

## Plant-Insect Interactions: The Hackberry Nipple Gall

Jeff McDermott\*, Richard Meilan#, and Robert Thornburg\*+

\*Department of Biochemistry and Biophysics  
Iowa State University  
Ames, IA 50011

#Department of Biochemistry  
University of Missouri  
Columbia, MO 65211

+Correspondence should be addressed to:  
Robert Thornburg  
Phone Number: (515) 294-7885  
FAX Number: (515) 294-0453  
Email: [thorn@iastate.edu](mailto:thorn@iastate.edu)

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### ABSTRACT

The [hackberry](#), *Celtis occidentalis*, is infested by the [insect](#), *Pachypsylla celtidis-mamma*, which produces growths on the leaf tissues termed [nipple galls](#). We have examined the proteins that accumulate in the leaf and the gall, and have identified several proteins that accumulate specifically in the gall. Even adjacent non-galled leaf tissues do not show the expression of the gall-specific proteins. Furthermore, several proteins that accumulate to high levels in normal leaf tissues show reduced levels in the gall tissues. One of the proteins that decreases in the leaf galls was shown by western blot analysis to be ribulose biphosphate carboxylase. In addition, the major protein that accumulates specifically in the galls was purified and antiserum was raised against this protein. When we tested for the presence of this protein in both leaf and gall by Western blot analysis, we found that the 37 kDa protein apparently arises from a 59 kDa protein by at least two proteolytic steps. A similar protein also is present in the leaf tissues, implying that gall

formation induces specific proteolytic degradation of the 59 kDa protein. The levels of cytokinins were determined for the unfested leaf tissues as well as for the gall tissues. The galls showed a 50 fold higher levels of isopentenyl adenosine than the leaf tissues.

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## INTRODUCTION

[The hackberry, \*Celtis occidentalis\* L.](#), is a large tree species (12 to 15 meters) that occurs in upland woods chiefly along streams in the midwestern region of North America. These trees are extensively used as windbreaks and shade trees throughout their range.

One entomological problem that affects hackberries in the midwestern United States is infestation by the jumping plant lice. This is a group of insects belonging to the family Psyllidae. These insects look much like adult cicadas, except that the psyllids are much smaller (4 to 5 mm long). The psyllids are often severe insect pests of hackberry trees, causing a variety of [galls on the foliage](#) of the trees. One species in particular, *Pachypsylla celtidis-mamma* Fletcher, causes the infestation known as the hackberry nipple gall [\(1\)](#).

The hackberry nipple gall maker usually has one generation of insects per year, with the adults emerging from crevices in the rough bark of the hackberry where they overwinter. Mating and egg laying occurs over a 2 to 3 week period beginning when the leaves emerge in the spring. The eggs hatch after 7 to 10 days and the nymphs begin feeding on the foliage. The feeding causes morphological changes in the cells of the leaf of the hackberry which results in the growth of a pouch or a gall that grows up around the nymphs. The nymphs live within the gall throughout the summer and they emerge as adults in September [\(2, 3, 4\)](#).

Gall forming insects, thus have the ability to alter the development of plant tissues to cause the formation of tumor-like growths that surround the insect to protect it from the environment and supply it with a source of food. However, the mechanisms of gall formation by plants in response to the insect attack remain largely unknown. In addition, the stimuli which trigger gall formation are also unknown. This work was undertaken to begin to study the initial events of gall formation by first identifying proteins in the gall that differ from those in the leaf.

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## MATERIALS AND METHODS

Reagents for PAGE were from Sigma, St. Louis, MO. The tritiated trialcohols of zeatin riboside ([<sup>3</sup>H]-ZRTA) and isopentenyl adenosine ([<sup>3</sup>H]-IPTA) were prepared according to the protocol of Weiler and Spanier [\(5\)](#). The [<sup>3</sup>H]-NaBH<sub>4</sub> was obtained from New England Nuclear (12.6 Ci/μmol). All other materials were obtained locally and were of the highest quality available.

### Plant Tissue

The nipple gall tissue and the hackberry leaves were obtained from several trees (*Celtis occidentalis*) at various locations on the campus of Iowa State University and the surrounding community of Ames, Iowa.

### Preparation of protein extracts

Plant tissues were frozen in liquid nitrogen and ground with sample buffer (1% SDS, 48% urea, 1% 2-mercaptoethanol, 0.0625% phosphoric acid, pH 6.8 with Tris base) in a mortar and pestle. Samples were prepared at a protein concentration of 1 mg/mL as estimated by Bradford's method (6). Xylene cyanol was added as a tracking dye.

### Preparation of anti-gall p37 antiserum

The 37 kDa protein was carefully excised from preparative 15% SDS PAGE gels, and stored at -70°C until use. To prepare antiserum, a portion of the gel, corresponding to approximately 25 mg of protein was ground in a mortar and pestle, then resuspended in 1 mL of 0.85% NaCl. This saline solution was combined with 1 mL of Freund's complete adjuvant and homogenized until a stiff emulsion formed. This emulsion was injected at several locations into the flanks of a male New Zealand White rabbit (7). After 14 days and again after 31 days, this procedure was repeated except that incomplete adjuvant was used to make the emulsion. Western blot analysis demonstrated that positive anti-p37 antiserum was produced after 31 days of immunization.

### Gel Electrophoresis and Western Blotting

SDS PAGE was conducted according to the method of Laemmli (8). Western blots were performed as described in Sambrook et al., (9).

### Cytokinin Analysis

#### Extraction

All tissue was removed from the tree, returned to the laboratory and frozen in liquid nitrogen. Tissues were stored at -80°C until used. The tissue was ground in a mortar and pestle with liquid nitrogen, transferred to a 50 mL polypropylene tube and extracted with 10 mL methanol containing 200 mg/L diethyldithiocarbamic acid and 500 mg/L butylated hydroxytoluene (BHT). The extracts were spiked with 64,000 cpm for [<sup>3</sup>H]-ZRTA and 60,000 cpm of [<sup>3</sup>H]-IPTA. The tubes were incubated on ice for 30 minutes with occasional mixing and centrifuged at 3000 X g for 15 min. The supernatant was transferred to a 15 mL polypropylene tube. The pellet was re-extracted with an additional 5 mL of methanol, centrifuged again and the supernatants were combined. The supernatants were evaporated to near-dryness *in vacuo*. This process was repeated three times using 2.0 g of leaves and 0.5 g of gall material.

#### Immunoaffinity chromatography

The extract residue was dissolved in 0.5 mL dimethylsulfoxide to which 14.5 mL of 40 mM ammonium acetate (adjusted to pH 7.0 with glacial acetic acid) was added. The samples were then percolated through a DEAE cellulose column (12 mL bed volume) connected in tandem to a mixed immunoaffinity column containing 1 mL each of clone 16 (anti-zeatin riboside) and clone 12 (anti-isopentenyl adenosine) conjugated to microcrystalline cellulose. Both the DEAE and immunoaffinity columns were pre-equilibrated together with at least 10 column volumes of 40 mM ammonium acetate. After the sample was applied to the column array, the columns were washed with 15 mL of 40 mM ammonium acetate. The DEAE column was then discarded and the immunoaffinity column was washed with an additional 20 column volumes of 40 mM ammonium acetate. Finally, the cytokinins were eluted with 10 mL methanol which was evaporated *in vacuo*.

### **HPLC Purification and Radioimmunoassay**

The immunoaffinity-purified cytokinins were fractionated on an Ultrasphere (ODS, particle size = 5 mm) HPLC column (4.6 mm X 250 mm, Beckman) eluted at a flowrate of 1.0 mL/min. with a three-step, linearly increasing gradient of acetonitrile (10% to 15% over 20 min., 15% to 30% over 20 min., and 30% to 100% over 1 min.) in water buffered with 40 mM triethylamine adjusted to pH 4.5 with glacial acetic acid. Fractions (0.5 mL) were collected and dried to completion *in vacuo*, after adding 5 ml of triethylamine (neat) to each. Ten percent of those fractions bracketing the retention times of the tritiated internal standards were counted in 2 mL Formula 963 (DuPont) scintillation cocktail to determine internal standard recoveries.

Each HPLC fraction was divided into thirds and assayed separately. The cytokinins in each sample were quantified using radioimmunoassay (10).



## **RESULTS AND DISCUSSION**

The hackberry nipple gall represents a unique plant insect interaction that occurs when the trees respond to infestation by insect pests of the genus *Pachypsylla*. A typical gall is a small dome approximately 8 mm tall that protrudes from the leaf surface (Figure 1, panel A). The fully formed galls typically contain several tissue types that are organized in layers (11). The outermost layers are the plant's epidermis, and inside the epidermis is a layer composed of thick-walled, highly lignified cells, which serves to protect the young insect (see Figure 1, panel B). Inside of this protective layer, is a layer of nutritive cells lining the central chamber. These cells provide a rich source of proteins, sugars and other nutrients needed for insect growth and development (12). Because the morphology of the gall is quite different from the leaf, we expected there to be differences in the proteins expressed in the gall and normal leaf tissues. Therefore, we initially examined the proteins of the galls and of uninfested hackberry leaves.

Because the galls contain insect nymphs (Figure 1, panel C), the gall tissue was harvested from the leaves of hackberry trees and the insect larvae were dissected out of the tissue prior to extracting the proteins. We compared the proteins from the gall and leaf tissues under two different polyacrylamide concentrations (15% and 6%) so that we could compare both small and large molecular weight proteins. As shown in Figure 2, the protein profile of the gall tissue does indeed differ from the protein profile of the normal leaf. Many of the major leaf proteins are expressed at reduced levels in the galls. Further, there are several proteins that are expressed in the gall tissue and are not strongly expressed in the leaf blade (see arrows). Those proteins that accumulate preferentially in the galls rather than in the leaves include a set of three large molecular weight proteins (between 85 kDa and 110 kDa) as well as a protein of 37 kDa. There was also a 31 kDa protein that was found to be at higher levels in the gall than in the leaf. However, this protein was also expressed in the normal leaf, and was of less interest to us than the 37 kDa protein.

To demonstrate that the proteins expressed in the galls are different from those present in the insect, we examined galls after removal of the insect nymphs and the insect nymphs after removal from the galls. The results of this are shown in Figure 3. There is little similarity between the protein profiles of the insects and the galls. Therefore, those proteins which are present in the gall and not in the leaf are newly induced plant proteins and not insect proteins.

In addition to those proteins that are expressed at higher levels in the gall, several leaf

proteins show reduced expression in the gall. Two of these are the two major leaf proteins which have molecular weights of 57 kDa and about 12 kDa. Because ribulose biphosphate carboxylase (RUBISCO) shows large and small subunits of these same sizes, we decided to probe the presence of this enzyme in the leaf and gall tissues. [Figure 4](#) shows a western blot analysis of leaf and gall tissues probed with antiserum raised against the large subunit of Amaranth RUBISCO (this antiserum was kindly provided by Dr. Basil Nikolau, Iowa State University). The anti-Amaranth RUBISCO does indeed show good cross-reactivity to the hackberry large subunit in leaf tissues (lane 1). However, RUBISCO was expressed in gall tissues at significantly reduced levels (lane 2). When RUBISCO was quantitated by cutting and counting the bands from the blot, we found the level of RUBISCO present in the gall was about 5% of the level in the leaf. Therefore, RUBISCO expression is significantly reduced in the gall tissues relative to the leaf tissues.

In order to better characterize the proteins that accumulate specifically in the gall, we prepared antiserum against the 37 kDa protein and examine its expression in leaf and gall tissues by western blot analysis. Antiserum against the 37 kDa protein was prepared as described in Materials and Methods. When the antiserum was tested in western blot assays against the gall extracts, it recognized the 37 kDa protein ([Figure 5](#), lane 2), but it also recognized a 52 kDa protein and a 59 kDa protein.

When the proteins were extracted from leaves without galls, we also found cross-reacting material in uninfested leaves ([Figure 5](#), lane 1). Thus, gall formation appears to result in a unique proteolysis of a protein that is normally present in the leaf. This proteolytic processing gives rise to the 37 kDa protein. Because the 37 kDa protein does not accumulate in uninfested leaves, gall formation may induce a unique proteolytic pathway. Because the 52 kDa intermediate protein also accumulates, it appears that proteolytic degradation proceeds in a two step process, first removing 7 kDa, and subsequently removing an additional 15 kDa. Alternatively, it is possible that the 57 kDa protein in leaves is normally turned over very rapidly and that gall tissues show altered proteolytic degradation of this protein which allows the 52 and 37 kDa proteins to accumulate. It is not yet clear which of these alternatives is occurring. Future studies will address this problem.

The structure of the nipple gall indicates that cell proliferation is likely to be involved in its development. Cytokinins are known to be involved in the control of plant cell division. In addition, they have also been shown to stimulate the production of certain mRNAs and proteins ([13](#), [14](#)). We therefore sought to determine the levels of cytokinins present in the leaf and the gall tissues. We utilized the immunological purification and quantification system of MacDonald and Morris ([10](#)) for these studies. Both uninfested leaf tissue (2.0 g) and nipple gall tissue (0.5 g) were extracted and the cytokinins were purified by immunoaffinity chromatography and fractionated by HPLC. Those HPLC fractions which eluted at the same time as authentic cytokinin standards were assayed in triplicate by radioimmunoassay. In both the leaf and the gall tissues, cytokinins were detected only in those HPLC fractions corresponding to the retention time of isopentenyladenosine (iPA). The average iPA level in uninfested hackberry leaf tissue was low. However, in marked contrast to this, the average iPA level in the hackberry nipple gall tissue was nearly 50 fold higher than the uninfested leaf tissues (see [Table I](#)).

Whether these elevated levels of cytokinins are responsible for the altered protein patterns observed between the leaf and gall has not yet been determined. Nor is it known whether the source of the cytokinin is the plant or the insect. It would also be of interest to examine whether exogenous application of cytokinin could result in the either the production of 37 kDa protein or in growth of gall-like structures on uninfested hackberry leaf tissues.



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