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EXPLOITATION OF PLANT PATHOGENESIS-RELATED PROTEINS FOR ENHANCED PEST RESISTANCE IN CITRUS

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Abstract. **Among the various defense mechanisms that plants exhibit in response to attack by pests is the expression of a number of proteins collectively referred to as 'pathogenesis-related proteins'. We are interested in these proteins in citrus with the long term goal of enhancing resistance to fungal and insect pests by developing improved germplasm and the use of elicitors to induce these proteins on demand. Two classes of enzymes, chitinases and β-1,3-glucanases, which are known to have antifungal characteristics are active in all citrus tissues studied (roots, leaves, blossoms, and fruit). The amount of activity varies with tissue, tissue age, and in some cases cultivar. Chitinase and β-1,3-glucanase activities increase with age in leaves whereas chitinase activity decreases and β-1,3-glucanase activity increases with age in flavedo. Infection of fruit by *Penicillium digitatum* induced increased activities of these enzymes. Feeding by sugar cane rootstock borer weevil (*Diaprepes abbreviatus*) larvae induced increased chitinase activity substantially in roots of 2 of 8 cultivars that we examined and in leaves of sour orange; in contrast to chitinase, β-1,3-glucanase activity is reduced by weevil feeding. Treatment of grapefruit trees with gibberellic acid, salicylic acid, or Keyplex 350 resulted in significant, although transient increases in both enzymes.**

Increased concern by consumers and regulatory agencies over the use of pesticides in agriculture has resulted in the need to develop alternative methods of plant protection. Plants have several mechanisms for defense against fungal and insect pests. Among the defense mechanisms is a group of proteins referred to collectively as "pathogenesis-related" (PR) proteins (Stintzi et al. 1993). Chitinase and β-1,3-glucanase are two enzymes that are classified as PR proteins. Chitinase hydrolyzes chitin, a β-1,4-linked polymer of *N*-acetylglucosamine, a structural component of fungal cell walls and the exoskeleton of arthropods. Chitin is not present in higher plants (Flach et al. 1992). β-1,3-Glucanase hydrolyses β-1,3-linked glucans, also structural components of fungal cell walls, and found in higher plants as callose (Stone 1984). Chitinases and β-1,3-glucanases are typically expressed constitutively at low levels in most plant tissues (Kombrink et al. 1988). Levels of chitinase and β-1,3-glucanase are typically regulated in a coordinated manner in a variety of tissues both developmentally (Mauch et al. 1988a,b; Felix and Meins 1986) and in response to various stimuli including ethylene treatment (Abeles et al. 1970; Boller 1985; Vogeli et al. 1988) and fungal infection (Mauch et al. 1988a). Chitinase and β-1,3-glucanase often act synergistically to inhibit fungal growth (Leah et al. 1991; Melchers et al. 1993). Transgenic tobacco plants engineered to express proteins which have the ability to inhibit fungal growth in vivo show enhanced resistance to the fungus *Rhizoctonia solani* (Broglie et al. 1991).

Because citrus is a long-lived perennial crop, it would be advantageous to enhance levels of PR proteins in established trees; the potential of chemical elicitors for this purpose is therefore of interest. Treatment of citrus with gibberellic acid is known to increase resistance to some fungal (Ferguson et al. 1982) and insect (Greany et al. 1994) pests, but the mechanism of the resistance is not known. Salicylic acid induces the accumulation of PR proteins in many plants and may enhance resistance to pests.

We are interested in developing an understanding of the roles of chitinase and β-1,3-glucanase in pest resistance in citrus. To this end, we have conducted studies to determine if chitinases and β-1,3-glucanases are expressed in citrus and if the enzymes are affected by challenges by fungal and insect pests. In this report we document levels of chitinases and β-1,3-glucanases in various citrus tissues, the effects of fungal and insect challenges, and chemical treatments on these enzymes.

Materials and Methods

Activity of chitinases and glucanases in various tissues. Leaves, blossoms, and fruit were sampled from mature field-grown 'Valencia' orange trees. Tissues were frozen and held at -20C until proteins were extracted.

Effects of fungal infection. 'Marsh' grapefruit were harvested at commercial maturity, washed in soapy water and then rinsed in deionized water. Fruit were inoculated using a syringe to a depth of 2 mm with 5 µL of either sterile distilled water or a suspension of *Penicillium digitatum* spores (1×10^5 spores/mL). The fruit were held at 21C and near 100% relative humidity. Flavedo samples were collected 4 days after inoculation from 3 sites at increasing distance from the lesion on each fruit; single tissue samples were collected from the noninoculated fruit. Tissue was stored frozen at -20C until used for protein extraction.

Effects of sugarcane rootstock borer weevil larval feeding. Eight citrus rootstocks (*Citrus grandis* (L.) Osb. × *Poncirus trifoliata* (2N) (L.) Raf. (pummelo 2N); *C. grandis (L.)* Osb. × *P. trifoliata* (4N) (L.) Raf. (pummelo 4N); *P. trifoliata* × *C. grandis* (Flying dragon× Nakon); *C. paradisi* Macf. × *P. trifoliata* (L.) Raf.

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(Swingle citrumelo); *C. macrophylla* (Christm.) (*C. macrophylla*); *C. reticulata* Blanco (Cleopatra mandarin); *C. sinensis* (L.) Osb. × *P. trifoliata* (Carrizo citrange); *C. aurantium* (L.) (sour orange) were used in this experiment. Root samples were taken from trees that were grown, maintained, and infested with sugarcane rootstock borer weevil larvae as described by Shapiro and Gottwald (1995). Roots were collected, washed to remove adhering soil, and frozen until used for protein extraction.

Effects of chemical treatments. 'Marsh' grapefruit trees (6-8 years old on sour orange rootstock) were sprayed prior to color break of the fruit to run off with a solution containing 10 ppm gibberellic acid (GA) (Abbott Laboratories, N. Chicago, Ill.) and 0.05% Silwet L-77 (OSi Specialties, Danbury, Conn.) surfactant. Single trees served as replicates with 4 replicates per treatment.

'Ruby Red' grapefruit trees (4 to 5 years old on 'Swingle' rootstock) were sprayed in July with 7,600 mL of a solution containing 1.3 mL Kinetic (Helena Chem. Co., Memphis, Tenn.) surfactant plus either 1.6g per liter salicylic acid (SA) (Sigma, St. Louis, Mo.) or 3.95 mL per liter Keyplex 350 (Morse Enterprises, Miami, Fla.). Replicates consisted of 2 trees with 5 replicates per treatment. Leaf samples were collected prior to treatment and at 1 week intervals thereafter for a period of 4 weeks.

Protein extraction and quantification. In each experiment, tissue samples were freeze-dried and then ground to a fine powder. Powdered tissue was suspended in extraction buffer (sodium phosphate, 0.1M, pH 7; ca. 1g per 15 mL buffer). Suspensions were filtered and either dialyzed against water or passed through a column (Econ-Pac 10DG, BioRad, Hercules, Calif.) equilibrated in water. Desalted extracts were then lyophilyzed and subsequently dissolved in 2 mL water. Protein was quantified by the method of Bradford (1976) using bovine serum albumin as the standard.

Enzyme assays. Chitinase was assayed either radiometrically using radiolabeled chitin (Osswald et al. 1992) or colorimetrically using dye-labeled chitin (Loewe Biochemica, Munich, Germany) as the substrate (Wirth and Wolf 1990). β-1,3-Glucanase was assayed using the method of Abeles and Forrence (1970) with laminarin (*Laminaria digitata*, Sigma) as the substrate. Reactions were run at pH 5 at 50C.

Electrophoresis. Samples were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Proteins were visualized by staining with Coomassie blue or blotted to nitrocellulose membranes.

Western blotting. Proteins were transferred to nitrocellulose membranes (BioRad) following SDS-PAGE using a semidry transfer cell according to manufacturers instructions (BioRad).

Immunostaining. Antisera raised against chitinase or glucanase that had been purified from citrus callus tissue (Mayer et al. 1995) were used as primary antibodies. Goat anti-rabbit immunoglobulin-alkaline phosphatase conjugate (BioRad) was used as secondary antibody. Serological reactions were detected by the colorimetric method with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Results

Activity of chitinases and glucanases in various Citrus cultivars and tissues. Activities of chitinase and β-1,3-glucanase in blossoms and young and old leaves of 'Valencia' oranges are presented in Fig. 1. Chitinase activities did not differ among the three tissues at the initial sampling time (March 9). At later sampling times chitinase activity decreased in blossoms, remained fairly constant in young leaves, and increased in old leaves. Chitinase activity was highest in old leaves, intermediate in young leaves, and lowest in blossoms at the final sampling time (April 13). β-1,3-Glucanase activity, in contrast to chitinase activity, was significantly different among the three tissues at each sampling time; being lowest in blossoms, intermediate in young leaves, and highest in old leaves. In addition, there was a trend towards increasing β-1,3-glucanase activity in old leaves.

Activities of chitinase and β-1,3-glucanase in 'Marsh' grapefruit flavedo during fruit development are presented in Fig. 2. Chitinase activity was highest in fruit harvested prior to September, but decreased rapidly as fruit developed. By mid October (Julian date ca. 280) the level of chitinase activity was low, but still detectable and thereafter remained fairly constant. In contrast to chitinase, β-1,3-glucanase activity was lowest in young fruit and increased in an irregular pattern during fruit development to levels greater than four times those found in initial samples.

Results of immunodetection of chitinase and β-1,3-glucanase on western blots of crude 'Marsh' grapefruit flavedo extracts following SDS-PAGE are presented in Fig. 3. A single protein band at 30 kDa crossreacted with antibody to citrus chitinase. A protein band at 32 kDa crossreacted with antibody to citrus ß-1,3-glucanase in young fruit and a second band at 34 kDa appeared late in fruit development. Changes in activities of chitinase and β-1,3-glucanase are reflected in the total amount of immunospecific protein for each enzyme.

Effects of fungal infection. Infection of 'Marsh' grapefruit with *P. digitatum* lead to significant increases in chitinase and β-1,3-

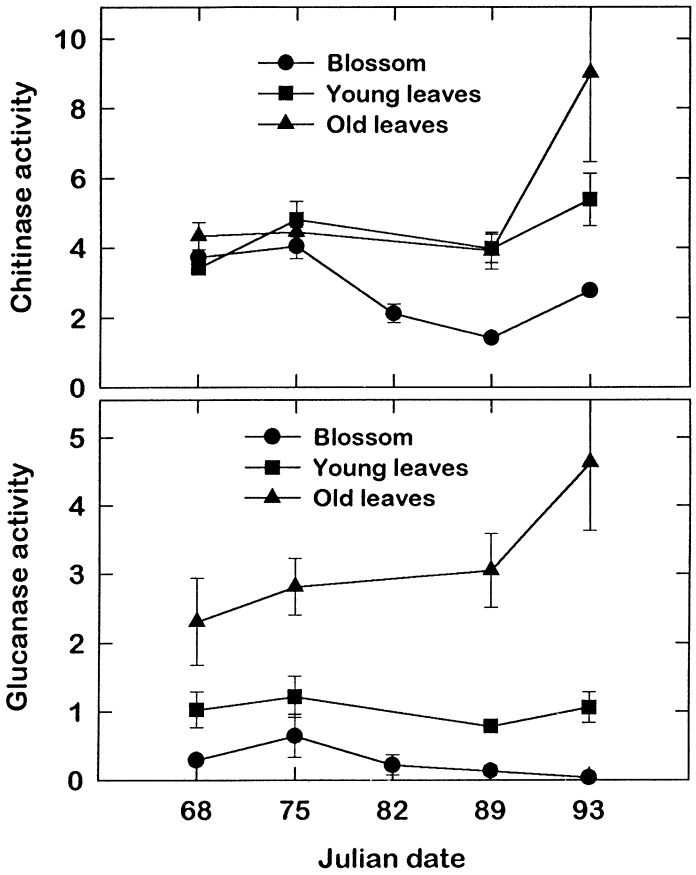

Figure 1. Chitinase (top) and glucanase (bottom) activities in blossoms, and young and old leaves of 'Valencia' oranges. Each data point represents the mean of six samples, vertical bars represent SE of the mean.

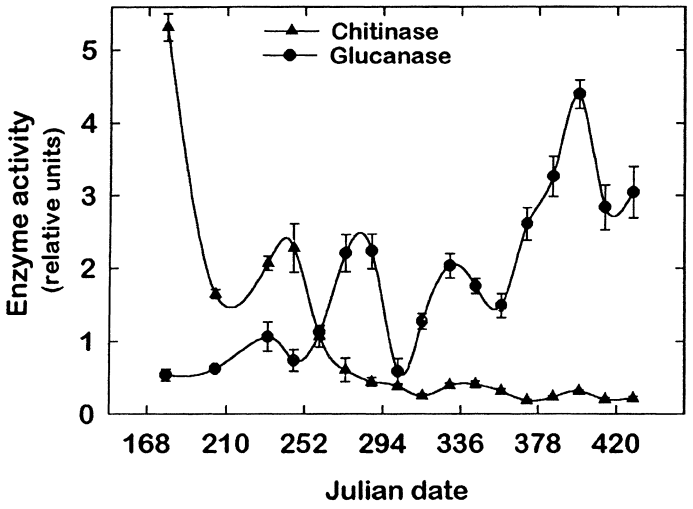

Figure 2. Chitinase and glucanase activities in 'Marsh' grapefruit flavedo during development. Each data point represents the mean of 6 samples, vertical bars represent SE of the mean.

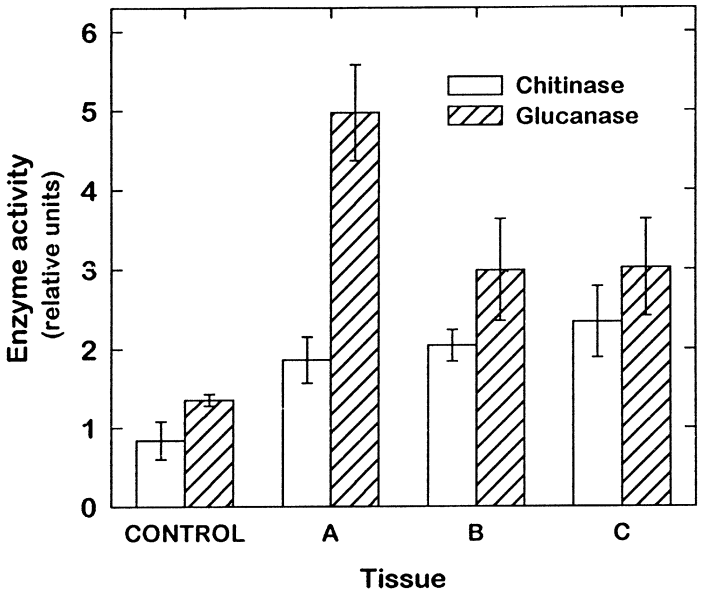

Figure 4. Effects of *P. digitatum* infection on chitinase and glucanase activities in 'Marsh' grapefruit flavedo. Control tissue was not infected, A = tissue closest to the lesion, B = tissue intermediate, and C = tissue furthest from the lesion. Each bar represents the mean of 3 samples, SE of the mean is indicated.

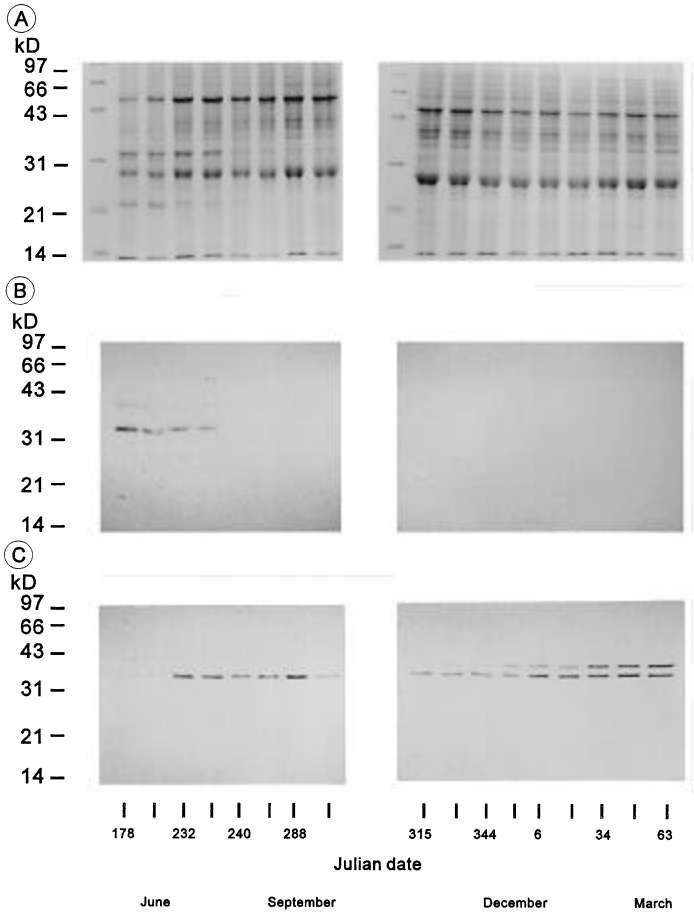

Figure 3. SDS-PAGE and immunodetection of chitinase and glucanase in crude protein extracts from 'Marsh' grapefruit flavedo. Following electrophoresis proteins were either visualized by staining with Coomassie brilliant blue (A) or electroblotted to nitrocellulose membranes and probed with antibody raised against citrus chitinase (B) or citrus glucanase (C).

glucanase activities (Fig. 4). Although the levels of activity of both enzymes increased in response to infection with *P. digitatum*, the pattern of increase differed. Chitinase activity increased with increasing distance from the lesion, whereas as β-1,3-glucanase activity was highest in the tissue closest to the lesion and decreased in tissue distant from the lesion.

Effects of root weevil feeding. Effects of feeding by *D. abbreviatus* on activities of chitinase and β-1,3-glucanase in roots of the eight citrus rootstock cultivars is presented in Fig. 5. A significant rootstock by root weevil interaction was detected for chitinase activity. In two of the rootstocks (Cleopatra and *C. macrophylla*), there was substantially higher chitinase activity in the infested than non-infested roots; however, Pummelo (2N and 4N) and Carrizo had slightly higher chitinase activity in non-infested than in infested roots. Root weevil feeding also resulted in an increase in chitinase proteins in the leaves of sour orange as revealed by immunodetection with potato leaf chitinase antibody (a generous gift of Dr. E. Kombrink) (Fig. 6). In contrast to chitinase, β-1,3-glucanase activity did not differ significantly among the cultivars, but was significantly reduced by root weevil feeding.

Effects of chemical treatments. Treatment of 'Marsh' grapefruit trees with gibberellic acid resulted in significant, but transient increases in chitinase and β-1,3-glucanase activities in flavedo (Fig. 7). Chitinase activities in flavedo from treated trees were significantly higher than controls for eight weeks following treatment. β-1,3-Glucanase activities in flavedo from treated trees were higher than from nontreated for six weeks following treatment.

Both Keyplex 350 and SA treatment of 'Ruby red' grapefruit trees resulted in significant, although transient increases in the levels of chitinase and β-1,3-glucanase in leaves compared with nontreated controls (Fig. 8). Chitinase activities in SA- and Keyplex 350- treated leaves were significantly higher than in control leaves two, three, and four weeks after treatment. Two and three weeks after treatment levels of chitinase were higher in SA-treated than in Keyplex 350-treated leaves, whereas at four weeks after treatment

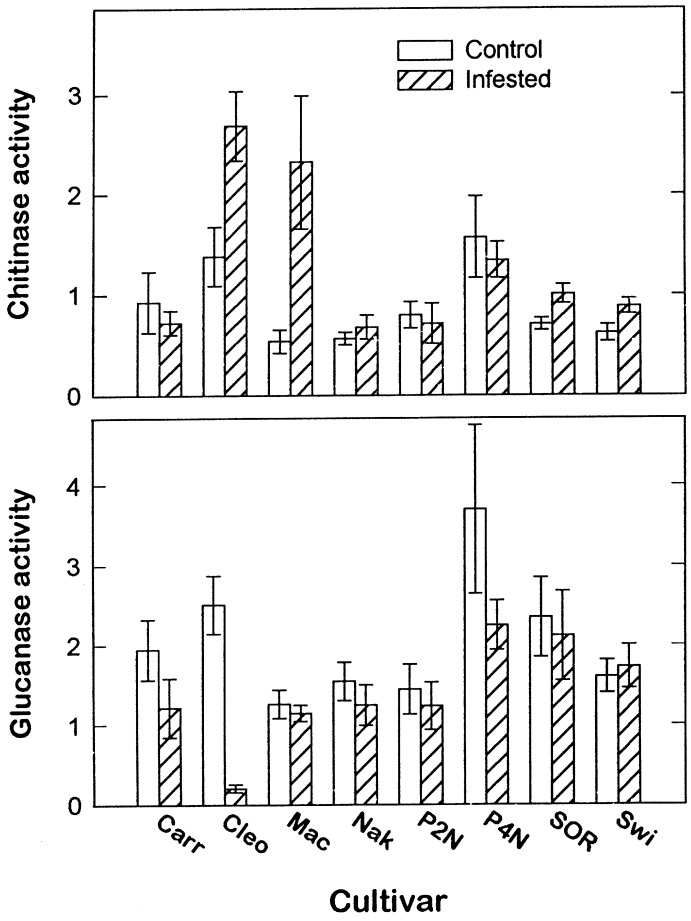

Figure 5. Effects of feeding by sugarcane rootstock borer weevils (*D. abbreviatus*) on chitinase (top) and glucanase (bottom) activities in 8 citrus rootstocks. Carr = Carrizo; Cleo = Cleopatra; Mac = *Citrus macrophylla*; Nak = Nakon × Flying Dragon; P2N = Pummelo, 2N; P4N = Pummelo 4N; SOR = Sour orange; Swi = Swingle.

chitinase was higher in the Keyplex treated leaves than in the SA-treated leaves. β-1,3-glucanase activities were higher in SA- and Keyplex 350-treated leaves than in controls two and three weeks after treatment, but differences between SA- and Keyplex 350-treated leaves were not significant.

Discussion

Results of the experiments presented in this report confirm that citrus plants produce chitinases and β-1,3-glucanase, two PR-proteins believed to be involved in plant resistance to fungal (Stintzi et al. 1993) and perhaps insect (Mayer et al. 1995) pests. The different levels of enzyme activity among tissues and stages of development (Figs. 1, 2, and 3) suggests that production of chitinases and β-1,3-glucanases are differentially regulated and may be amenable to genetic manipulation.

Chitinases and β-1,3-glucanases may also be part of a citrus plant's natural response to fungal and insect pests. Infection of grapefruit with *P. digitatum* leads to an increase in activities of both enzymes (Fig. 4), and feeding by *D. abbreviatus* larvae leads to an increase in chitinase in the roots of some of the cultivars we evaluated (Fig. 5) and in the levels of a chitinase protein in the leaves of sour orange (Fig. 6). The appearance of new chitinases in sour orange leaves in response to *D. abbreviatus* larval feeding may have potential as a diagnostic test for the pest in the field.

Immunodetection of chitinases in
Sour Orange leaves +/- *D. abbreviatus*

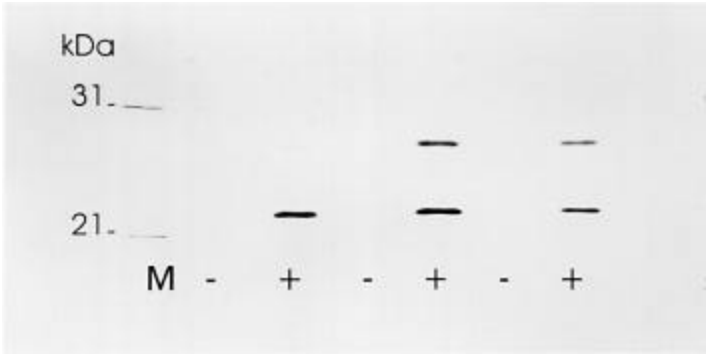

Figure 6. Effects of feeding by citrus root weevils on immunodetection of chitinase in 'Sour orange' leaves.

We also found that levels of the two enzymes can be increased by treatment with either gibberellic acid (Fig. 7), or salicylic acid or Keyplex 350 (Fig. 8). Different citrus varieties will probably respond differently to chemical treatment since the types and levels of these enzymes vary between varieties and tissues. It has been reported that GA treatment increases resistance of citrus fruit to both fungal (Ferguson et al. 1982) and insect pests (Greany et al. 1994);

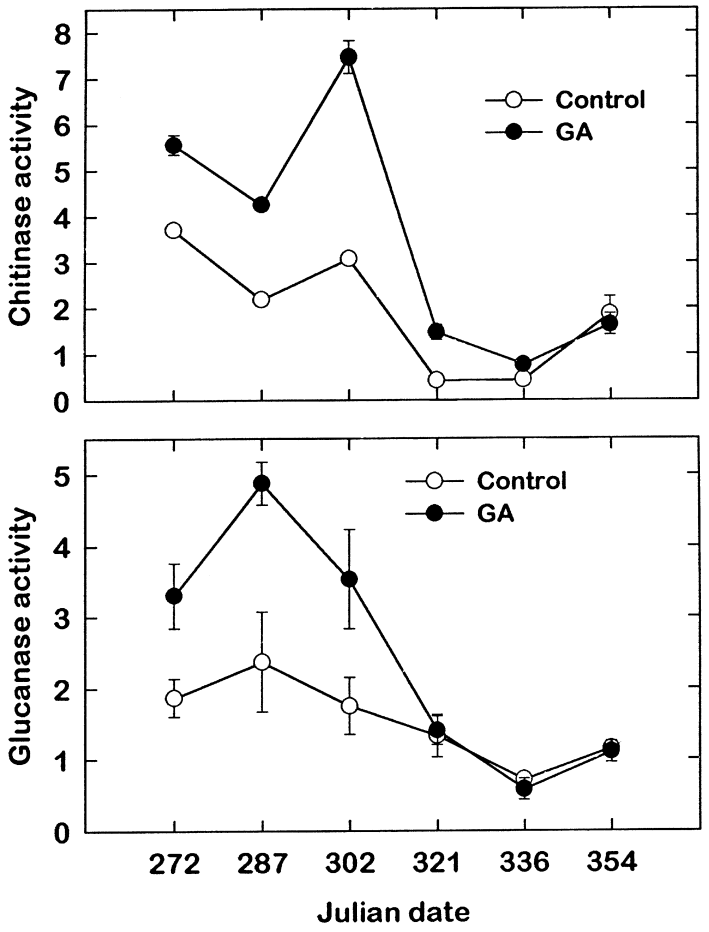

Figure 7. Effects of gibberellic acid treatment on chitnase (top) and glucanase (bottom) activities in 'Marsh' flavedo. Each data point represents the mean of 5 samples, SE of the means are indicated.

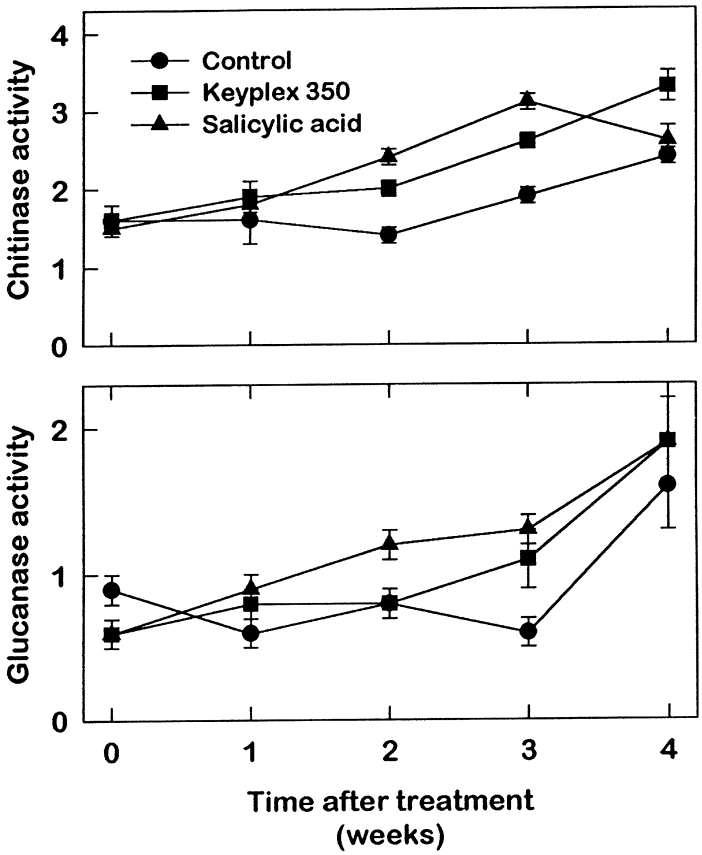

Figure 8. Effects of salicylic acid treatment on chitinase (top) and glucanase (bottom) activities in 'Ruby Red' leaves. Each data point represents the mean of 5 samples, SE of the means are indicated.

it is possible that the increase in chitinase and β-1,3-glucanase induced by such treatment is related to the increase in resistance.

Increased levels of chitinase and β-1,3-glucanase in citrus may be accomplished by genetic engineering technology, chemical treatments, or both. The former approach will require more time for development of superior germplasm while the later can be used with existing plant material. The eventual outcome of this research may be that losses due to pests can be reduced without increasing the use of traditional chemical pesticides.

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