Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 3737–3741, June 1984
Botany

Wound signals in plants: A systemic plant wound signal alters plasma membrane integrity

(pest attacks/protoplasts/proteinase inhibitors/tomato and potato leaves)

MARY WALKER-SIMMONS, HEIKE HOLLÄNDER-CZYTKO*, JULIE K. ANDERSEN, AND CLARENCE A. RYAN†

Institute of Biological Chemistry and the Biochemistry/Biophysics Program, Washington State University, Pullman, WA 99164

Communicated by P. K. Stumpf, February 29, 1984

ABSTRACT **Within 4 hr after wounding the lower leaves of young potato and tomato plants, a rapid and remarkable change is induced in the cells of upper undamaged leaves that results in extensive lysis of protoplasts during their isolation. Protoplast yields from unwounded upper leaves, 4 hr after wounding a lower leaf by crushing with a hemostat, decreased 25% below yields from leaves of unwounded plants. From 8 to >20 hr after wounding, protoplast yields were less than half of those from control plants. Multiple woundings decreased yields even further, as did chewing of the lower leaves by tobacco hornworms over a period of several minutes. In addition, within 4 hr of excising young tomato plants at their base with a razor blade, a 90% decrease in leaf protoplast yields was recorded. The major loss of protoplasts induced by wounding was primarily due to an increased cell lysis during protoplast isolation. Cell lysis was apparently due to a weakened cell membrane, because newly recovered protoplasts released from leaves of wounded plants were extremely fragile and exhibited 70% lysis during low speed centrifugation, compared to 20% lysis of protoplasts recovered from control plants. We conclude that a signal is released by wounding that is rapidly transmitted or transported through the plants to induce a profound change in the leaf cell membranes that renders them fragile during protoplast isolation. It is proposed that this signal may play a role in inducing cellular changes in the plant cells as part of their responses to environmental stress such as pest attacks.**

Systemic induced immunity in plants to viruses, bacteria, and fungi has been well documented over the past 4 decades (1), and numerous reports of induced insect resistances that are systemically mediated have appeared within the past 5 years (2). Only a few of these systemic resistance responses toward microorganisms and insects have been studied at the biochemical or molecular biological levels. However, three systemic wound signals have been identified and studied in some detail: (*i*) a lipopolysaccharide fragment from bacterial cell walls appears to play a role in systemic induced immunity toward bacterial infections in tobacco plants (3), although the molecular basis of the resistances are not clear; (*ii*) a pectic polysaccharide, a fragment of the plant cell wall, has been shown to induce the synthesis and accumulation of antinutrient proteinase inhibitor proteins in excised tomato and potato leaves that are systemically regulated in these plants by wounds from insects (4); and (*iii*) electrical signals have been implicated in systemic responses induced by mechanical wounding (5). Little is known of the intracellular mechanisms that accompany the amplification or reception of any of these signals, and nothing is known of their mechanism(s) of gene activation.

In this communication, we report that systemic signals resulting from mechanical damage to tomato and potato leaves profoundly change properties of the cells of undamaged leaves many centimeters away. This change is manifested in strikingly decreased yields of protoplasts isolated from these cells. We suggest that the wound-induced changes in the properties of the leaf cells may be an early recognition event of the plant's responses to pest attacks.

MATERIALS AND METHODS

Tomato plants (var. Bonnie Best) and potato plants (var. A6595-3) were grown from seeds in a growth chamber under 30% humidity with a daily schedule of 17 hr of light (150×10^{-6} einsteins/m^2 per sec) at 30°C and 7 hr of dark at 22°C. Tomato plants were used for experiments 15–18 days after planting. They were ≈3 cm tall and possessed 2–3 expanding leaves. Potato plants were used 4–5 weeks after planting. They were ≈5 cm tall and possessed 4–5 expanding leaves.

Plants were wounded by crushing the appropriate leaves across the midvein with a hemostat. The lowest terminal leaflet of tomato plants and two to three lower terminal leaflets of potato plants were wounded. The upper nonwounded leaves from the plants were used to prepare protoplasts.

Tomato plants excised and supplied with proteinase inhibitor inducing factor (PIIF) were used ≈20 days after planting and had two expanding leaves and a small spiral leaf. The plants were excised just above the soil level and allowed to take up solutions of 0.05 M sodium phosphate (pH 6.0) or PIIF at 1 mg/ml dissolved in the same buffer for 60 min. The plants were then supplied with water for the appropriate times at 150×10^{-6} einsteins/m^2 per sec at 30°C and assayed for inhibitor I or used for protoplast isolations.

Tobacco hornworm larvae (*Manduca sexta*) in the fourth instar stage were allowed to feed on one lower leaf of 17-day-old tomato plants for 15–30 min or until approximately one-third of the leaf had been consumed. The plants were maintained insect free in light as described above for 20 hr when protoplasts were prepared from the adjacent upper undamaged leaves.

Protoplast Preparation. The epidermis of the selected leaves were abraded with a plastic brush and the leaves were cut into 2- to 4-mm sections. The leaf tissue (0.45 g) was placed in 10 ml of a solution of 0.3 M mannitol containing 0.3% (wt/vol) cellulase RS/0.1% (wt/vol) macerozyme R-10 (both from Yakult Pharmaceutical Industries, Nishinomiya, Japan)/0.3 mM dithiothreitol/0.3 mM $CaCl_2$/25 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.6, and incubated in a gyratory shaker at 15 rpm for 3.5 hr at 27°C in the dark. The digested material was filtered through a polypropylene screen with a 100-μm opening (Tetko, Elmsford, NY). The remaining leaf debris was washed once with 0.3 M mannitol buffer as described above without enzymes (one-half of the original volume) and filtered again. The filtered solutions

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: PIIF, proteinase inhibitor inducing factor.

*Present address: Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität Bochum, D-4630 Bochum 1, F.R.G.
†To whom reprint requests should be addressed

were pooled and protoplast numbers were monitored using a hemocytometer. For purification of protoplasts, the solution was spun at 50 × *g* for 7 min. The precipitate was resuspended in 5 ml of 10% (wt/vol) Ficoll (Sigma) containing 0.3 M mannitol/0.3 mM dithiothreitol/0.3 mM $CaCl_2$/25 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.6, and overlayered with 5 ml of a 5% (wt/vol) Ficoll solution (containing the same components and concentrations as the 10% Ficoll solution). After centrifugation at 50 × *g* for 15 min, purified protoplasts were collected from the upper surface of the 5% Ficoll layer.

Mannosidase was assayed as described by Boller and Kende (6). One unit of activity is that amount of extract that hydrolyzes 1 nmol of *p*-nitrophenyl α-D-mannopyranoside per min. Chlorophyll was assayed according to Strain *et al.* (7).

Tomato leaf proteinase inhibitor I was assayed by radial immunodiffusion as described by Ryan (8), and the data were plotted as described by Trautman *et al.* (9).

Tomato PIIF was prepared from tomato leaves as described (10).

RESULTS AND DISCUSSION

Our observations of decreased leaf protoplast yields from wound-damaged potato and tomato plants resulted from our research on the wound induction of two proteinase inhibitors in plant leaves (11, 12). Two inhibitors (inhibitors I and II) accumulate in substantial quantities in leaves of potato and tomato plants in response to a wound-released systemic signal. (13). The inhibitor proteins were shown by electron microscopy to be sequestered as protein aggregates in the central vacuoles of leaf cells (14). These observations were later confirmed when vacuoles were isolated from leaves of wounded tomato plants and were shown quantitatively to contain the two inhibitors (15).

During the isolation of vacuoles, we noticed consistently lower yields of protoplasts and vacuoles from unwounded leaves of wounded plants than from leaves of control plants. We have investigated the possible reasons for the lower protoplast yields from wounded plants. In this communication, we report that a systemic signal released from wounded leaves induces cells from unwounded leaves throughout the plant to become more fragile during protoplast isolation. This phenomenon is readily apparent when young potato and tomato plants are used.

A time course of the loss in yields of protoplasts from potato and tomato leaves during the period after wounding is shown in Fig. 1. The percent loss in yield is calculated by comparing yield of protoplasts prepared from the upper nondamaged leaves of wounded plants with that of unwounded control plants, all maintained in the same environments of

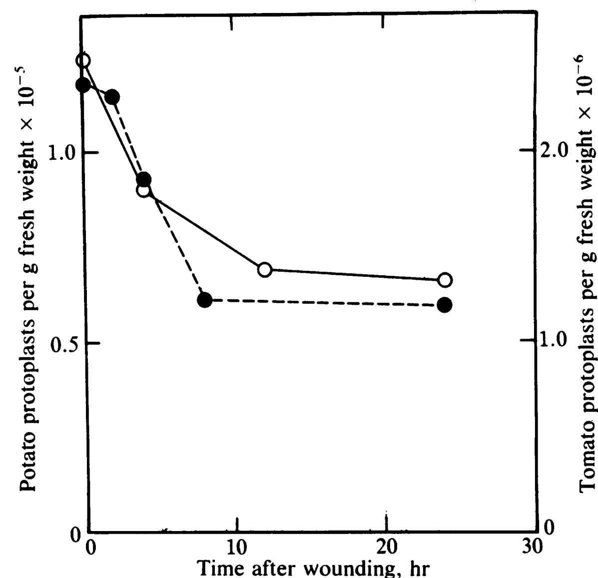

FIG. 1. Protoplast yields from upper nonwounded leaves of potato plants (○) and tomato plants (●) wounded twice on the lower leaf.

light, temperature, and humidity. As seen in Fig. 1, the wound-induced loss in protoplast yields from both potato and tomato leaves is time dependent. Within 3–4 hr of wounding, the yields of protoplasts dropped to 75% of controls, and after 8–10 hr the yields dropped to <50% of control plants.

The magnitude of the decreased yields of protoplasts from wounded plants depends on the severity of wounding. In Table 1, data are presented for protoplast yields and two cellular markers from unwounded, upper leaves of once- and twice-wounded tomato plants. The yields from plants wounded only once, 4 hr before isolation, were 74% those of control plants, whereas those wounded twice (4 and 3 hr before isolation of protoplasts) were 53% of controls. Chlorophyll and mannosidase levels per protoplast from each treatment were similar, indicating the consistency of the protoplast preparations. In Table 1, the relationship of single and double wounds was also compared with the levels of proteinase inhibitor I that were induced to accumulate in leaves of tomato plants of similar age and size during a 24-hr period after wounding. These results show twice-wounded plants accumulating about 2 times the quantity of inhibitor I induced by a single wound, confirming observations previously reported (16).

Decreased yields of protoplasts after wounding appear to

Table 1. Effects of single and double wounding of leaves of tomato plants on protoplast yields and proteinase inhibitor I levels

Treatment	Protoplasts ($\times 10^{-6}$) per g fresh wt	Chlorophyll/mannosidase ($\times 10^{-4}$) in isolated protoplasts	Leaf content of proteinase inhibitor I, μg per ml of leaf juice
Unwounded	2.33 (100%)	55	0.6
Wounded once	1.72 (74%)	51	28.3
Wounded twice	1.24 (53%)	55	53.5

Once-wounded tomato plants were wounded across the midvein 4 hr before protoplast preparation. Twice-wounded plants were wounded 4 and 3 hr before protoplast preparation. Protoplasts were prepared from the opposite nonwounded leaves of the plant. Two sets of plants were treated for each set of experiments. One set was used to isolate protoplasts, and a second set was incubated under light (150×10^{-6} einsteins/m^2 per sec) for 24 hr and the levels of inhibitor I were determined immunologically to assess the effectiveness of the wounding. All data represent assays from five separate experiments. Chlorophyll values were evaluated as μg per g fresh weight of leaf tissue, and mannosidase activity was evaluated in units per g fresh weight. The levels in both mannosidase and chlorophyll per protoplast remained constant in all of the experiments.

Table 2. Comparison of chlorophyll, mannosidase, and intact protoplasts initially released from leaves of wounded and control potato and tomato plants during protoplast isolation

Leaf tissue	Total components (intact plus lysed protoplasts)			
	Intact protoplasts ($\times 10^{-6}$) per g fresh wt	Chlorophyll, μg per g fresh wt	Mannosidase ($\times 10^{-4}$), units per g fresh wt	Chlorophyll/mannosidase
Potato*				
Control	1.34 ± 0.12	459 ± 52	25.4 ± 8.9	18.1
Wounded	0.64 ± 0.16	462 ± 192	26.1 ± 9.6	17.7
Tomato†				
Control	21.8	604	9.0	67
Wounded	8.96	533	9.3	57

Leaf tissue (0.9 g) was incubated in 20 ml of mannitol solution containing cell-wall digesting enzymes. After 3.5 hr, each leaf tissue digest preparation was filtered through a 100-μm mesh polypropylene screen. Undigested leaf material remaining on the screen was resuspended in 10 ml of the mannitol solution and was refiltered through the polypropylene screen and the two filtrates were combined. Intact protoplasts were counted and the levels of chlorophyll and mannosidase in the digest were assayed.

*Average of four experiments, each with five replicate protoplast counts. The lower leaves of the potato plants were wounded 20 hr before harvest, and upper nonwounded leaves were used for the experiment.

†One experiment with five replicate protoplast counts. One leaf of each tomato plant was wounded 19 and 20 hr before harvest. The opposite, nondamaged leaves were used for the experiments.

be due to protoplast lysis, as judged by analysis of total cellular components released into the medium during protoplast isolation. After incubation of leaf tissue with cell-wall digesting enzymes for 3.5 hr, the protoplasts, together with organelles, small fragments of broken tissue, and soluble components from lysed cells were separated from undigested leaf tissue by filtering through a polypropylene screen with a 100-μm mesh opening. In Table 2, the yields of protoplasts and the two markers, chlorophyll (a choloroplast marker) and mannosidase (a vacuolar marker), in the filtrates from leaves of wounded and control plants are compared. The yields of the two markers from both wounded and control plant digests are similar, indicating that about the same number of cells were released from both digests. Since the yields of intact protoplasts from wounded plants are only half of those obtained from control plants, the decreased yield appears to have resulted from either lysed protoplasts and whole cells or from incomplete conversion of released whole cells to protoplasts.

A major concern was that wounding may have caused a change in the plants that decreased the numbers of whole cells that were being converted into protoplasts during digestion with protoplast-releasing enzymes. For example, Geballe and Galston (17) have reported the development of a resistance of oat leaves to cellulolytic digestion in response to wounding. In tomato protoplast preparations from wounded plants, an increase in whole cells was noted over preparations from unwounded plants. This increase in whole cells could account for only 27% of the loss of protoplast yields. In potato protoplast preparations from wounded plants, we can detect no increase at all in whole cells over those from unwounded plants. Thus, it appears that the major losses in protoplasts from both tomato and potato leaves are from protoplast and/or cell lysis.

The induced fragility of the newly isolated protoplasts was particularly pronounced during centrifugation of the protoplasts at low speed. In Table 3, the results of an experiment are shown in which newly released protoplasts from unwounded leaves from both wounded and unwounded plants were centrifuged at low speed. Recoveries of protoplasts from wounded plants immediately after incubation with the cell-wall digesting enzymes were ≈67% of control yields. However, on centrifugation at 25 × g for 7 min, ≈70% of the protoplasts from wounded plants were broken compared to 20% breakage of control protoplasts. When the newly harvested protoplasts were centrifuged at 100 × g, ≈70% of the protoplasts from wounded plants were again broken, but at this speed only 40% of the protoplasts from control plants had lysed. This experiment indicates that two populations of protoplasts may exist in leaves of wounded plants: one population that is easily lysed on centrifugation, and a second, stable population, that remains unbroken even under the higher 100 × g forces.

Inhibitor I has been shown to accumulate in excised young

Table 3. Effect of centrifugal stress on protoplast recovery from leaves of wounded and control plants

Treatment	Control plants		Wounded plants	
	Protoplasts ($\times 10^{-6}$) per g fresh wt	% recovery	Protoplasts ($\times 10^{-6}$) per g fresh wt	% recovery
Initial protoplast recovery	12.8	100	8.56	100
Initial protoplasts				
Centrifuged at 25 × g				
Pellet	1.44		0.78	
Supernatant	8.89		1.83	
Total	10.33	80	2.61	31
Centrifuged at 100 × g				
Pellet	6.83		2.28	
Supernatant	0.89		0.44	
Total	7.66	60	2.72	32

Unwounded leaves from tomato plants wounded twice at 19 and 20 hr previously or from control plants were incubated with hydrolytic enzymes to release protoplasts. After the initial incubation of 3.5 hr, the protoplast preparation was divided into equal aliquots and was treated as described in the table. Centrifugation was for 7 min.

tomato plants when supplied with a purified preparation of PIIF (18). Excision alone induced little or no inhibitor when a razor blade was used to cut the base of the main stem (18). We therefore isolated protoplasts from leaves of excised young tomato leaves 4 hr after supplying them with buffer or PIIF, and we compared the yields of protoplasts from the leaves of these plants with those from leaves of intact plants. The accumulation of inhibitor I in similarly treated leaves was also recorded 24 hr after excision. Fig. 2 shows that an 80%–90% decrease in protoplast yields resulted from excision. The decrease occurred whether buffer or PIIF was supplied to the leaves. However, inhibitor I accumulated only in the leaves of plants induced with PIIF. Thus, in excised leaves the induction of inhibitor I synthesis is not obligatorily linked to the process that decreases protoplast yield, suggesting that separate signals may trigger the two phenomena. The data do not, however, rule out the possibility that some change in the cellular organization of the leaves may be occurring that is predisposing the cells to receive the information carried by PIIF. If this important change in protoplast fragility due to excision occurs in other plant genera, then such changes should be taken into account whenever excised plants are used for experimental study.

Effects of Insect Attacks. Insect attacks have been shown to be effective in inducing proteinase inhibitor accumulation in leaves of tomato plants (13). In fact, the continuous chewing of leaf-consuming insects may be the most effective method of inducing the accumulation of proteinase inhibitors I and II in plants. We have often found levels of inhibitors to be higher in insect-infested field-grown plants than we can induce in the laboratory. We allowed *M. sexta* larvae, which are avid feeders on tomato leaves, to briefly feed on the lower leaves of young tomato plants until they had consumed about one-third of a lower leaf of a plant. After a 20-hr incubation under normal growing conditions, we compared the yields of protoplasts from undamaged leaves with those of control plants. The larvae decreased protoplast yields to 43% of controls. At the time of protoplast isolation (20 hr after insect attacks) the plants had accumulated >60 μg per g of tissue of inhibitor I in the undamaged leaves. Both chlorophyll and mannosidase assays again ensured us that the protoplasts were of the highest purity. Thus, even a short period of continual feeding by an insect is sufficient to induce significant losses in protoplast yields in leaves of tomato plants.

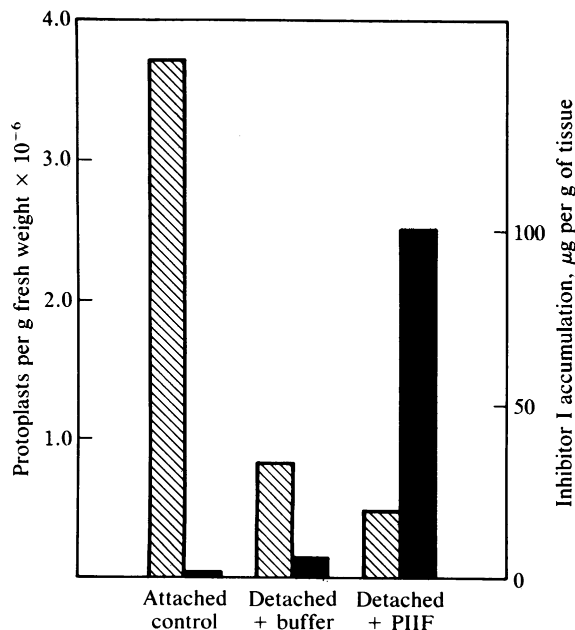

FIG. 2. Protoplast (▧) yields from excised tomato leaves supplied with buffer and tomato PIIF 4 hr before protoplast isolation. The yields are compared with proteinase inhibitor I (■) induced to accumulate during a 24-hr incubation under constant light.

The cumulative data lead us to speculate that the lysis of protoplasts results from a systemic signal that triggers a change in the protoplast membrane. This could be an enzymic process resulting from changes in the leaf cytosol and/or a conformational or compositional change in the membrane that renders the protoplast membrane more susceptible to lysis when released into the digestion media. This response is not understood from a functional standpoint, but it shows that injury of tissues by mechanical damage or insect attacks can cause profound changes in the properties of plant cell envelopes many centimeters away. We suggest that these changes may be involved in mobilizing plant defenses in these cells. The systemic signal is apparently different from the signal PIIF that induces proteinase-inhibitor synthesis and accumulation in potato and tomato plants (4, 10, 18), but it could be part of an overall communication system that predisposes the receptor cells to receive information conveyed by PIIF or other signals. It is not clear whether the signal that decreases protoplast yields is electrical or chemical. Electrical signals have been measured in wounded plants (5) and may be involved with a rapid wound-induced polysome formation in tomato plants (19). In addition, Theologis and Laties have observed a rapid induction of phospholipase activity that can act on membrane lipids in wounded potato tubers (20). This latter signal has not been identified, but membranes in cells several millimeters from the wound site display lipolysis within 15 min after slicing. Corn roots have also been shown to respond to wound signals originating from mechanical injury. After cutting, root plasma membrane H^+/K^+ ATPase decreases, accompanied by an increased membrane permeability, which causes ion influx (21). The cutting caused a rapid (within 30 min) partial collapse of the electrogenic H^+/K^+ ATPase of the plasma membrane in tissues 2 cm away (22). Nothing is yet known of the signals that induce this response or of the mechanisms that cause the changes in the membrane properties.

In contrast to the aforementioned examples, the systemic effects observed in this report occur several centimeters away in distal tissues. Nevertheless, it is possible that all of these processes are related through the same signals or through similar biochemical responses. Analysis of membranes of wounded and unwounded plants for both composition and chemical properties may provide clues toward understanding the biochemical and chemical processes that contribute to the systemic wound-induced changes in leaf cells reported here.

We thank Richard Hamlin and Sally Combelic for growing the plants used in this research; Dr. Mark Martin of the U.S. Department of Agriculture (Prosser, WA) for the gift of potato seeds; and Dr. David Moffett, Department of Zoology (Washington State University), for a gift of tobacco hornworms. This research was supported in part by National Science Foundation Grants PCM 8023285 and 8309344, U.S. Department of Agriculture Cooperative Research Service Grant 81-CRCR-1-0697, and a scholarship from the Deutsche Forschungsgemeinschaft (to H.H.-C.). This is College of Agriculture Research Center, Washington State University, Scientific Paper 6684, Project 1791.

1. Kuć, J. (1982) *Bioscience* **32,** 854–860.
2. Rhoades, D. F. (1982) in *Variable Plants and Herbivores in Natural and Managed Systems*, eds. Denno, R. F. & McClure, M. S. (Academic, New York), pp. 155–220.
3. Sequeira, L. (1981) in *Plant Disease Control*, eds. Staples, R. C. & Toenniessen, G. H. (Wiley, New York), pp. 143–153.
4. Ryan, C. A., Bishop, P., Pearce, G., Darvill, A. G., McNeil, M. & Albersheim, P. (1981) *Plant Physiol.* **68,** 616–618.

5. Van Sambeek, J. W., Pickard, B. G. & Ulbright, C. E. (1976) *Can. J. Bot.* **54,** 2651–2661.
6. Boller, T. & Kende, H. (1979) *Plant Physiol.* **63,** 1123–1132.
7. Strain, H. H., Cope, B. T. & Svec, W. A. (1971) *Methods Enzymol.* **23,** 452–476.
8. Ryan, C. A. (1967) *Anal. Biochem.* **19,** 434–440.
9. Trautman, R., Cowan, K. M. & Wagner, G. G. (1971) *Immunochemistry* **8,** 901–906.
10. Bishop, P. D., Makus, D. J., Pearce, G. & Ryan, C. A. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 3536–3540.
11. Ryan, C. A. (1980) *Curr. Top. Cell. Regul.* **17,** 1–23.
12. Ryan, C. A. (1978) *Trends Biochem. Sci.* **3,** 148–150.
13. Green, T. R. & Ryan, C. A. (1971) *Science* **175,** 776–777.
14. Shumway, L. K., Rancour, J. M. & Ryan, C. A. (1970) *Planta* **93,** 1–14.
15. Walker-Simmons, M. & Ryan, C. A. (1977) *Plant Physiol.* **60,** 61–63.
16. Nelson, C., Walker-Simmons, M., Makus, D., Zuroske, G., Graham, J. & Ryan, C. A. (1982) in *Mechanisms of Plant Resistance to Insects*, ed. Hedin, P. (Am. Chem. Soc., Washington, DC), Monogr. 208, pp. 103–122.
17. Geballe, G. T. & Galston, A. W. (1982) *Plant Physiol.* **70,** 781–787.
18. Ryan, C. A. (1974) *Plant Physiol.* **54,** 328–332.
19. Davies, E. & Schuster, A. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 2422–2426.
20. Theologis, A. & Laties, G. G. (1981) *Plant Physiol.* **68,** 53–58.
21. Zocchi, G. & Hanson, J. B. (1982) *Plant Physiol.* **70,** 318–319.
22. Gronewald, J. W. & Hanson, J. B. (1980) *Plant Sci. Lett.* **18,** 143–150.