence of the 14 kb fragment compared to ANU845, but was not restored to wild type ANU843 levels on pea. This suggests that products of the 14 kb nod gene region contribute to peroxidase elicitation, but that other genes on the Sym plasmid, or the interaction between the nod genes with others on the Sym plasmid, have a more profound effect on the elicitation of peroxidase. Strain 845pRT032Δ910 contains the common genes nodLJDABC but lacks the hsn genes nodFERLMN and elicits less peroxidase activity than does 845pRT032, with the level similar to the

elicited by pSym$^-$ ANU845. This indicates that the common nod genes alone are insufficient to elicit the level of peroxidase activity observed after inoculation of pea with 845pRT032. The hsn genes themselves or the interaction between the hsn and common nod genes may control the contribution of the nod gene region in the heterologous elicitation of peroxidase activity. In clover, the responses to 845pRT032Δ910, 845pRT032, ANU845, and ANU843 are all similar. This may reflect the insensitivity of the spectrophotometric assay for a mixture of peroxidase isozymes from clover roots.

The mutation of a single gene, nodE, can extend the host range for both bv. trifolii and bv. viciae. If our hypothesis is correct and heterologous elicitation of peroxidase activity hinders penetration by rhizobia, then those mutants with extended host range should no longer elicit increased peroxidase activity. Therefore, the nodE::Tn5 mutants of both ANU843 and 5039 were tested for their ability to elicit peroxidase activity. Strain 5039nodE::Tn5 on clover and 843nodE::Tn5 on peas each elicited significantly less peroxidase activity than the corresponding heterologous wild type strains. These data are consistent with our hypothesis and correlate with the nodulation phenotype of these mutants in that 5039nodE::Tn5 forms small white, presumably Fix$^-$ nodules on clover (Salzwedel, unpublished) and Djordjevic et al. (1985) report that an 843nodE::Tn5 mutant has extended its host range to peas (Nod+Fix-). Therefore, we believe that the pivotal host range gene, nodE, functions as an avirulence gene in which the loss of function leads to increased host-range (virulence). The nodE gene product may affect the production of the bacterial factor which elicits peroxidase activity in the heterologous host which in turn affects the efficiency

of successful nodulation.

A Tn5 mutation in the nodE gene of bv. viciae does not affect the level of peroxidase elicited on pea; however, 843nodE::Tn5 on clover elicits more peroxidase activity than ANU843. This increased elicitation of peroxidase may contribute to the delayed nodulation phenotype for 843nodE::Tn5 on clover. The level of peroxidase activity elicited by the 843nodE::Tn5 mutant and the 845pRT032 strain on pea are similar. Since the 14 kb fragment in pRT032 contains nodE but lacks the rest of pSym outside the 14 kb region, and the 843nodE::Tn5 mutant contains the entire Sym plasmid with only the nodE gene interrupted, the implication is that the interaction of nodE with some as yet uncharacterized region of pSym is essential to the expression of the elicitor of peroxidase activity.

The nodE gene is transcribed in the nodFERL operon of pSym in bv. trifolii. We tested whether a mutation in nodE had a polar effect on the expression of genes located downstream in the operon by determining the effect of an 843nodL::Tn5 mutant on the elicitation of pea root peroxidase. The nodL mutant elicited a reduced level of pea root peroxidase similar to the level elicited by the nodE mutant. This indicates that either the Tn5 insertion in nodE has a polar effect on the expression of nodL which then is the key gene controlling the elicitation of peroxidase activity, or both nodE and nodL are needed for the elicitation of peroxidase activity.

The host range of Rhizobium can also be extended by introducing homologous hsn genes into a heterologous wild type background. Strain Rl300pRT290 is able to nodulate white clover (Djordjevic et al., 1986) and the reciprocal strain 843pKTK85 becomes Hac^+ on vetch (Squartini,

unpublished). Strain Rt843pKTK85 containing the bv. viciae nodDFELMN genes on a multicopy plasmid elicits substantially less peroxidase activity on peas than does the parental wild type ANU843 suggesting the presence of the bv. viciae hsn genes modifies the expression of the elicitor encoded by the ANU843 genes. Strain Rt843pKTK85, however, did not elicit greater peroxidase activity than the wild type bv. viciae strains on white clover. This suggests either that heterologous hsn genes do not code for an elicitor themselves or that the wild type bv. trifolii genes are dominant. The recombinant Rl300pRt290, and wild type bv. viciae Rl300 elicited similar levels of peroxidase activity in clover roots. This again may reflect the insensitivity of the spectrophotometric assay to detect changes in a single peroxidase isozyme within a mixture of isozymes from clover roots.

To determine whether the elicitor of peroxidase activity in clover roots was an excreted metabolite, the cell-free washings from plate-grown Rl300 were tested for elicitation of peroxidase activity. Such washings from the heterologous bv. viciae on clover elicited an increase in peroxidase from the root surface within 24 hr just as did the live bacteria. However, only the washing from heterologous Rl300 grown in the presence of a flavone (DHF or naringenin) will elicit an increase in peroxidase activity (Fig. 8). Therefore, the bacterial factor must be an excreted metabolite whose production, modification, or export is dependent on a flavone-inducible gene. The known nod genes are induced by flavone, but the 14 kb nod gene region in 845pRT032 is not sufficient to elicit wild type levels of peroxidase. The flavone-induction of the elicitor from Rl300 may depend on hsn genes in part; however some other genes, perhaps also induced by flavone, may be found outside the nod

gene region and may have a large impact on the elicitation of peroxidase. The dominant flavone from the clover rhizosphere, DHF, also induces the heterologous Rl300 elicitor and thus it is feasible that the elicitor is produced in the interaction of field-grown clover and soil-borne bv. viciae cells.

This elicitor from Rl300 is heat-stable since eliciting activity is retained after autoclaving (Fig. 9). Both the ethanol-soluble and ethanol-insoluble fractions of the cell-free wash fluid elicited increased peroxidase activity with the greatest elicitation by the ethanol-soluble fraction. The ethanol-insoluble precipitate (primarily EPS) may have some inherent eliciting activity, however there may also be a small ethanol-soluble molecule caught in the polysaccharide matrix to account for the eliciting activity of that fraction.

Bioassays were performed to test whether the cell-free washing which elicited peroxidase could also influence infection of root hairs. Treatment of white clover with sterile cell-free washing from homologous ANU843 followed by inoculation with ANU843 cells increased the number of infected root hairs compared to the control. This is consistent with previous studies in which curling factors and biologically active polysaccharides were found in homologous culture supernatant (Faucher et al., 1988; Abe et al., 1984). In contrast, the washing from the heterologous Rl300 decreased the number of root hairs infected by ANU843 on clover (Table 3). The number of nodule initiations formed, however, was not affected by heterologous culture supernatant. We believe that the excreted elicitor from Rl300 acts at the stage of root hair penetration and that those few bacteria which overcome the peroxidase response elicited by the supernatant are unimpaired in the initiation of

nodules. Growth of ANU843 in broth-culture was not affected by addition of the culture supernatant from R1300 (data not shown). Root length was not affected by the treatment indicating the supernatant did not exert negative effects on plants. Thus, the correlation between elicitation of peroxidase and the decrease in number of infections after treatment with heterologous culture supernatant supports the hypothesis that the heterologous biovar elicits a plant defense response which is a determinant of host specificity.

To conclude that Rhizobium elicits a plant defense response, the following minimum criteria must be met: 1. the response is elicited to a greater extent by the incompatible than the compatible microorganism. 2. The response occurs in tissue where the microorganism is located. 3. The response must occur rapidly at a time prior to ingress by the microbe. 4. The response must contribute to the exclusion of the microbe. The results presented here meet these criteria. We therefore propose that heterologous rhizobia produce an excreted metabolite whose synthesis or modification is dependent on flavone-inducible genes present on the Sym plasmid and which elicits increased peroxidase activity on the roots of heterologous legumes. We further propose that this rapid increase in peroxidase activity acts on cell wall polymers to increase the integrity of the root hair wall and thereby hinder its penetration by the bacteria. In contrast, the transient suppression of root hair peroxidase activity by homologous rhizobia contributes to successful infection by delaying the normal polymerization of wall polymers and thus facilitates penetration by the bacteria. We conclude that the increased peroxidase activity elicited by heterologous rhizobia is a plant defense response which contributes to expression of host

specificity during the infection process in the Rhizobium-legume symbiosis.

CHAPTER TWO

RHIZOBIUM LEGUMINOSARUM BV. *TRIFOLII* AND ITS PURIFIED LPS STIMULATES A RAPID NEUTRALIZATION AT THE SURFACE OF CLOVER ROOT HAIRS

Abstract

Inoculation of white clover seedlings with homologous *Rhizobium leguminosarum* bv. *trifolii* results in the rapid (30 min) increase in the pH of the medium from 6.5 to 6.7 at the surface of individual root hairs as measured by pH microelectrodes. Inoculation with bv. *viciae* or *R. meliloti* results in a only a slight rise in pH above that of the uninoculated control. Mutant derivatives of bv. *trifolii* strain ANU843 with a Tn*5* insertion in the regulatory gene *nodD* or the host specificity gene *nodL* fail to stimulate the neutralization response. The *nodE*::Tn*5* mutant is slightly impaired in its ability to stimulate alkalinization compared to the wild type strain. Purified LPS from bv. *trifolii* strain ANU843 and strain 0403 also stimulates the neutralization at the surface of root hairs while the LPS from *R. meliloti* 102F28 does not. Excreted LPS may serve as a bacterial signal to which the plant responds and facilitates development of the symbiosis. This neutralization response may confer an advantage to homologous rhizobia in the rhizosphere by providing a localized pH environment which allows infection to proceed past the acid-sensitive step. Neutralization at the surface of root hairs may represent an uptake of H^+. Since homologous (compatible) rhizobia are stimulatory, this response is fundamentally different than

the H^+ uptake observed during the hypersensitive response elicited by incompatible pathogens.

Introduction

The movement of ions across cell plasma membranes is essential for numerous activities in plant and animal cells. These fluxes are mediated by integral membrane proteins such as H^+-ATPases, symports, antiports, carrier proteins, and voltage-gated ion channels (Hedrich and Schroeder, 1989). In roots, net ATP-dependent H^+ efflux drives the uptake of sugars, amino acids, and cations (Marschner, 1986). With the application of patch-clamp technology to plant cell membranes, single ion channel measurements have been possible. In particular, channels for Cl^- and Ca^{++} transport have been studied, as well as several types of plasma membrane channels for K^+ transport (Hedrich and Schroeder, 1989).

Studies on cell elongation in barley and maize led to the acid-growth hypothesis in which indole-acetic acid (IAA) treatment is believed to induce H^+ extrusion which acidifies and loosens the cell wall to allow turgor-driven cell elongation (Cleland, 1976). The most recent studies, however, demonstrate that the low pH (3.0-3.5) required to achieve loosening of the cell wall is not biologically feasible with IAA application and state that the acid-growth hypothesis has yet to be confirmed (Schopfer, 1989).

Even though H^+ flux across membranes is necessary to maintain homeostasis for actively metabolizing cells, there are predictions that pH could serve as the second messenger of external stimuli in plant tissue. Analogs to animal cell second messengers (e.g. cAMP) have been sought but not found in plant tissues (Felle, 1989). Although the

regulation of enzyme activity depends on appropriate cytoplasmic pH, and normal metabolism is constantly affecting internal pH, Felle (1989) proposes that pH functions as a second messenger by interacting with vacuolar Ca^{++} and thus is perceived diffently than normal metabolic changes in pH.

The plant-defense response known as the hypersensitive reaction (HR) is induced by incompatible plant pathogenic bacteria. An early event in suspension-cultured tobacco cells during the HR is a net H^+ uptake and concomitant K^+ efflux (Atkinson et al., 1985). This phenomenon is significantly reduced by ATPase inhibitors and apparently requires an electrochemical gradient across the plasmalemma (Atkinson and Baker, 1989). Neither the precise mechanism of the H^+/K^+ exchange nor the relationship between H^+ uptake and HR cell death are known.

Ion fluxes which generate electric currents play a role in orienting plant growth during development (Jaffe and Nuccitelli, 1977; Weisenseel et al., 1979). Miller et al. (1988) describe these cellular currents as a 3-part system consisting of an internal current loop, an external current loop, and the interface of the two at the plasmamembrane. Ion replacement studies with barley roots and root hairs have shown that such external currents are carried by H^+ (Weisenseel et al., 1979). Currents have also been studied in tobacco, maize, and white clover roots using a non-invasive vibrating microelectrode. For each type of root system, the direction of current flow is inward for meristematic and elongating regions and for the tips of actively growing root hairs, whereas mature epidermal cells (post-elongation region) have an outward current (Miller et al., 1986, 1988, Miller, 1989).

In the soil environment, such external currents are thought to affect the interaction of the plant root with the rhizosphere. The sites of inward current flow in tobacco roots, including growing root tips, sites of emerging laterals, and wound sites, are precisely the same areas to which Phytophthora parasitica zoospores are attracted (Miller et al., 1988). Miller et al. (1988) suggest that the zoospores are electrotactic, as well as chemotactic, thus allowing the organism to distinguish between living and dead host cells. The surface of rhizobia carries a net negative charge at pH 7 (Marshall, 1967). It has been suggested that currents in white clover root hairs could play a role in attracting the bacteria to the proper sites for infection (Miller et al., 1986).

Plasmamembrane potential in cortical cells and root hairs is affected by treatment with rhizobia or their supernatants. Érsek et al. (1986) measured changes in transmembrane potential for soybean cortical cells after inoculation with homologous and heterologous rhizobia. After 1 d, both homologous Bradyrhizobium japonicum and the heterologous Rhizobium meliloti reduced the transmembrane potential (depolarized), with R. meliloti eliciting the greatest reduction. Neither heat-killed rhizobia nor Pseudomonas fluorescens decreased the membrane potential. They conclude that host cells do respond physiologically to heterologous inoculation and suggest that living rhizobia increase membrane permeability in root cells, thus giving rise to the altered potential difference similar to that seen in cells undergoing HR. Long et al. (1991) report that the transmembrane potential of single alfalfa root hairs may be measured. Root hairs impaled with an electrode and then exposed to the flavone-induced supernatant of wild type R. meliloti

show a rapid depolarization within 1-2 min followed by recovery and slight hyperpolarization in 10-15 min. Supernatants from a nodA::Tn5 derivative did not induce the depolarization. Purified bacterial factor NodRm-1 which induces alfalfa root hair deformation also stimulated the rapid depolarization.

Previously, external pH around roots was measured in the bulk medium or by pH sensitive dyes in agar plates. Actively growing white clover root hairs generate endogenous electrical currents which are carried by H^+ and which might influence rhizosphere microoganisms. The objective of this study was to determine the effect of inoculation with homologous and heterologous rhizobia on the H^+ concentration at the surface of individual clover root hairs.

Materials and methods

Bacterial cultures.

The wild type Rhizobium strains R. leguminosarum bv. trifolii ANU843 and R. leguminosarum bv. viciae 300 were supplied by Barry Rolfe, Australian National University. R. meliloti strain 1021 was provided by Jean Dénarié, INRA-CNRS Toulouse, France. Tn5 mutants derived from ANU843 (from Barry Rolfe) included strain ANU851 nodD::Tn5, ANU258 nodE::Tn5, and ANU251 nodL::Tn5. Bacteria were grown for 4 d at 30 C on BIII agar plates (Dazzo, 1982) for wild types or BIII agar containing 100 ug/ml kanamycin for Tn5 mutants. Bacterial inoculum was prepared by suspending the bacteria in nitrogen-free Fahraeus medium (-NF, Dazzo, 1982) to a density of klett 100 (ca. 10^9 cells/ml).

LPS isolation.

Rhizobia were grown in shaken flasks containing BIII broth at 30 C. R. leguminosarum bv. trifolii strains ANU843 and 0403 (Rothamsted

Experimental Station, Harpenden, U.K.) and R. meliloti strain 102F28 (Joseph Burton, The Nitragin Co., Milwaukee, WI) were each grown to a turbidity of 90 klett units (early stationary phase, ca. 9 x 10^8 cells/ml) as measured with a Klett-Summerson colorimeter. Cells were pelleted by low speed centrifugation, resuspended in 0.5 M NaCl, and then stirred rapidly for 1 h to remove capsular polysaccharide. Following centrifugation at 10000 x g, the LPS was extracted from the cell pellet with hot phenol/water and the aqueous portion dialyzed against water. Purification of LPS was achieved by a combination of ion exchange chromatography on AG 1X1, DNase and RNase treatment, ultracentrifugation, gel filtration through Bio-Rad A1.5M, and then through Sepharose 4B in EDTA-triethylamine to yield an LPS peak with a constant ratio of total carbohydrate, 2-keto-3-deoxyoctulonic acid, and heptose (Carlson et al. 1976; Hrabak et al. 1981). LPS peak fractions were pooled, dialyzed against deionized water, and lyophilized. LPS was dissolved in -NF medium and filter-sterilized before assay.

Preparation of pH microelectrodes.

Liquid membrane microelectrodes were prepared daily. Glass micropipettes with tip diameters of 0.8-1.5 um were made from Pyrex microcapillaries (Corning no. 7740, Corning, NY) using a PP-83 Narishige micropipette puller. Capillaries were then cleaned for 15 hrs in nitric acid, rinsed with double distilled water and distilled methanol, dried under a stream of argon to evaporate the solvent, further dried at 200 C for 16 hrs, and stored over silica gel (Ammann et al., 1981).

Prior to filling, micropipettes were silanized with N,N-Diethyl trimethylsilylamine and dried again at 200 C for 40 min. The H^+-

selective neutral carrier was prepared by mixing double distilled tri-n-dodecylamine as the H^+-selective ligand (final concentration 10 wt%) with sodium tetraphenylborate (0.7 wt%) in o-nitrophenyl octyl ether, and then incubated at room temperature for 18 hrs under 100% CO_2 (Ammann et al., 1981).

Micropipettes were filled with the H^+-selective carrier by capillary action to a column height of 600 um from the tip with the remainder of the pipette back-filled with a buffer containing 40 mM KH_2PO_4, 15 mM NaCl, and 23 mM NaOH (pH 7.0). Microelectrodes were inserted into a holder with a Ag/AgCl wire immersed directly into the microelectrode buffer solution, which in turn was connected to a high-impedance 614 Keithly electrometer and one of the channels of an 8800 Dagan Total Clamp System with specific adjustments for ion-selective electrode measurements. The Ag/AgCl pellet of the reference electrode was in contact with the seedling bathing solution by an agar bridge. During measurements, all electrodes and high impedance components were located inside a Faraday cage.

Measurement of root hair pH.

Surface sterilized seeds of white clover cv. Dutch White were germinated in the dark on -NF agar plates at 23 C day/20 C night. For experiments, single 3-d old seedlings were anchored to small 5.5 cm plastic petri dishes with a drop of molten agarose, and then submerged in 2 ml of -NF medium, pH 6.5. Seedlings were allowed to equilibrate for 5 min prior to inoculation or addition of LPS treatment solutions. Bacteria were inoculated by adding 100 ul of the klett 100 bacterial suspension to the seedling bathing solution. The purified LPS from Rm102F28, ANU843, and 0403 was dissolved in -NF medium and applied to

seedling bathing solution at a final concentration of 5 ug/seedling. Microelectrodes were positioned roughly perpendicular to and midway along the surface of single root hairs with a micromanipulator and inverted microscope. Changes in electrical potential due to fluctuations in H^+ concentration were recorded every 5 min for up to 60 min and later converted to pH each day by measurement of electrode response to pH standards.

Results

The pH at the surface of root hairs on uninoculated seedlings did not vary significantly from the pH of the original bathing solution over 60 min (Fig. 1). Addition of heterologous rhizobia (Rl300, Rm1021) raised the pH slightly, whereas the homologous bv. trifolii ANU843 stimulated a significant rise in pH and neutralization of the medium at the surface of root hairs beginning 30 min after inoculation. A 60 min incubation of ANU843 in -NF medium lacking a seedling did not affect the pH of the medium (data not shown).

Mutants with Tn5 insertions in either nodD (a positive regulatory gene) or nodL (a host-specific nodulation gene) did not stimulate a pH change (Fig. 2). The nodE::Tn5 mutant was slightly impaired its ability to elicit the neutralization as compared to the homologous wild type.

The pH at the surface of root hairs rose within 20 min after treatment with purified LPS from both homologous Rhizobium strains (ANU843 and 0403) (Fig. 3.). The heterologous Rm102F28 LPS did not elicit this neutralization.

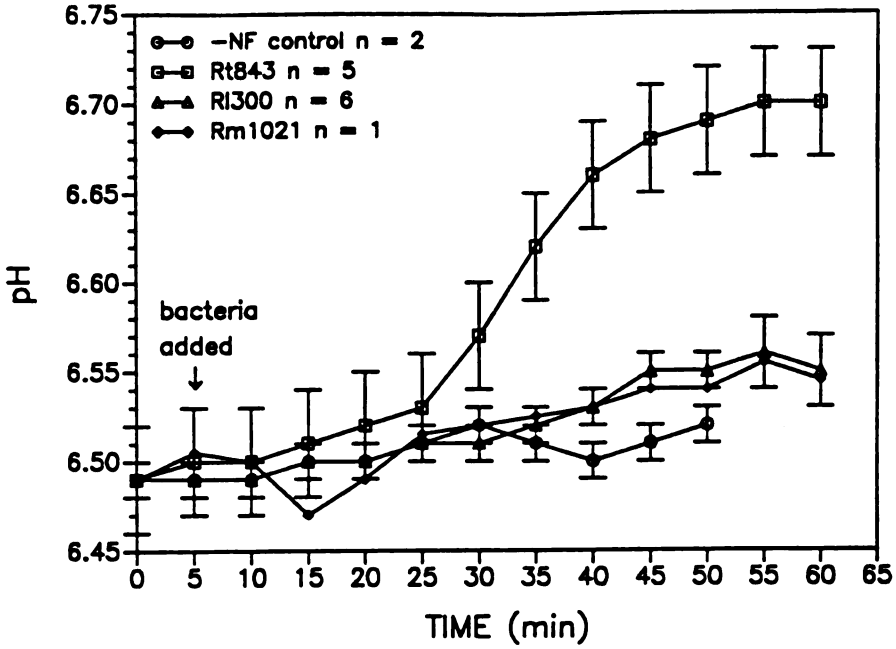

Fig. 1. pH at the surface of single white clover root hairs inoculated with wild type rhizobia. Bars = SE.

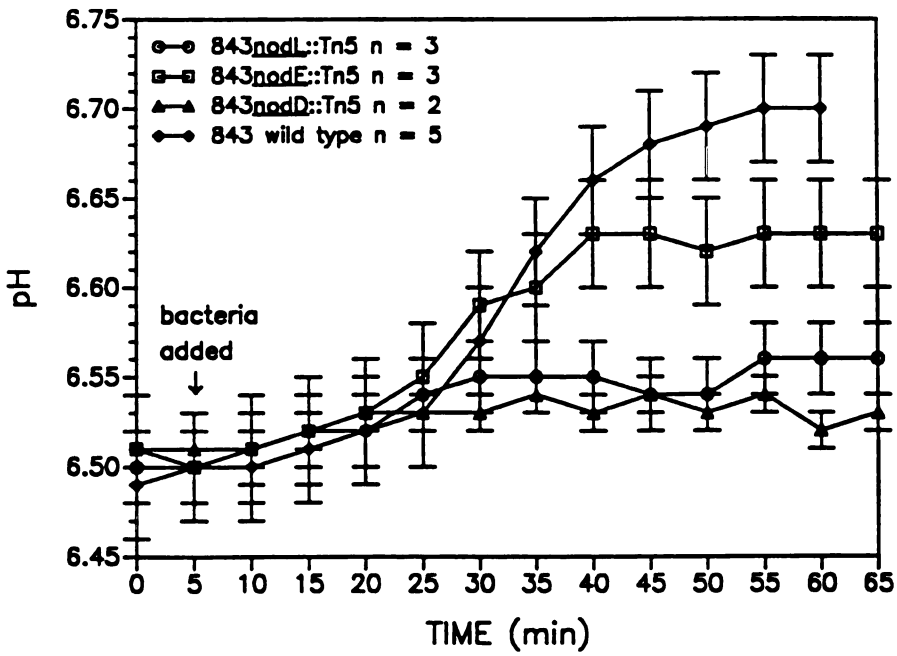

Fig. 2. pH at the surface of single white clover root hairs inoculated with Tn5 mutants derived from Rt843. Bars = SE.

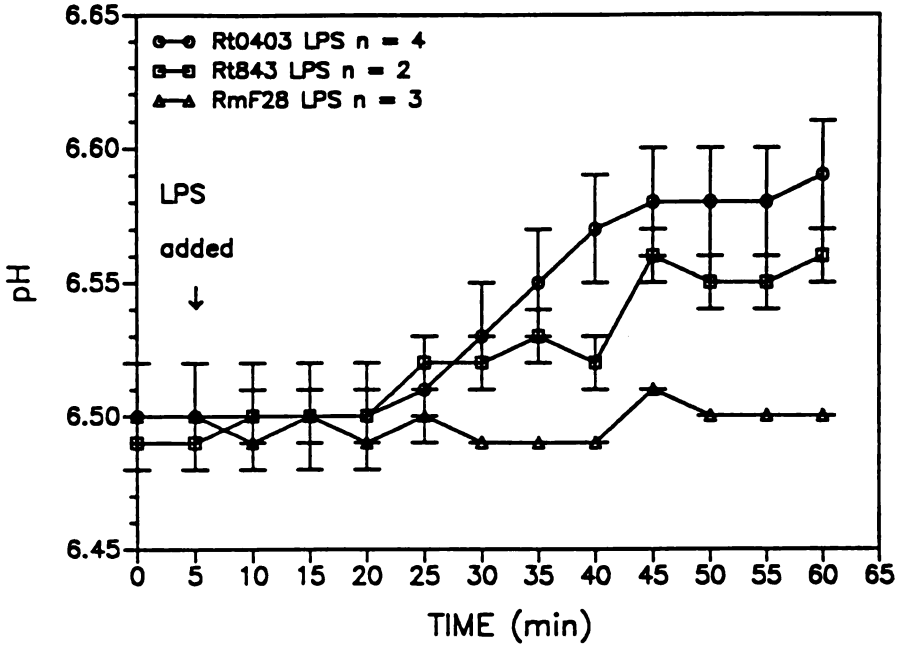

Fig. 3. pH at the surface of single white clover root hairs treated with purified LPS. Bars = SE.

Discussion

Bulk measurements of the medium around roots shows acidification in several days, especially after inoculation with Azospirillum (Bashan, 1990). By measuring pH at the surface of single root hairs, the microenvironment at the sites important to infection were analyzed. Our results using non-invasive electrodes and wild type bacteria are more representative of what must occur during competition in the rhizosphere. Previous studies have mapped current paths of single root hairs and showed that there are sites of outward and inward flux at the root surface (base of root hairs) and the root hair tip, respectively (Miller et al., 1986). In our study, the electrode was positioned midway between the points of outward and inward flux in order to measure the net change in H^+ concentration.

Nitrogen-free Fahraeus medium was used in experiments to maintain the osmotic balance within root hairs. This medium contains 10 mM phosphate and thus provides some buffering which might affect the magnitude of pH change observed. We therefore believe that our measurements represent the minimum pH change possible and any differences could be enhanced in a natural environment.

The increase in medium pH after treatment with homologous Rhizobium or purified LPS can be interpreted as an uptake of H^+ by the root hair. Alternatively, the external change in pH may be an effect of the efflux of cations. The charge imbalance caused by such an efflux in the aqueous solution could easily be compensated by the association/dissociation of water molecules resulting in a net change in pH (Good, 1986). Using a K^+-specific microelectrode, we also observed a 35% increase in external K^+ at the surface of root hairs 20 min after

inoculation with strain ANU843 (data not shown). This efflux of K^+ coupled with the rise in pH (low H^+ concentration) is suggestive of a H^+/K^+ antiport such as those reported in a number of systems (Behl and Raschke, 1987; Sze, 1985).

In Sinapis alba root hairs, the cytoplasmic pH is 7.3 (Bertl and Felle, 1985). If pH of the cytoplasm in clover root hairs is similar, the external medium (pH 6.5) would supply a higher concentration of H^+ outside of the root hair than was present in the cytoplasm. It is possible that the Rhizobium-induced neutralization of the external medium would not require energy input since the H^+ gradient favors uptake rather than efflux. The requirement for ATP to pump H^+ out across the membrane of root cells is well characterized (Marschner, 1986).

Nodulation by homologous rhizobia can be blocked by inoculating roots in a medium with a pH of 5.0 or less. Munns (1968) identified an early acid-sensitive step in the infection process which occurred prior to infection thread formation. Subsequent work showed that acidic pH inhibited the activity of host polygalacturonase which is a possible factor in the softening of root hair walls during homologous infection (Munns, 1969; Ljundgrenn and Fåhraeus, 1961). Dazzo and Hubbell (1975) found that components of the bv. trifolii CPS likely to be recognized by the host plant lectin are very acid labile. Bacteria treated with buffer at pH 5.0 or less no longer bound the antibody made against the cross-reactive clover antigen. Growth of plants at pH <5.0 decreased the net nod gene-inducing activity of root exudate from subterrranean clover but not white clover (Rolfe et al., 1988). There may be additional infection-related events sensitive to pH including the

interaction of pectic carboxyls or concentration of Ca^{++} which would affect cell wall structure. Thus the neutralization at the surface of white clover root hairs may provide a localized pH environment favorable to homologous infection and alleviate the potential acid-inhibition of infection.

This Rhizobium-induced response in root hairs is significant because it is a rapid biovar-specific phenomenon which is affected by mutations in bacterial nod genes. Strain ANU851 nodD::Tn5 is mutated in its regulatory gene giving a Nod^- phenotype on any host. Strain ANU851 is also unable to induce the wild type neutralization response, suggesting that the action of some gene under the regulation of nodD is essential to stimulate the response. The operons nodFERL and nodMN represent the 8 kb of the ANU843 symbiotic plasmid designated host specific nodulation (hsn) genes. A Tn5 insertion in nodE results in expanded host range while retaining some nodulation ability on white clover (Djordjevic et al., 1985). The phenotype for a nodL mutant is delayed nodulation on white clover. In addition, inoculation of the nodL mutant on white clover severely impairs cytoplasmic streaming in developing root hairs (Dazzo and Appenzeller, unpublished observation). Although a limited number of mutants were tested, we find that mutation of nodE only slightly diminishes the stimulation of the neutralization, however a Tn5 in nodL abolishes the response. This may represent a function for nodL which contributes to nodulation efficiency.

Rhizobium LPS may be part of the complex chemical signaling which must take place to form a successful symbiosis. Rhizobium leguminosarum bv. trifolii LPS is excreted in the clover rhizosphere and pretreatment of clover roots with purified LPS at low concentration enhances the

number of infection threads formed by wild type R. leguminosarum bv. trifolii (Dazzo et al., 1991). Flavones released from legume roots signal the Rhizobium to express necessary genes. The LPS could be a species-specific signal from the bacteria which elicits neutralization at the root hair surface which in turn provides a favorable environment for infection. Whether the neutralization of the external medium results in cytoplasmic or cell wall acidification which then causes direct chemical effects or serves as a further internal signal cannot be determined from these measurements. The site of interaction between LPS and clover root hairs may be the cell wall or more likely the cell membrane. In any event, it is significant that homologous rhizobial LPS can stimulate the clover root hair response while heterologous LPS does not.

The uptake of H^+ and concomitant efflux of K^+ has been observed for plant tissue culture cells undergoing the hypersensitive reaction (HR) in response to incompatible pathogens (Atkinson et al., 1985). Based on electron micrographs, Djordjevic et al. (1988) have suggested that a mutant of strain NGR234 causes a hypersensitive-like response on Macroptillium. Our results, however, indicate that the wild type Rhizobium strain equivalent to an incompatible pathogen, the heterologous biovar, does not elicit the H^+ uptake response. Instead, it is the homologous rhizobial strain (analogous to the compatible pathogen) which elicits the response. Thus the plant's response to wild type rhizobia is the opposite of what has been observed for the HR. Therefore the Rhizobium-stimulated change in H^+ concentration does not indicate a resistance response but more likely is a contributing factor in successful symbiosis. These results do not rule out the possibility

that other plant defense-like responses contribute to the determination of host specificity.

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