JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Relationships between Salicylic Acid Content, Phenylalanine Ammonia-lyase (PAL) Activity, and Resistance of Barley to Aphid Infestation

MERCEDES E. CHAMAN,† SYLVIA V. COPAJA,‡ AND VICTOR H. ARGANDOÑA*,§

Departamento de Ciencias Biológicas, Facultad de Ciencias Biológicas, Universidad Nacional de Trujillo, Peru; Depàrtamento de Química, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile; and Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile

It has been suggested that salicylic acid (SA) is a signal in acquired resistance to pathogens in several plants. Also, it has been suggested that infestation of plants causes an increase in the activity of phenylalanine ammonia-lyase (PAL), a key phenolic biosynthesis enzyme. The purpose of this work was to investigate whether the induction of SA and PAL activity is related to the susceptibility of barley to aphid infestation. The induction of free and conjugated SA in two barley cultivars that differ in susceptibility to aphids was analyzed. Analyses of several physiological parameters showed that cv. UNA-80 was more susceptible to the aphid *Schizaphis graminum* than cv. LM-109. Salicylic acid was not detected in noninfested plants. Levels of free and conjugated SA in cv. LM-109 and of conjugated SA in cv. UNA-80 increased with aphid infestation, whereas the levels of free SA in cv. UNA-80 remained high under all infestation degrees. Maximum values reached in both cultivars were not significantly different. With respect to PAL activity, cv. LM-109 showed a significantly higher specific activity than cv. UNA-80, the more susceptible cultivar. The relationship between the susceptibility of a plant to aphid and SA induction and PAL activity is discussed.

KEYWORDS: ***Hordeum vulgare*; greenbug; *Schizaphis graminum*; salicylic acid; PAL**

INTRODUCTION

Physical, chemical, and biological stresses induce defensive mechanisms in plants (*1*, *2*). Among the defensive responses induced by pathogens are cell-wall appositions, accumulation of pathogenesis-related (PR) proteins, production of H_2O_2, hypersensitive cell death, and accumulation of callose, phenolic compounds, and lignin around the site of infection (*3*, *4*). An increase in the ethylene evolution and induction of soluble and cell-wall peroxidases by aphid infestation has also been described (*5*, *6*). Pathogen-infected leaves of tobacco, cucumber, and *Arabidopsis thaliana* accumulate salicylic acid (SA) (*7*−*9*). Several authors suggest that SA plays a role in the accumulation of H_2O_2 and PR proteins and that it is involved in systemic acquired resistance (SAR) (*10*−*13*). It has been proposed that SA inhibits catalase activity, resulting in accumulation of H_2O_2 and induction or activation of defense genes (*14*, *15*). However, other authors have provided evidence for an SA-independent biological induction of resistance (*16*). Other results about hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus support the hypothesis that H_2O_2, but not SA, may play a role in defense against fungi (*17*). Studies on insect−plant interaction suggest that phenyl-propanoid compounds are involved in plant defenses against pests (*18*). Phenylalanine ammonia-lyase (PAL) is a key enzyme in the synthesis of phenolic compounds. Aphids need to penetrate with their stylets into plants to reach the phloem sieve elements, the sap of which forms their food source. Also, probing plays an important role in food plant tissue selection. The greenbug affects the vegetative growth of barley by destruction of photosynthetic areas.

In contrast to pathogen attack, herbivore attack is frequently associated with wounding and also allows plants to use defenses that would be ineffective against pathogens. For example, plants use secondary metabolites. Both herbivore feeding and mechanical damage induce systemic responses propagated throughout the plant. Systemic responses require mobile signals; these could be electrical, hydraulic, and chemical (*19*). In this paper, we report the effect of aphid infestation on SA accumulation and PAL activity in two barley cultivars that differ in their susceptibility to the aphid *Schizaphis graminum*.

MATERIALS AND METHODS

Plants and Aphids. Barley (*Hordeum vulgare* L.) cultivars LM-109 and UNA-80 were grown in a glasshouse, in pots with vermiculite and kept at field capacity with Hoagland nutrient solution at 25 °C,

* Author to whom correspondence should be addressed (telephone 56-2-6787324; fax 56-2-2717580; e-mail vargando@uchile.cl).
† Universidad Nacional de Trujillo.
‡ Depàrtamento de Química, Universidad de Chile.
§ Depàrtamento de Biología, Universidad de Chile.

10.1021/jf020953b CCC: $25.00
Published on Web 03/06/2003

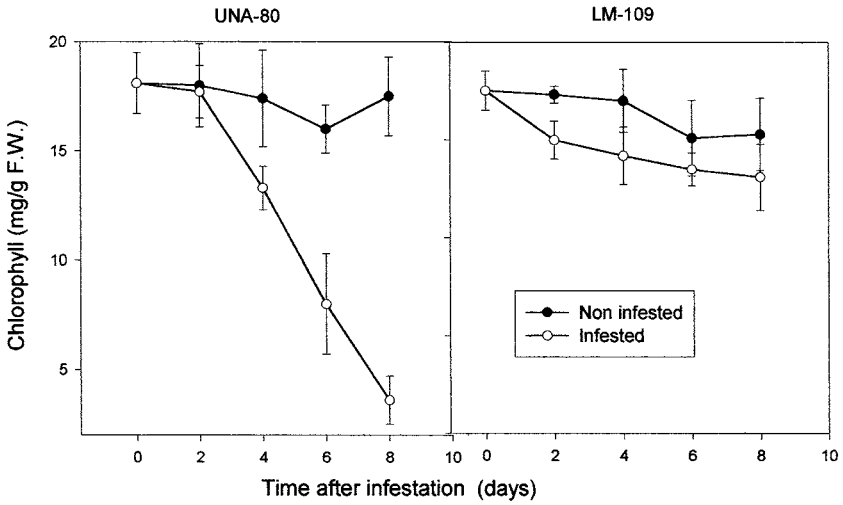

Figure 1. Effect of aphid *S. graminum* infestation on chlorophyll content in leaf of *H. vulgare* cv. UNA-80 and LM-109. Two groups of seedlings, 10 days old, of each cultivar were used for experiments. Controls were without infestation, and infested groups were initially infested with 10 aphids. Chlorophyll content was measured after 2, 4, 6, and 8 days. Each value is the mean of three samples ± SE.

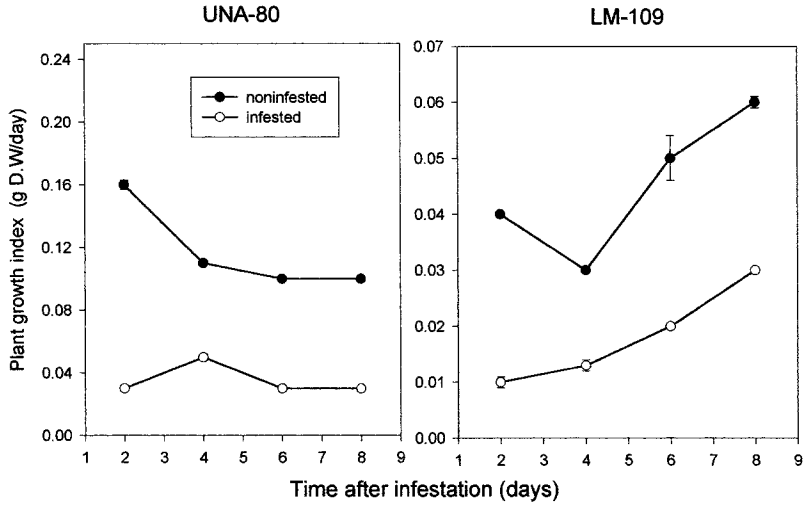

Figure 2. Effect of aphid *S. graminum* infestation on growth of *H. vulgare* cv. UNA-80 and LM-109. Ten-day-old seedlings were infested with 10 aphids each and maintained in a growth chamber under 25 ± 3 °C and with 14/10 h light/darkness. Dry weight of 10 plants was obtained every 2 days. Samples were dehydrated in a stove at 50 °C to obtain a constant weight. The relative growth index (RGI) was calculated with the expression RGI = (dry weight final − dry weight initial)/no. of days. Each value is the mean of three independent measurements ± SE. The significance of differences was determined by one-way ANOVA.

with a photoperiod of 14/10 h light/darkness. The aphids used in the assays were *S. graminum* (biotype C) obtained from colonies maintained in the greenhouse at temperatures of ∼28 °C and under a photoperiod of 12/12 h light/darkness. The age of the plants was measured after sowing.

Analysis of Physiological Parameters. Barley seedlings of 10 days of age of cv. LM-109 and cv. UNA-80 were infested with 10 apterous aphids each. After 2, 4, 6, and 8 days, chlorophyll content (ChlC), plant growth index (PGI), and the reproduction of aphids (IR) were measured. Chlorophyll content was measured by extracting plant leaves with 96% (v/v) EtOH. The extract was filtered through Whatman II paper, and the absorbance of the extract was measured at 649 and 665 nm as described in ref *20*. The equation used for determinations of total chlorophyll was as follows: total Chl (μg of Chl mL^{-1} of extract) = $18.08A_{649} + 6.63A_{665}$). The relative growth index of plants was calculated as (dry weight final − dry weight initial)/number of days. The reproduction index of aphids was calculated as (final number of aphids − initial number of aphids)/number of days.

Analysis of Free SA. One gram of fresh weight of frozen material was macerated in a mortar with a pestle with 3 mL of methanol (HPLC grade) three times. Samples were stirred in a vortex and then centrifuged at 1650*g* for 20 min. The supernatant was transferred to a new centrifuge tube. The residue (pellet) was resuspended in 3 mL of methanol, stirred in a vortex, and centrifuged for another 20 min at 1650*g*. All of the supernatants were mixed and centrifuged once again for 20 min. The liquid was transferred to a glass bottle, and the solvent was evaporated with vacuum at <40 °C. Before this step, samples were frozen for subsequent treatment. Frozen residues were resuspended in 2.5 mL of 5% trichloroacetic acid (TCA), stirred for 1 min in a vortex, and ultrasonicated for 10 min; the solution was then centrifuged for 15 min at 1400*g*. Five milliliters of ethyl acetate/cyclopentane/isopropyl alcohol (50:50:1 v/v, all of them of HPLC grade), was added to the samples. These samples were transferred to a separator funnel. After shaking, the organic phase was left in a vial and evaporated with nitrogen. The residue was frozen for subsequent analysis.

Analysis of Conjugated SA. The aqueous phase from the separation with the organic solvents mixture was adjusted to pH 1.0 with 0.1 N HCl and boiled for 30 min. Then the samples were treated with the same procedure as for free SA.

Chemical Analysis. Residues were resuspended in 1.5 mL of 0.01 N H_2SO_4. HPLC was performed with a reverse-phase column [LiChroCART 125-4, LiChrosfer 100 RP-18 (5 μm)]. Elution was carried out isocratically with methanol/water (pH 3.0; 0.1 N H_2SO_4) (55:45). The flow rate was 1.0 mL min^{-1}. Detection was carried out at 280 nm. The

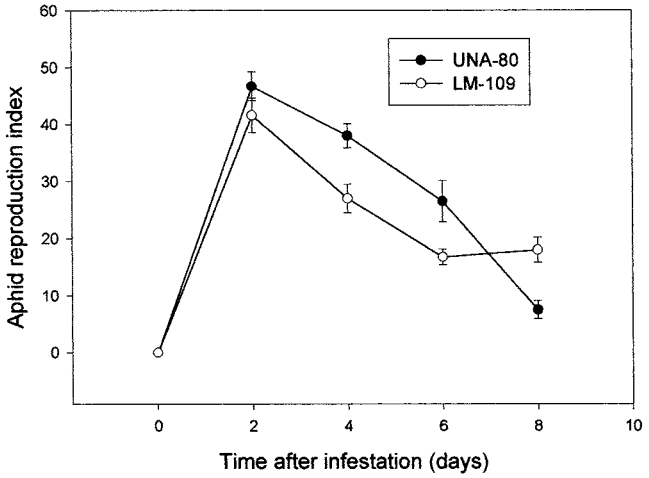

Figure 3. *S. graminum* reproduction index on cv. LM-109 and UNA-80. Ten-day-old seedlings were infested with 10 aphids each. The reproduction rate was measured after 2, 4, 6, and 8 days. Aphid reproduction index was calculated with the expression IR = (final no. of aphids − initial no. of aphids)/no. of days. Each value is the mean of three independent measurements ± SE.

sample injection volume was 50 μL. Identification of free and conjugated SA was made by their retention time and coincidence with the pure standard.

Preparation of Extracts and Quantification of PAL Activity. PAL activity was evaluated by measuring *trans*-cinnamic acid formation in a crude extract of proteins (*21*). Proteins were extracted from 1 g of fresh leaves using 3 mL of borate (0.1 M, pH 8.8), containing β-mercaptoethanol (0.01 M) and phenylmethanesulfonyl fluoride (PMSF) (3 mM). The homogenized tissue was centrifuged at 1000*g* for 10 min at 4 °C. PAL activity was assayed with an aliquot from supernatant (100 μL), 10 mM L-phenylalanine, and borate in a 3 mL final volume. The control was without L-phenylalanine. The reaction product was measured at 290 nm in a Shimadzu UV-240 over 1 h. The enzymatic activity was expressed in pKat (picomoles of cinnamic acid formed per second). Soluble protein concentrations were measured using Bradford reactive and measuring the absorbance at 595 nm. Calibration curves were done with bovine serum albumin (BSA).

Statistical Analysis. Descriptive statistics (means and standard errors) was used to characterize the data on a plant basis. A one-way ANOVA was used to determine significant aphid effect on the different parameters analyzed.

RESULTS

Effect of Aphid Infestation on Barley Seedlings. The ChlC of cv. LM-109 was not significantly affected; however, in cv. UNA-80 it decreased significantly after 4 days of infestation, reaching an 80% loss after 8 days (**Figure 1**). The PGI of cv. UNA-80 was more affected than that of cv. LM-109 (**Figure 2**). After 2 days of infestation, the PGI of cv. UNA-80 decreased 87% and that of cv. LM-109 decreased 75%. After 4 days of infestation, the PGI of cv. UNA-80 and cv. LM-109 decreased 75 and 50%, respectively. The IR of *S. graminum* at 2 days was not significantly different in either cultivars (**Figure 3**). The IR of *S. graminum* at 4 and 6 days in cv. LM-109 was lower than in cv. UNA-80 (**Figure 3**). After 6 days, the IR in cv. UNA-80 decreased considerably because the plants were in poor condition due to infestation.

SA Induction from Two Infested Barley Cultivars. Barley seedlings (cv. LM-109 and cv. UNA-80) of 10 days of age were infested with 10, 20, and 40 nymphs of third- and fourth-instar of the aphid *S. graminum* (Rondani). Another group of barley seedlings was not infested as a control. Free and conjugated SA levels were measured after 3 days of infestation and were expressed as micrograms per gram of fresh weight (FW). SA was not detected in noninfested plants (**Figure 4**). In cv. LM-109 the concentration of free and conjugated SA increased as a function of infestation level, reaching values of 12 and 18 μg/g of FW, respectively, in plants infested with 40 aphids. In cv. UNA-80, the more susceptible cultivar, the maximum values of free SA concentrations were detected in plants infested only with 10 aphids. Conjugated SA was first detected in plants infested with 20 aphids, reaching a concentration of 9.1 μg/g in plants infested with 40 aphids. The maximum values of accumulation of free SA were not significantly different in either cultivar ($P < 0.05$).

Effect of Aphid Infestation on PAL Activity in Barley. There was a significant increase in PAL activity as a function of infestation degree (**Table 1**). The values of total activity did not show a significant difference between cultivars. However, cv. LM-109 showed a significantly higher specific activity than cv. UNA-80, the more susceptible cultivar.

DISCUSSION

Its genetic composition and the environment in which it is grown determine a plant's phenotype. Molecular markers have

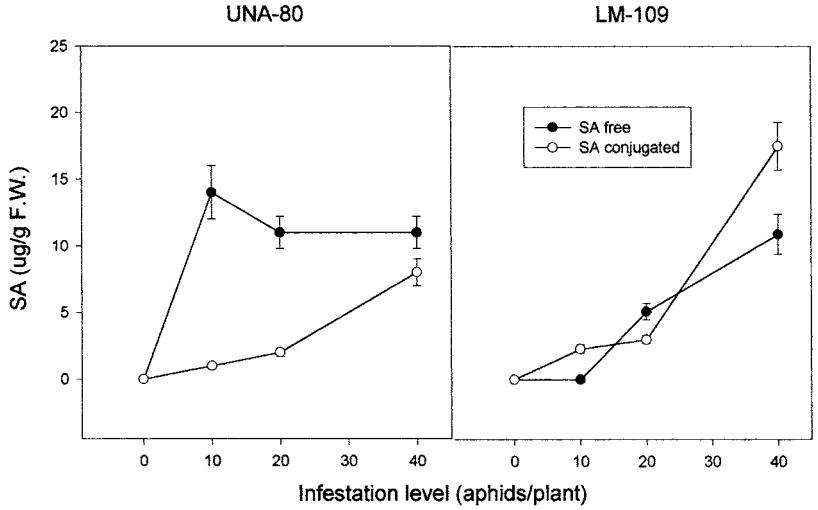

Figure 4. Effect of aphid *S. graminum* infestation on salicylic acid content in leaf of *H. vulgare* cv. LM-109 and UNA-80. Ten-day-old primary leaves of the barley cultivars were infested with 10, 20, and 40 aphids. At 72 h the leaves were cut and free and conjugated SA levels were determined by HPLC. Each point represents the mean of five samples ± SE.

Table 1. Effect of Aphid Infestation on PAL Activity in Barley

no. of aphids	specific activity (pkat mg^{-1} of protein)		total activity (pkat/g of fw)	
	UNA-80	LM-109	UNA-80	LM-109
0	4.1 ± 0.30	3.5 ± 0.22	5.22 ± 0.21	4.20 ± 0.48
10	5.7 ± 0.62	4.9 ± 0.29	7.03 ± 0.35	8.14 ± 0.52
20	8.2 ± 0.29	9.3 ± 0.42	9.20 ± 0.92	12.52 ± 0.39
40	9.1 ± 0.26	10.8 ± 0.23	9.45 ± 0.39	13.64 ± 0.58

[a] Ten-day-old seedlings were infested with 10, 20, or 40 aphids each. PAL activity was measured 72 h later. Each value is the mean of five samples ± standard error.

been used to map genes for insect resistance in most major crop species. Often, the effect of the environment masks genotypic effects. In practice, the phenotypic selection for insect resistance will depend on a comparison of economic and logistical advantages offered by the different systems (*22*). The greenbug is known to cause breakdowns of the cell membranes, including chloroplasts and mitochondria damage. Chlorosis has been identified as the main cause for the reduced photosynthesis and the decrease in plant growth. The differences seen in the ChlC and PGI between noninfested and infested plants and the differences seen in the IR of aphids in both cultivars show that cv. LM-109 was more resistant than cv. UNA-80. Resistance in plants involves intricate relationships between the plant that is being attacked and the organism that is doing the attacking. Resistance and susceptibility are relative concepts. A plant may be moderately or extremely more susceptible than another. Resistance depends on some structural or physiological characteristics of plants. Some of these characteristics may be present before the invasion of the parasite; others can be induced by parasite attack.

When an insect attacks a plant, it induces several morphological and physiological changes (*6*, *23*, *24*). Some of these changes are related to tissue repair, others with the synthesis of metabolites that cause noxious effects against insects and pathogens. In the present work, we provided evidence that accumulation of free SA in barley is related with damage level caused by aphid infestation. Free SA is assumed to be the active form (*25*). The differences between conjugated and free SA concentrations was higher in cv. UNA-80 than in cv. LM-109, with the exception of plants infested with 40 aphids per plant. Studies of SA metabolism in rice show that SA is synthesized from cinnamic acid, a precursor for *p*-coumaric acid and ferulic acid from which lignin can be synthesized. Also, other phenolic compounds have been related to defensive mechanisms of plants because they cause toxic effects against aphids. PAL gene expression has been associated with abiotic stress, wounding, and infection (*26*). It has been found that wheat infestation induces an increase in the PAL activity and in the content of phenolic compounds (*27*). Analysis of the results suggests that the differences in susceptibility between cv. UNA-80 and cv. LM-109 appear to be independent from free SA accumulation. It is likely that SA accumulation occurs in response to the damage caused by aphids. The smaller reproduction index of aphids in cv. LM-109 is probably due to the greater presence of phenolic compounds and other defensive characteristics.

ACKNOWLEDGMENT

We thank INIA Cajamarca y Programa de cereales de la Universidad Agraria La Molina Perú for donation of seeds.

LITERATURE CITED

(1) Larcher, W. *Physiological Plant Ecology*, 3rd ed.; Springer-Verlag: Heildelberg, Germany, 1995.

(2) Somssich, I. E.; Hahlbrock, K. Pathogen defense in plants—a paradigm of biological complexity. *Trends Plant Sci.* **1998**, *2*, 86−90.

(3) Thordal-Christensen, H.; Zhang, Z.; Wei, Y. D.; Collinge, D. B. Subcellular localization od H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J.* **1997**, *11*, 1187−1194.

(4) Benhamou, N. B. Elicitor-induced plant defense pathways. *Trends Plant Sci.* **1996**, *7*, 233−240.

(5) Argandoña, V. H.; Chaman, M.; Cardemil, L.; Muñoz, O.; Zúñiga, E.; Corcuera, L. J. Ethylene production and peroxidase activity in aphid-infested barley. *J. Chem. Ecol.* **2001**, *27*, 53−68.

(6) Chaman, M.; Corcuera, L. J.; Zúñiga, G. E.; Cardemil, L.; Argandoña, V. H. Induction of soluble and cell-wall peroxidases by aphid infestation in barley. *J. Agric. Food Chem.* **2001**, *49*, 2249−2253.

(7) Malamy, J.; Carr, J. P.; Klessig, D. F.; Raskin, I. A likely endogenous signal in the resistance response of tobacco to viral infection. *Science* **1990**, *250*, 1002−1004.

(8) Metraux, J. P.; Singer, H.; Ryals, J.; Ward, E.; Wyss-Benz, M.; Gaudin, J.; Raschdorf, K.; Schmid, E.; Blum, W.; Inverardi, B. Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* **1990**, *250*, 1004−1006.

(9) Uknes, S.; Winter, A. M.; Delaney, T.; Vernooij, B.; Morse, A.; Friedrich, L.; Nye, G.; Potter, S.; Ward, E.; Ryals, J. Biological induction of systemic acquired resistance in *Arabidopsis*. *Mol. Plant−Microbe Interact.* **1993**, *6*, 692−698.

(10) Shirasu, K.; Nakajima, H.; Rajasekhar, V. K.; Dixon, R. A.; Lamb, C. Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. *Plant Cell* **1997**, *9*, 261−270.

(11) Delaney, T. P.; Uknes, S.; Vermooij, B.; Friedrich, L.; Weymann, K.; Negretto, D.; Gaffney, T.; Gut-Rella, M.; Kessmann, H.; Ward, E.; Ryals, J. *Science* **1994**, *266*, 1247−1249.

(12) Gaffney, T.; Friedrich, L.; Vernooij, B.; Negrtto, D.; Nye, G.; Uknes, S.; Ward, E.; Kessmann, H.; Ryals, J. Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* **1993**, *261*, 754−756.

(13) Vernooij, B.; Friedrich, L.; Morse, A.; Reist, R.; Kolditz Jawhar, R.; Ward, E.; Uknes, S.; Kessmann, H.; Ryals, J. Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plant Cell* **1994**, *6*, 959−965.

(14) Bradley, D. J.; Kjellbom, P.; Lamb, C. J. Elicitor and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel rapid defense response. *Cell* **1992**, *70*, 21−30.

(15) Wu, G.; Shortt, B. J.; Lawrence, E. B.; León, J.; Fitzsimmons, K. C.; Levine, E. B.; Raskin, I.; Shah D. M. Activation of host defense mechanisms by elevated production of H_2O_2 in transgenic plants. *Plant Physiol.* **1997**, *115*, 427−435.

(16) Wei, Y.; Zhang, Z.; Andersen, C. H.; Schmeizer, E.; Gregersen, P. L.; Collinge, D. B.; Smedegaard-Petersen, V.; Thordal-Christensen, H. An epidermis/papilla specific oxalate oxidase-like protein in the defense response of barley attacked by the powdery mildew fungus. *Plant Mol. Biol.* **1998**, *36*, 101−112.

(17) Hückelhoven, R.; Fodor, J.; Preis, C.; Kogel, K. Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with hydrogen peroxide but not with salicylic acid accumulation. *Plant Physiol.* **1999**, *119*, 1251−1260.

(18) Dixon, R. A.; Paiva, N. L. Stress induced phenylpropanoid metabolism. *Plant Cell* **1995**, *7*, 1085−1097.

(19) Kessler, A.; Baldwin, I. T. Plant responses to insect herbivory; The emerging molecular analysis. *Annu. Rev. Plant Biol.* **2002**, *53*, 299−328.
(20) Lichtenthaler, H. K.; Wellburn, A. R. Determination of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. *Biochem. Soc. Trans.* **1983**, *11*, 14.
(21) Zucker, M. Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. *Plant Physiol.* **1965**, *40*, 779−784.
(22) Yencho, G. C.; Cohen, M. B.; Byrne, P. F. Applications of tagging and mapping insect resistance loci in plants. *Annu. Rev. Entomol*. **2000**, *45*, 393−422.
(23) Argandoña, V. H. Effect of aphid infestation on enzyme activities in barley and wheat. *Phytochemistry* **1994**, *35*, 313−314.
(24) Cabrera, H. M.; Argandoña, V. H.; Zúñiga, G. E.; Corcuera, L. J. Effect of infestation by aphids on water status of barley and insect development. *Phytochemistry* **1995**, *40*, 1083−1088.
(25) Dempsey, D'M. A.; Klessig, D. F. Salicylic acid active oxygen species and systemic acquired resistance in plants. *Trends Cell Biol.* **1994**, *4*, 334−338.
(26) Leyva, A.; Jarillo, J. A.; Salinas, J.; Martinez-Zapater, J. M. Low-temperature induces the accumulation of phenylalanine ammonia-lyase and chalcone synthase mRNAs of *Arabidopsis* in a light-dependent manner. *Plant Physiol.* **1995**, *108*, 39−46.
(27) Leszczynski, B. Changes in phenols content and metabolism in leaves of susceptible and resistant winter wheat cultivars infested by *Rhopalosiphum padi* (L) (Hom. Aphididae). *Z. Angew. Entomol.* **1985**, *100*, 343−348.

Received for review September 12, 2002. Revised manuscript received December 13, 2002. Accepted December 13, 2002. This research was supported by Red Latinoamericana de Botánica Proyecto BINAC 99-2.

JF020953B