Short Communication

Phospholipids and Lipid Acyl Hydrolase (Phospholipase) in Leaf Galls (Hymenoptera: Cynipidae of Black Oak [Quercus robor L.])

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ABSTRACT

Phosphatidylcholine, phosphatidylethanolamine, and cardiolipin are the major phospholipids in young leaves of black oak (Quercus robor L.). Except for phosphatidylcholine, young, developing cynipid-galls on black oak leaves, i.e. the insect-transformed tissues, contain less phospholipid than normal leaf tissues. Lipid acyl hydrolase activity determined by the cleavage of fatty acids from a labeled phospholipid substrate is higher in the tissue extracts from galls than from leaves. The increase in enzyme activity and the altered phospholipid composition are discussed in relation to expected membrane modifications and transport phenomena in insect-transformed tissues.

The morphology and histology of highly organized insect galls (cecidia) have been studied in detail for over 100 years (for recent review, see 22), yet the insect-elicted cecidogens (gall-inducing factors) remain unknown. Substances such as amino acids, auxins, and other hormones, and digestive enzymes of the insect's saliva and guts, have been detected in a few plant galls (1, 11, 16, 28, 29); reports on the physiological or biochemical alterations in plant galls are rare (19, 20, 26). That the nutrient tissues of various oak galls accumulate a great amount of neutral lipids (especially triacylglycerol, the most abundant lipid storage form) correlates with the cytological features of gall nutrient cells (21). The composition of membrane phospholipids, known to be responsible for the modifications at cellular membranes of stressed plant tissues and of neoplastic cells (5, 14), has not been determined for insect-transformed tissues. Changes in the cellular membrane systems are thought to be responsible for the modifications in the transport of water, nutrients, and assimilates, as well as for phytohormone binding in insect-transformed tissues (13, 15, 23, 30). This paper describes the first time alterations in the major membrane-associated phospholipids and the activity of membrane-associated lipid acyl hydrolase in insect galls.

MATERIALS AND METHODS

Plant Material. Leaf galls of Neuroterus quercus baccarum L. (Hymenoptera: Cynipidae) were collected between mid-May and the beginning of June from black oak (Quercus robor L.). This spring form of the gall is induced by the bisexual generation of the cynipids. Galls, containing living young larvae, measured about 0.5 to 1 cm in diameter and had an average weight of 0.4 g (minus the larva). Normal leaf tissue was excised either from the corresponding opposite area of the uninfected leaf blade or from leaves belonging to the same leaf succession as those containing galls. One g each of fresh tissue from galls and from normal leaf tissue (excluding the midrib and veins) were used for the extraction of phosphoglycerides and for the lipid acyl hydrolase assay. The term lipid acyl hydrolase, given to an enzyme that deacylates a wide range of lipids including phospholipids and galactolipids and that is most likely responsible for the phospholipase activity (10), is used in this paper. Inasmuch as we measured only the hydrolysis of 14C-labeled fatty acids from a phospholipid substrate, the enzyme activities detected will most likely be due to plant endogenous phospholipase.

Total Lipids. Total lipids from galls and leaves were extracted according to the method of Bligh and Dyer (4) by quickly homogenizing 10 g of tissue in a chilled mortar in a mixture of cold chloroform:methanol (1:2). The amount of lipid in the chloroform phase was estimated gravimetrically (4).

Phospholipid Extraction. Plant phospholipids were extracted either with methanol-chloroform or with hot isopropanol. The procedure of Folch et al. (9) was adapted to small sample sizes; fresh tissue was cut up, ground in the cold, and extracted in chilled chloroform:methanol (50:16.2) for 30 min at 4°C. All subsequent steps were carried out at 4°C using chilled glassware and solutions. The mixture was filtered through cheesecloth three times with subsequent washes in chloroform:methanol:ddH2O (1:1:0.1) containing 50 mM KCl (12). After centrifugation at 3600 rpm for 5 min, the biphasic supernatant was collected and spun at 2000 rpm 5 min. The upper (methanol-H2O) and lower (chloroform) phases were collected separately. FFA and lipids were extracted in the chloroform phase (24). This phase contained large amounts of Chl from the leaf extracts which, however, did not interfere with the chromatographic separation and specific stains used for phospholipid detection. Gall extracts were relatively Chl-free due to the reduced number of chloroplasts in these tissues. Fractions were dried under a stream of N2 at 35°C within 10 min, taken up in chloroform:methanol (2:1), and transferred to TLC glass plates (Silica gel H plates, Analabs, Inc.). In the second extraction procedure, hot isopropanol was used.
for phospholipid solubilization (6). One g of fresh plant tissue was cut up into small pieces and immediately added portionwise to 6 ml hot isopropanol (80°C). The tissue was ground up in a mortar, washed 2 times in 3 ml hot isopropanol and 2 times in chloroform/isopropanol (1:1 v/v). The combined supernatant fractions were concentrated in vacuo (40°C), and the lipid residues were dissolved in chloroform:methanol (2:1 v/v) and quantitatively assayed for their phosphorus content (1). Phospholipids were separated by two-dimensional TLC. Solvent 1 contained chloroform:methanol:7 N NH₄OH (195:90:12); solvent 2, chloroform: methanol:acetic acid:dd water (170:25:25:3) (6). Commercial phosphoglycerides were run as controls on separate TLC plates together with the extract plates; the location of individual phospholipid spots was recorded after staining with molybdenum blue (8) or in iodine vapor (12). The following standards (all from Sigma) were used as controls: PC, PE, PG, PI, PS, sphingomyelin, CL, lysophosphatidylethanolamine, lysophosphatidylserine, and phosphatic acid.

**Phosphorus Assay.** Total plant phospholipids and phospholipid spots scraped from TLC plates after chromatography were quantitatively assayed for their phosphorus content with the Fiske—SubbaRow reagent (2). Controls were in the range of 0.02 to 0.12 μmol phosphate (Sigma's inorganic phosphorus standard solution). The optical density at 830 nm was recorded with a Beckman photometer.

**Enzyme Assay.** (a) Preparation of substrate for the phospholipase assay was carried out according to the procedure described by Bayer et al. (3). In short, [¹⁴C]palmitic acid (16:0) and [¹⁴C]oleic acid (18:1) were mixed with fatty acid-free BSA (2 mg/ml) and unlabeled oleic acid (18:1). This dispersion was mixed with 1 ml of a growing culture of *Escherichia coli* B (4 × 10⁶ cells) and 9 ml nutrient broth. The culture was harvested after 1 to 2 h incubation (37°C) by centrifugation and two further washes in nutrient broth. The pellet was taken up in 0.5 ml of 0.15 M NaCl, autoclaved for 30 min at 120°C, washed twice in sterile saline solution, and suspended in 0.5 ml of the same solution; 0.1-ml aliquots were frozen at −70°C.

(b) *Extraction of Lipid Acyl Hydrolyase from Galls and Leaf Tissue.* One g each of fresh gall and fresh leaf tissue were cut as described above and extracted in 0.1 M phosphate buffer plus 0.1 M NaCl containing fatty acid-free BSA at pH 7.5 for 30 min at 4°C (10). Gall tissue was extracted in 1 ml, leaf tissue in ~2 ml of buffer in order to obtain equal final volumes of the extracts. After centrifugation at 3,000 rpm, 10 min at 4°C, the extracts were reduced in volume by Amicon concentration and used for the enzyme assay.

(c) **Phospholipase Assay.** The activity of lipid acyl hydrolyase was determined according to the procedure of Scott et al. (27) as described by Bayer et al. (3): 250 μl of the plant extracts were added to 200 μl of 100 mM Tris-chloride buffer (pH 7.5), 25 μl of 200 mM CaCl₂, and 25 μl of the phospholipid labeled *E. coli* substrate (containing approximately 4 × 10⁵ cpm/ml). After incubation for 2 h at 37°C, the reaction was stopped by adding 3 ml of a mixture of chloroform:methanol (2:1 v/v). Phospholipids and FFA were extracted overnight in the biphasic mixture of chloroform:methanol (12) and separated by TLC on silica gel H plates in a solvent system of petroleum ether:diethyl ether:glacial acetic acid (80:20:1) (31). In control experiments, the substrate was incubated with commercial phospholipase A₂ (from snake venom, Sigma); in these controls, the recovery of [¹⁴C]FFA was 87%. On the same thin-layer chromatogram plates, oleic acid, palmitic acid, PE, and PC were run as controls. Plates were stained (as described above), the location of stained FFA and other phospholipid derivatives recorded, the individual sections scraped off, and their radioactivity measured in a LS-233 liquid scintillation counter (Beckman Instruments, Inc.).

## Results

Phospholipids and lipolytic hydrolase activities, which reside in cell membrane components (17), were detected in whole cell extracts containing the plasma membrane, the tonoplast, and the membrane systems of cell organelles (chloroplast, mitochondria, and ER). No attempt was made to purify these cell membrane components, but rather, emphasis was placed on a comparative analysis of the total phospholipid and lipase content in galls and in normal tissues.

As shown in Table I, the total extractable lipid content (including neutral as well as polar lipids) is considerably lower in the oak galls than in leaves, *i.e.* 2.4 and 38.5 mg/g fresh weight, respectively. The number of chloroplasts per cell is about 10 to 15 times larger in leaves than in galls and contributes to the higher lipid concentration in leaves. The study of Rey et al. (21) had shown that storage lipids, essentially neutral lipids, were distributed in larger amounts in the nutrient cell layer of several oak galls. The relatively low content of total lipids and of phosphoglycerides detected by us in *Neuroterus* galls derives from the more mature developmental stage of these Cecidia (the nutritive layer is partly atrophied) as well as from their low content of total membrane material (few chloroplasts, generally larger cell size).

**Phospholipids.** The major membrane phospholipid extracted from *Neuroterus* galls was identified by one- and two-dimensional chromatography as phosphatidylethanolamine (0.52 μg P/mg lipid); about the same amount of this phospholipid was present in normal leaf tissues (0.51 μg P/mg lipid) (Table II). PG and PE were the major phospholipids in normal leaves (0.85 and 0.70 μg P/mg lipid). The content of free fatty acids was relatively low in both normal tissues and in galls.

**Table I. Lipid and Phospholipid Content of Gall and Leaf Tissues**

<table>
<thead>
<tr>
<th></th>
<th>Total Lipids</th>
<th>Phosphorus Content in Total Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/g fresh wt</td>
<td>μg/mg lipid</td>
</tr>
<tr>
<td>Gall</td>
<td>2.4</td>
<td>1.75 ± 0.15</td>
</tr>
<tr>
<td>Leaves</td>
<td>38.5</td>
<td>3.35 ± 0.6</td>
</tr>
</tbody>
</table>

**Table II. Phospholipids in Neuroterus Galls and in Leaves**

Phospholipids were identified by 2-dimensional TLC. Phospholipid assay of phospholipids was according to Bartlett (2).

<table>
<thead>
<tr>
<th></th>
<th>Gall</th>
<th>Leaf</th>
<th>G:L</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>0.52</td>
<td>0.51</td>
<td>1:1</td>
</tr>
<tr>
<td>PG</td>
<td>0.30</td>
<td>0.85</td>
<td>1:2.8</td>
</tr>
<tr>
<td>PE</td>
<td>0.17</td>
<td>0.70</td>
<td>1:4.1</td>
</tr>
<tr>
<td>CL</td>
<td>0.34</td>
<td>0.60</td>
<td>1:1.7</td>
</tr>
<tr>
<td>PI</td>
<td>0.17</td>
<td>0.30</td>
<td>1:1.7</td>
</tr>
<tr>
<td>PS</td>
<td>0.26</td>
<td>0.35</td>
<td>1:1.3</td>
</tr>
</tbody>
</table>

Total P Phospholipid: 1.76:3.30:1:1.8

**Table III. Phospholipase Activity in Tissue Extracts of 1 Gram Gall or Leaf Tissue Each**

The data are the average of six experiments and are expressed as percentage of substrate phospholipid hydrolysis.

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Total Substrate Hydrolyzed</th>
<th>FFA (Oleic and Palmitic Acids)</th>
<th>Mono- and Diglycerides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gall</td>
<td>10.2 ± 2.1</td>
<td>5.2 ± 1.4</td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td>Leaf</td>
<td>2.7 ± 0.4</td>
<td>1.4 ± 0.5</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>Control:ddH₂O</td>
<td>1.6 ± 0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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PHOSPHOLIPIDS, PHOSPHOLIPASE IN GALLS

µg P/mg lipid, respectively). In gall tissues, only 0.3 and 0.17 µg P/mg lipid, respectively, were detected. The ratio of phospholipid species in gall and leaf tissues is expressed in Table II. The amount of PE is about 4 times as great in normal tissues than in galls. In leaves, the increase in PG (the major phospholipid of chloroplasts [17]) is due to the large amount of chloroplasts in these tissues. Similarly, the amount of the mitochondrial phospholipid CL and one of the major membrane phospholipids, PI, are higher in leaf than in gall cells. PS, a minor but generally distributed phospholipid, was present in smaller amounts in leaf and in gall tissues.

Lipid Acyl Hydrolase Activity. Enzyme activity was determined by measuring the amount of 14C at the position of oleic and palmitic acid on TLC plates. The rate of phospholipid hydrolysis by plant extracts is given in Table III. Higher lipolytic activities were detected in gall extracts, whereas only very little activity occurred in leaves. FFA were not detected in controls.

In order to determine the ratio of fatty acids (16:0, 18:1) hydrolyzed by plant phospholipase, bacterial substrates were prepared (as described in “Materials and Methods”) with either palmitic or oleic acid as the labeled acyl side chain of the phospholipid moiety. Phospholipases in extracts of gall and leaf tissues released twice as much [14C]oleic acid than [14C]palmitic acid from the substrate. No attempt was made to study the hydrolysis of phosphoglycerides by phospholipases C and D; we were concerned here only with the cleavage of free fatty acids from the labeled phospholipid substrate.

DISCUSSION

The cellular membranes and organelles of numerous plant species contain the major phospholipids PC and PE (17). The amount of PC in Neuroterus galls is not much different from that in normal leaves, but the galls contained less PE than the normal tissues. The elevated PG concentration in leaves is most likely due to the 10 to 15 times larger number of chloroplasts per cell. Also, the concentrations of CL and PI are slightly higher in leaf cells. Such differences in phospholipid composition may be comparable with variations in the phosphoglyceride content detected in stressed and pathogen-infected plant tissues (5, 14). The increase in lipolytic hydrolase activity detected in young oak galls coincides with earlier observations on defects in the regulation of fatty acid synthesis and on the deranged control of enzyme biosynthesis in neoplastic cells (32). It has to be added, however, that the phospholipid degradation can, at least in part, be interpreted by the activity of phospholipase C or D followed by lipase activity. In view of the altered phospholipid content and the increased activity in lipid acyl hydrolase in Cecidia, we conclude that the insect transformed cells are not only characterized by their histological specialization, but also by biochemical changes of their cellular membranes. Alterations in one or more of the membrane systems in normal and diseased plants are known to influence solute transport and phospholipid-auxin binding (7, 18, 25, 33). Developing Cecidia which contain only little Chl and exhibit a low photosynthetic capacity, are attracting sinks for assimilates from the leaf blade (13). The observed alteration in phospholipid ratio and the increased lipolytic activity in Neuroterus galls may reflect their involvement in the source-sink relationship between leaf and gall.

Acknowledgments—I acknowledge with thanks the technical assistance in the preparation of the labeled substrate by Greg Costello and the excellent typing of the manuscript by Gerri Zygmunt.

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