UNIVERSITY OF CALIF - SANTA BARBARA

FINAL TECHNICAL REPORT **DE-FG03-95ER-20192**

During the period of DOE funding, we synthesized several PRP peptides, generated rabbit antisera against two PRP repeats found in early nodulin PRPs, and developed confocal microscopy methods for root immunohistochemistry. Using the anitbodies, we completed extensive descriptive studies of PRP deposition in medic and alfalfa roots showing that PRPs deposition is developmentally regulated in roots and spatially restricted within the walls of specific root tissues. Domain-specific antibodies were isolated from polyclonal sera using peptide affinity chromatography and were then used to demonstrate that nodule-specific epitopes are shared by several nodule-specific proteins. The following provides a more detailed summary of this work:

A. Generation of anti-PRP peptide antibodies. Pilot syntheses of four peptides (with increasing numbers of POVYK repeats, Table 1: Peptides 1-4) were conducted by sequential synthesis on a Millipore 9050 Peptide Synthesizer using f-MOC chemistry. Peptides were purified by reverse phase HPLC and characterized by MS. Despite the high content of proline and hydroxyproline, these syntheses were completed with excellent yields and without complication, although double-coupling of the imino acid residues was required and some cyclization occurred during the addition of the third amino acid (the Lys-Pro coupling) in each peptide. Each of the additional peptides listed in Table 1 were subsequently synthesized and purified.

Table 1. PRP peptides synthesized and purified for this project.

Peptides 1-4	$(POVYK)_nPO$ n=1, 2, 4, 8
Pep 5	C-$(POVYK)_3PO$
Pep 6	C-$(POVEK)_3PO$
Pep 7	C-$(POVHK)_3PO$
Pep 8	C-$(POHKE)_3PO$

Pep5 and Pep6 represent the repeated pentapeptides found in PRP1 and PRP2, while Pep7 and Pep8 represent pentapeptides found in PRP4 and ENOD12 (Pep7) or only in ENOD12 (Pep8), but not found in PRP1 or PRP2 (thus representing potential "nodule-specific" PRP repeats). The N-terminal Cys of Pep7 and Pep8 were reduced with TCEP, covalently coupled to keyhole limpet hemacyanin (KLH) using Sulfo-SMCC, a water-soluble hetero-bifunctional crosslinking reagent from Pierce, and the resulting KLH-peptide conjugates were injected into white New Zealand rabbits. Peptides 5, 7 and 8 were also covalently coupled to Sulfo-Link resin (Pierce), and the resulting resins were used to purify and fractionate anti-PRP antibodies using affinity chromatography.

Antibodies against Pep7 were generated and characterized first. Three rabbits were immunized with the (POVHK)-KLH conjugate, and a Pep7-Sulfolink column was used to affinity-purified antibodies from crude rabbit antisera. Affinity-purified anti-POVHK antibodies from two rabbits recognized the same pattern of immunoreactive proteins in medic roots as a well-characterized antiserum produced against purified SbPRP2 (Kleis-San Francisco and Tierney, 1990), while antiserum from the third rabbit recognized a 110 kD nodule-specific PRP with a much higher affinity. These results validated the proposed strategy of using synthetic PRP repeats as antigens to generate immunological reagents. Since the affinity purified anti-POVHK antibodies recognized

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PRP1 and PR2 from roots with very high affinity, they were useful for the immunolocalization of PRPs in root tissues (Section III.B.). Antibodies were subsequently raised against Pep8-KLH in three rabbits. Affinity purified antibodies from two rabbits (#449, 450) recognized several different nodule-specific wall proteins.

B. PRP deposition in *Medicago* seedlings. As a prelude to detailed studies on the regulation and function of PRPs during early nodule development, we investigated PRP deposition during the growth and development of uninoculated *Medicago* (medic and alfalfa) roots. Completion of these "background" studies was more involved than was originally anticipated, including both the production and characterization of the affinity-purified anti-peptide antibodies and the development of confocal microscopy methods for plant root tissues. Briefly,

- Genomic DNA blots showed that PRP1 and PRP2 represent two of six members of the PRP family in *Medicago truncatula*, and transcripts for these two PRP genes accumulated to high levels in roots as compared to aerial plant organs.
- Antibodies raised against purified soybean PRP2 protein (generously provided by Dr. Mary Tierney) recognized 33 kD PRP1 and 55 kD PRP2 immunoreactive proteins, along with a third less abundant 65 kD PRP, in high-salt or SDS extracts of medic root cell walls.
- PRP1 and PRP2 transcript levels changed during early seedling growth, and levels of the two major soluble PRPs changed in parallel with transcript levels (Fig. 3).
- Both PRPs were oxidatively insolubilized by H_2O_2 in medic roots, with soluble half-lives of 9 and 23 min, respectively, indicating that both major root PRPs are secreted into cell walls as soluble monomers representing the biosynthetic precursors of insoluble cell wall PRP networks.
- Two major alfalfa PRP transcripts were identified by hybridization with medic cDNA sequences at high stringency. Both alfalfa PRP genes were expressed at high levels in roots, and two major immunoreactive PRPs (36 kD and 72 kD) were solubilized from alfalfa root cell walls using salt or SDS, along with four minor 50 - 60 kD immunoreactive proteins (Fig. 3).
- PRPs accumulated differentially during root development in both medic and alfalfa seedlings, with very low levels detected in the distal root tip region (that includes the root cap, meristem, and cellular elongation zone), high levels of PRP2 in the emerging root hair (ERH) zone, and high levels of both PRP1 and PRP2 in the mature root hair zone and older regions of roots.
- Immunolocalization studies using affinity-purified anti-POVHK antibodies (recognizing the major root PRPs) found the highest levels of PRPs in the intercellular junctions between root cortical cells, with lower levels detected in the root endodermis and pericycle, and in the differentiating protoxylem and outer phloem tissues (Fig. 4).
- In the ERH region, PRPs were detected only in the intercellular junctions in the root cortex. Tissue printing experiments showed that at least some of the PRPs in the intercellular junctions remained soluble.
- The spatial localization of PRPs in the root cortex is tightly restricted to the outside of the expanded middle lamellae between groups of three neighboring cells, thus forming a triangular cross-sectional pattern (Fig. 4). Previous work localized polygalacturonic acids to the inside of these expanded middle lamellae (Moore and Staehelin, 1988).

Thus, the intercellular junctions between root cortical cells have a discontinuous molecular composition with a pectin gel "core" surrounded by a cross-linkable PRP sheet. Based on these patterns, we proposed that intercellular junction regions of the root cortex function together as a supercellular system of triangular girders that integrate the structural properties of the root cortex and provide roots with strength needed to resist crushing during growth (Cooper et al. 1997a,b).

•HRGPs are also deposited in the root cortex, but the cross-sectional triangular pattern of HRGP deposition is distinctly different from the pattern of PRP deposition. Thus, it is unlikely that PRPs interact directly with HRGPs to form copolymer networks in the root cortex.

•In xylem, PRPs are deposited in differentiating protoxylem walls before lignification occurs. As lignin is deposited, the pattern of immunoreactive PRPs becomes restricted to a series of concentric rings or layers around the circumference of the vessel during vessel maturation. In longitudinal views, the spatial pattern of PRP deposition mirrors the pattern of lignin deposition. These data are consistent with potential roles for PRP networks as templates that spatially control the free-radical polymerization of phenylpropanoid monomers.

PRPs in developing root nodules. We have used RNA gel blot, protein immunoblot, and confocal microscopy experiments to investigate the symbiotic regulation of host cell wall PRPs. RNA blots demonstrated that PRP1 and PRP2 transcript levels were highly attenuated in wild-type medic nodules as compared to whole roots. In contrast, transcript levels in empty nodules produced by exopolysaccharide-deficient (EPS-deficient) mutants were very high. In situ hybridization experiments demonstrated that PRP transcripts accumulated in the outermost tissues of empty nodules, the same tissues where infection by the EPS mutants is known to be aborted. By comparison, in wild-type alfalfa nodules, the level of PRP2 transcript was greatly reduced while PRP1 transcripts were still present at significant levels. Although spot-inoculation experiments with one alfalfa cultivar (Ferry Morse AS13) showed that both PRP transcripts were undetectable in 4d old nodules, flood-inoculation experiments showed a decrease in only the PRP2 transcript in 4d old nodules as compared to whole roots (never the complete loss of PRP1 transcript). Subsequent spot-inoculation experiments with a different alfalfa cultivar (Ferry Morse GT13) also showed that the levels of PRP2 transcript were reduced in 4d old nodules.

Two possible explanations for these results might be that symbiotic signals directly regulate PRP transcript abundance in host root tissues, or that observed differences in PRP transcript levels simply reflect different tissue compositions of wild-type nodules compared to both roots and empty nodules formed by EPS-mutants. To test these possibilities, we isolated RNA from nodules and from the subtending root tissues following inoculation with wild-type and exo$^-$ *Rhizobium* strains. RNA was also isolated from roots inoculated with a deletion mutant of *R. meliloti* that lacks the common nodulation genes and is unable to synthesize Nod-factors (SL44) and from mock-inoculated roots. Since each of these "inoculated root" samples contains identical root tissues that were simply exposed to different symbiotic signals, differences in PRP transcript abundance cannot be explained by differences in tissue composition. Surprisingly, as shown in Fig. 5, root tissues that have been inoculated with wild-type *Rhizobium* expressed very high levels of the two PRP transcripts as compared to mock-

inoculated roots, and this induced increase did not depend on Nod-factor production but did depend on EPS. To the best of my knowledge, this is the first demonstration of a Nod-factor-independent/EPS-dependent change in host gene expression.

In contrast to PRP transcript levels in root nodules and inoculated roots, data from immunoblot experiments indicated that root nodules contained very high levels of the major root PRPs and several nodule-specific PRPs, while levels of soluble PRPs in inoculated root tissues attached to nodules were unaffected by *Rhizobium* inoculation. This result is consistent with post-transcriptional regulation of cell wall PRP deposition. Using spot-inoculation experiments, we found that an increase in soluble PRP levels is induced by *Rhizobium* early in nodule development, even though the PRP2 transcript levels are reduced to near background. Taken together, the above results indicate that the regulation of cell wall architecture by *Rhizobium* is complex and that symbiotic signals operate both to control PRP transcript levels and to post-transcriptionally regulate PRP biosynthesis, secretion, and/or crosslinking. One important goal of this project is to investigate the functional significance of these changes in nodule development.

Sherrier and VandenBosch reported that PRPs are components of the infection thread in pea nodules, but the anti-sbPRP2 antisera used contained antibodies that recognized a *Rhizobium* protein, thus greatly complicating the interpretation of their immunolocalization data. Importantly, we have found that the affinity-purified anti-POVHK antibodies have no cross-reactivity with any *Rhizobium* proteins. Immunolocalization experiments were recently completed using affinity-purified antibodies from rabbit #383 that recognize the major root PRPs and at least 6 nodulin PRPs. For these experiments, *Rhizobia* harboring a constitutive Green Fluorescent Protein gene were used as the inoculant in order to easily visualize the symbiotic bacteria using the 488 nm laser line on the confocal microscope. PRPs were localized to the wall of the infection thread of wild-type alfalfa nodules, confirming the results of Sherrier and VandenBosch, however the localization of specific PRPs in the infection thread wall required the purification of domain-specific antibodies.

E. Generation of domain-specific anti-PRP antibodies. An important objective of this project was to generate domain-specific anti-PRP antibodies for use in localizing individual PRPs in developing roots and root nodules. Affinity-purified antibodies from rabbit #383 were applied to a Pep5-Sulfolink column to remove antibodies recognizing epitopes found in most PRP repeat motifs. This strategy successfully fractionated the polyclonal antibodies into those recognizing common PRP epitopes and those recognizing domain-specific epitopes. Most (99%) of the affinity purified anti-POVHK antibodies bound to both POVHK and POVYK columns, while the domain-specific fraction represented only 1% (did not bind to the POVYK column). The "VHK-specific" antibodies primarily recognized the 110 kD PRP, demonstrating that the VHK-repeat is indeed a nodule-specific PRP domain. Localization experiments showed that the VHK-specific antibodies localized in the same nodule tissues as the common epitopes indicating that nodule-specific PRPs probably function together with PRP1 and PRP2.