Induction of Soluble and Cell Wall Peroxidases by Aphid Infestation in Barley

Mercedes E. Chaman,[†] Luis J. Corcuera,[‡] Gustavo E. Zúñiga,[§] Liliana Cardemil,[†] and Víctor H. Argandoña*,[†]

Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile; Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Casilla 160-C, Concepción, Chile; and Departamento de Ciencias Biológicas, Facultad de Química y Biología, Universidad de Santiago, Casilla 40, Correo 33, Santiago, Chile

Peroxidase enzymes have been found in soluble, ionically bound, and covalently bound forms and have been implicated in several physiological processes in plants. This paper investigates the effect of aphid infestation on soluble and bound-cell wall peroxidase activity and bound-cell wall isoform changes of barley plants. Peroxidase activity was measured in control plants and plants infested with the aphid *Schizaphis graminum* (Rondani). The activity of soluble peroxidases increased with time of infestation, older plants being more affected than younger ones. The increase in bound-cell wall peroxidase activity as a function of age was higher in infested than in control plants, being higher in ionically bound than in covalently bound peroxidases. When the aphids were removed from plants, the activities of both types of peroxidases decreased to control levels. Isoelectrofocusing analyses of the ionically bound peroxidases showed changes in the isoform pattern. A new isoform was induced by infestation. The activities of all covalently bound isoforms increased after infestation. The physiological implications of these changes are discussed.

Keywords: *Hordeum vulgare; Gramineae; greenbug; Schizaphis graminum; isoelectrofocusing*

INTRODUCTION

Plants are usually exposed to various types of physical, chemical, and biological stresses, which may induce defensive mechanisms (*1*, *2*). Among these mechanisms are biochemical and structural responses that lead to strengthening of cells (*3*, *4*). Some of the compounds involved in this process are hydroxyproline-rich glycoproteins, callose, phenolic compounds, and lignin (*5*). The accumulation of lignin-like material around the site of infection is one of the early responses of plants to wounding (*6*). Lignin is an heteropolymer formed from monolignols by oxidases (peroxidases and lacases) in the cell wall (*7*). In addition, cell wall peroxidases catalyze lignin and suberin synthesis (*8*, *9*), the cross-linking between hydroxyproline-rich and proline-rich glycoproteins with other cell wall polymers (*10*), the synthesis of flavonols (*11*), and the oxidative catabolism of auxin, participating in this way in the regulation of plant growth (*12*). In lignified walls, peroxidases promote covalent linking between lignin and other cell wall polymers and insolubilize proteins, which make the cell wall a more effective barrier against pathogens and insects (*3*, *4*).

In Gramineae, peroxidases are induced by cold (*5*), wounding (*3*, *4*, *6*), and pathogens and aphids (*7*, *8*, *13*). Peroxidase activity increases in plants of *Hordeum vulgare* L. infested by aphids with respect to noninfested plants (*8*, *13*). Because aphid infestation causes wound and water stress in barley plants (*13*), the increase in peroxidase activity observed in this crop may take place in the cell wall and be related to repair mechanisms of the injured walls and/or to reinforce cell walls to avoid water losses.

The purpose of this paper was to study the effects of aphid infestation of barley on peroxidases, determining changes in activity in soluble and cell wall bound peroxidases and patterns of peroxidase isoforms bound to the cell wall.

MATERIALS AND METHODS

Plant Material and Growth Conditions. *H. vulgare* L. cv. Frontera plants were grown in vermiculite and kept at field capacity with Hoagland nutrient solution at 25 °C, with a photoperiod of 14/10 h light/dark. Seedlings of 7, 10, 13, and 16 days of age were infested with 20 nymphs of third- and fourth-instar of the aphid *Schizaphis graminum* (Rondani) for 24, 48, and 72 h in the case of soluble peroxidases and for 48 and 72 h in the case of cell wall bound peroxidases. In another set of experiments the leaves of 10-day-old barley plants were infested with 20 aphids, and the aphids were removed after 48 h of infestation. In these experiments ionically bound and covalently bound peroxidase activities per gram of cell wall were measured at 12, 24, 48 (when aphids were removed), 72, 96, and 120 h.

Extraction and Measurement of Soluble Peroxidase Activity. Leaf tissue (1 g) was ground with a pestle in an ice-cold mortar with 10 mL of 50 mM sodium phosphate buffer, pH 7.0. The homogenate was centrifuged at 10000*g* during 10 min. The supernatant was used as the cytosolic fraction and assayed for peroxidase activity.

Cell Wall Extracts. Cell walls were extracted according to the procedure of Ros-Barceló et al. (*14*). Five grams of foliar tissue was frozen and ground in dry ice, with a buffer containing 0.1 M Tris-HCl, pH 7.2, 0.25 M sucrose, 1 mM

* Author to whom correspondence should be addressed (telephone 56-2-6787324; fax 56-2-2717580; e-mail vargando@abello.dic.uchile.cl).
[†] Universidad de Chile.
[‡] Universidad de Concepción.
[§] Universidad de Santiago.

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magnesium acetate, 2 mM benzamidine, and 0.05 g of quartz per gram of fresh weight. The crude extract was filtered and centrifuged at 1000*g* for 5 min. The pellet was resuspended in 15 mL of 0.05 M Tris-HCl−1% Triton X-100, pH 7.2, and centrifuged again. This pellet wash was repeated three times. After this, the pellet was washed with 0.1 M sodium phosphate, pH 6.0, and centrifuged at 2000*g* for 5 min. Again, this wash was repeated three times. The complete extraction procedure was performed at 4 °C.

Extraction of Cell Wall Peroxidases. To extract proteins ionically bound to cell walls, the pellet from the previous extract was incubated in 1 M KCl at 4 °C for 12 h under constant orbital agitation. This extract was centrifuged at 15000*g* for 30 min. Proteins ionically bound to cell wall were released into the supernatant by the previous procedure. Peroxidases covalently bound to cell walls were obtained by washing the pellet three times with 1 M KCl. The pellet was resuspended in 50 mM sodium acetate, pH 5.0, with 2.5% pectinase (w/v) (5 units/mg of protein, Sigma Chemical Co.), 0.65% cellulase (w/v) (0.3 unit/mg of solid, Sigma), 2 mM benzamidine, and 1 mM peptatin A and kept under agitation for 12 h at 25 °C. This extract was centrifuged at 15000*g* for 30 min.

Peroxidase Activity Determination. The activity of the enzyme was measured according to the method of Chance and Mahely (*15*) using either guaiacol as substrate or *o*-phenylenediamine (o-PDA) as substrate (*16*). When guaiacol was used as substrate, the assay mix contained 50 μL of 20 mM guaiacol, 2.9 mL of buffer phosphate, pH 7.0, and an appropriate amount of enzyme extract. The oxidation of guaiacol was measured by the increase in A_{470} ($\epsilon = 26.6$ mM^{-1} cm^{-1}) for 2 min. The reaction was started with 20 μL of 40 mM H_2O_2. When o-PDA was used as substrate, the reaction mixture contained 0.1 M sodium citrate, pH 4.5, 10 μL of enzyme extract, 5 mM o-PDA, and 0.002% H_2O_2 (v/v) in a 1 mL final volume at 25 °C. The activity was determined by measuring A_{450}, using $\epsilon_{450} = 1.01$ mM^{-1} cm^{-1}.

In both methods, one unit of peroxidase activity was defined as the amount of enzyme necessary to oxidize 1 μmol of guaiacol or 1 μmol of o-PDA per minute under the above conditions. No reaction was observed in the absence of H_2O_2 (*16*).

Isoelectrofocusing (IEF). Extracts of ionically and covalently bound proteins (see above) were obtained from 150 g of fresh weight of leaves. These extracts were concentrated and desalted using microconcentrators (Amicon Centricon 10), with an exclusion limit of 10 kDa. These extracts were subjected to IEF in polyacrylamide gels with a pH range of 3−10 (ampholites, Bio-Rad), using a mini IEF chamber (model III, Bio-Rad). The gels were stained for activity by placing them in a solution with 0.1 M sodium citrate, pH 4.5, 5 mM 4-methoxynaphthol, and 0.004% H_2O_2 (v/v) for 1−2 min and washing them with distilled water afterward. The gels were densitometrically analyzed at 660 nm (laser densitometer model Ultrascan XL). A contact electrode was used to determine the p*I* of the various protein bands.

Statistical Analysis of Data. Descriptive statistics [means and standard errors (SE)] were used to characterize the data on a plant basis. A one-way ANOVA was used to determine significant aphid effect on plant peroxidase activity.

RESULTS

Effect of Infestation on Soluble Peroxidases. The activity of soluble peroxidases increased with infestation time (Figure 1). This increase was more evident in older plants than in younger ones. For example, soluble peroxidase activity was 86% higher in 16-day-old plants infested for 72 h than in control plants ($P < 0.05$). Soluble peroxidase activity did not change with age in control plants ($P > 0.05$).

Effect of Infestation on Peroxidases Ionically and Covalently Bound to Cell Walls. The activity of peroxidases ionically bound to cell wall in barley leaves was higher in infested plants than in their respective controls ($P < 0.05$) (Figure 2A,B). This activity, as in the case of soluble peroxidases, increased in infested plants until 13 days of age, decreasing afterward. In the controls, the activity also increased with age, but to a lower degree. Covalently bound peroxidase activity

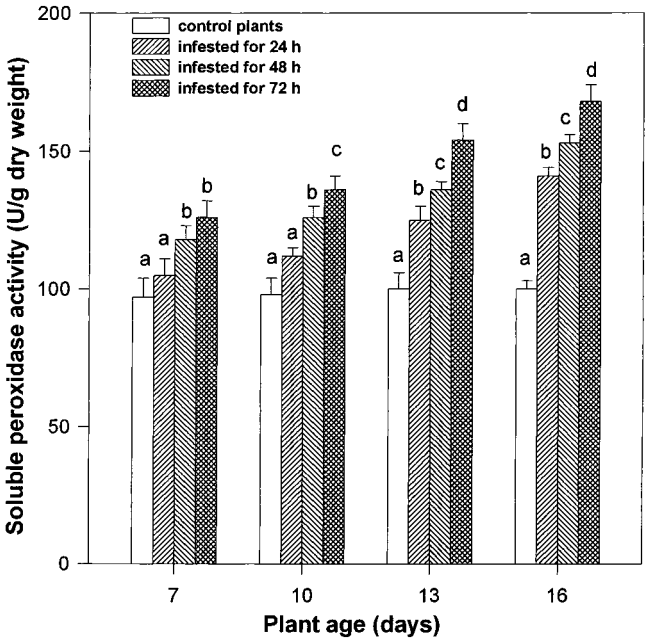

Figure 1. Effect of aphid infestation on soluble peroxidase activity in barley plants of different ages. The activity was measured by using the method of Chance and Mahely using guaiacol as substrate. Plants were infested with 20 aphids, and soluble peroxidase activity was measured at 24, 48, and 72 h. Each value is the mean of three samples ± 1 SE. The significance of differences (noted by different letters) was determined by one-way ANOVA.

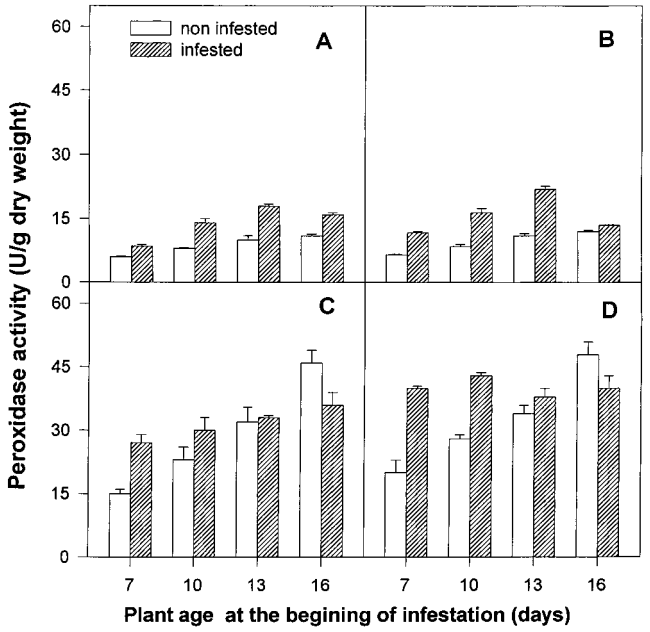

Figure 2. Effect of aphid infestation on the activity of peroxidases ionically and covalently bound to the cell wall of barley plants of different ages. Seedlings were infested with 20 aphids each. Ionically bound peroxidase activity (A and B) and covalently bound peroxidase activity (C and D) were measured at 48 h (A and C) and 72 h (B and D) with the same method but using o-PDA as substrate. Each value is the mean of three independent measurements ± SE. The significance of differences was determined by one-way ANOVA.

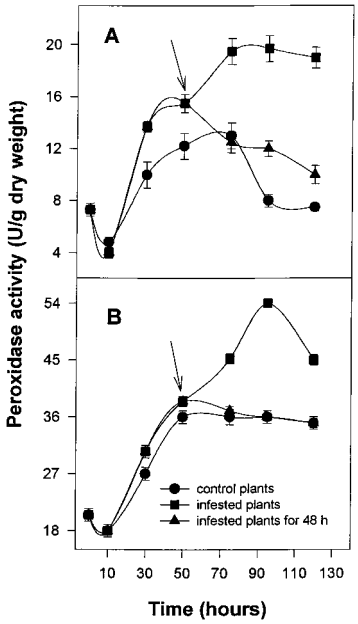

Figure 3. Effect of aphid infestation on peroxidase activity: (A) activity of ionically bound peroxidase to the cell wall; (B) activity of covalently bound peroxidase to the cell wall. Enzymatic activities were measured periodically in leaf extracts of three groups of plant of *H. vulgare* cv. Frontera. The first group was infested during all of the experiment with 20 nymphs of the aphid *S. graminum*. The second group was infested only during 48 h; after this time, the aphids were removed from the plants. The third group was the control (noninfested plants). The experiment was performed with 10-day-old plants. Arrows point to the times when the aphids were removed. Each value is the mean of three independent measurements ± SE. The significance of differences was determined by one-way ANOVA.

increased in infested plants with respect to the control only in the first 10 days of infestation (Figure 2C,D). The activity in control plants increased steadily with age, becoming higher than the activity of infested plants at the age of 16 days. In 13-day-old plants, the increase in ionically bound peroxidase activity (Figure 2A,B) was proportionally higher in both control and infested plants than the increase in the covalently bound peroxidase activity of plants of the same age (Figure 2C,D) ($P < 0.05$).

When the aphids were removed from the plants, the peroxidase activities returned to values similar to those of the control (Figure 3). Because the activity started to decrease immediately after the aphids were removed, it may be concluded that the presence of aphids induced the increase in peroxidase activity over the control plants.

Effect of Infestation on Peroxidase Isoforms. The pattern of ionically bound peroxidases in noninfested plants showed eight isoforms as detected by IEF with p*I* values ranging between 6.44 and 9.23 (Figure 4A). Infestation induced the transient expression of one isoform with a p*I* of 6.76, only in 7- and 10-day-old plants. The band of p*I* 7.75 was induced with the age of the plant. This band also increased strongly with infestation. An increase in the activity of the isoforms p*I* 6.96, 7.98, and 8.54 was also detected with age and infestation of the plants (Figure 4B).

The analysis of covalently bound peroxidase isoforms present in the cell walls of barley was done in 7-day-old plants and after 72 h of infestation, because at this age there is a higher increase in peroxidase activity.

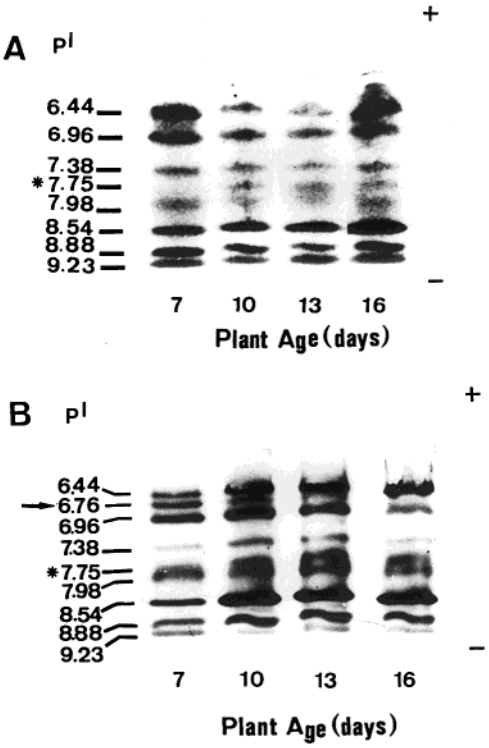

Figure 4. IEF of peroxidases ionically bound to cell walls of barley leaves: (A) extracts from leaves of noninfested plants (control); (B) extracts from infested plants taken 72 h after infestation. IEF was performed in polyacrylamide gels with a pH gradient from 3 to 10. Each well was loaded with 2 units of activity. The bands were stained by activity as described under Materials and Methods. The pH gradient along the gels was determined by a contact electrode. The arrow points to the new band induced by infestation. Asterisks (*) indicate the band present in control plants that is induced by age and increased after infestation.

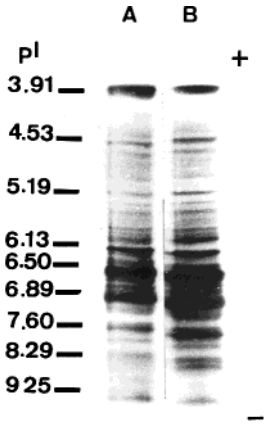

Figure 5. IEF of peroxidases covalently bound to cell walls of barley leaves: (A) extracts prepared by enzymatic digestion of leaves of 7-day-old noninfested plants (control); (B) extracts from infested plants taken 72 h after infestation. IEF was performed in polyacrylamide gels with a pH gradient from 3 to 10. Each well was loaded with 1.5 units of activity. The isoform bands were stained by activity as described under Materials and Methods.

Under infestation most of the covalently bound peroxidase isoforms increased. The increase was higher for those isoforms with p*I* 6.50, 6.89, and 7.60 (Figure 5).

DISCUSSION

An increase in the activity of soluble peroxidases seems to indicate that aphid infestation initiates a

general oxidative metabolism in barley, which is probably triggered by H_2O_2. The level of H_2O_2 increases rapidly after wounding (*17*) and during pathogenesis (*18*). H_2O_2 also increases in barley plants cv. Frontera after infestation with aphids (manuscript in preparation). Enzymes related to oxidative processes such as peroxidases and catalases appear in infested tissues (*18*). Among other oxidative enzymes are those which catalyze ethylene and jasmonic acid biosynthesis, which in turn induce defense responses in plants (*2*, *19*). The age-related increase in activity of ionically bound peroxidases in noninfested plants may be associated with the synthesis of lignin during growth and development, as has been noticed in other systems (*4*). For example, ionically bound peroxidase activity also increases with age in the epicotyls of *Cicer arietinum* L. cv. Castellana, with a higher activity found when growth ceased (*20*). Similarly, cell wall-bound peroxidases also increase in *Arachis hypogaea* L. when hypocotyl segments are cultivated with metafluorotyrosine (MFT), a growth inhibitor (*21*). A lower activity in cell wall peroxidases has been found in coleoptiles and roots of *Oryza sativa* L. (*22*) and leaves of *Festuca arundinacea* Schreb at the time of maximal growth rate (*23*). Cell wall peroxidases may be responsible for the cross-linking between cell wall polymers, which in turn increases the rigidity of cell walls. Cell wall plasticity is, therefore, decreased. For these reasons, it seems logical that cell wall peroxidase activity becomes maximal at the time the cells stop growth (*4*, *10*). Peroxidase activity also increases during pathogenesis and periods of environmental stress (*2*, *18*). Therefore, increases in activity of this type of peroxidases are the result of both age and infestation. Aphids delay the normal growth of barley plants; in more severe cases of infestation plant growth is arrested. Infestation anticipates the increment in activity of this kind of peroxidase, which normally occurs later with age. The decrease in peroxidase activity in infested 16-day-old plants could also indicate that in infested plants there are already sufficient phenolic compounds for lignification and reinforcement of cell walls. Some phenolic compounds could act as feeding deterrents or show toxicity against insects. Perhaps both defense mechanisms are present in the plants at this age (*24*).

The age-related increase in covalently bound peroxidase activity in control plants suggests that this type of peroxidases could also be involved in the regulation of growth, development, and cell wall lignification (*4*, *10*, *25*). Covalently bound peroxidases have also been related to fiber lignification, for example, in *Linum usitatissimum* L. (*26*). This may also be the case of barley and other Poaceae species, which develop sclerenchymatic tissue with lignified cell walls.

The proportionally higher increase in ionically bound activity than in covalently bound activity could suggest that ionically bound peroxidases have a more important protective role against infestation than the covalently bound peroxidases. Nonetheless, this remains to be shown.

The decrease in peroxidase activity after the aphids were removed suggests that cell wall peroxidases are more involved in defense against aphids than in repairing the wounded tissue. Peroxidases may also protect plants against the oxidative metabolism triggered in the cell wall by aphid infestation. Injury results in the presence of H_2O_2 in the cell wall, where active peroxidases decompose it (*2*, *17*, *27*). Among other oxidative enzymes are those that catalyze ethylene and jasmonic acid biosynthesis (*4*, *19*).

Changes in the peroxidase patterns have been reported in wounded plants or plants attacked by pathogens (*28*, *29*). Our work indicates that the increase in activity of ionically bound peroxidases produced by aphid infestation was mainly due to an increase in the activity of constitutive isoforms rather than to the induction of new isoforms. However, a new isoform having a p*I* of 6.76 was induced by infestation. The role that this isoform may play during the 3-day period when its activity was detected is unknown.

The IEF profile of the covalently bound peroxidases showed a higher number of isoforms compared with the isoforms of ionically bound peroxidases. Most of the isoforms were acidic, with p*I* values ranging from 3.91 to 6.89. The infestation also induced an increase in the activity of the isoforms already present in the control plants, principally the isoforms with p*I* values of 6.5, 6.89, and 7.60. No new isoforms of covalently bound peroxidases were detected upon infestation.

The main conclusion of this work is that aphid infestation increases soluble and cell wall-bound peroxidase activity in barley plants. Is the increase in peroxidase activity detected after infestation due to the activity of plant peroxidases or due to contamination by the aphids' peroxidases? It is known that *S. graminum* may inject enzymes to infested tissues (*30*). Thus, it is possible that aphids may contribute to the increase in soluble barley peroxidases with their own peroxidases. However, because many of the changes in activity were observed in cell wall-bound peroxidases, it is unlikely that they correspond to aphid-injected peroxidases. Moreover, IEF studies showed that many peroxidases that were present before infestation increased their accumulation in infested plants.

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