

# Systemin Potentiates the Oxidative Burst in Cultured Tomato Cells<sup>1</sup>

Michael J. Stennis, Sreeganga Chandra, Clarence A. Ryan, and Philip S. Low\*

Department of Chemistry, Purdue University, 1393 Brown Building, West Lafayette, Indiana 47907–1393 (M.J.S., S.C., P.S.L.); and Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164–6340 (C.A.R.)

Plants that have been wounded by insects or other herbivores may be more susceptible to infection by adventitious microbes. Wound-induced signal molecules, which serve to induce responses in the plant that retard further feeding, might also act to prepare a plant for possible pathogen attack. We have examined the effect of a wound-generated systemic messenger (systemin) on a pathogen-stimulated defense-response marker, the oxidative burst. We observed that neither systemin nor its inactive analog (A-17) was able to directly induce H<sub>2</sub>O<sub>2</sub> biosynthesis in suspension-cultured tomato (*Lycopersicon esculentum* L.) cells, regardless of the duration of exposure of the cells to the two peptides. Similarly, neither systemin nor A-17 was capable of modifying an oligogalacturonide-elicited oxidative burst, as long as elicitor addition occurred within minutes of treatment with systemin or A-17. In contrast, preexposure of the cell cultures to systemin (but not to A-17) led to a time-dependent enhancement of the oligogalacturonide-elicited oxidative burst. By 12 h of exposure, the H<sub>2</sub>O<sub>2</sub> biosynthetic capacity of systemin-treated cells exceeded that of the control cells by a factor of 16 ± 2. A similar up-regulation by systemin of a mechanically stimulated oxidative burst was also observed. Because the systemin-induced augmentation in oxidant synthesis is quantitatively prevented by coinubation with 2 μM cycloheximide, and because the oxidative burst of oligogalacturonic acid-elicited control cells (no systemin exposure) is unaffected by preincubation with cycloheximide, we conclude that systemin enhancement of the tomato-cell oxidative burst requires protein synthesis.

Tomato (*Lycopersicon esculentum* L.) cells respond to wounding/herbivore attack by releasing a highly mobile octadecameric peptide termed systemin (Pearce et al., 1991). Systemin, the first peptide hormone found in the plant kingdom, was originally synthesized as a 200-amino acid precursor protein called prosystemin (McGurl et al., 1992). After its proteolytic activation/release, the oligopeptide is rapidly translocated into unwounded tissues, where it is thought to bind a 50-kD plasma membrane receptor (Pearce et al., 1993; Schaller and Ryan, 1994). Subsequent to receptor activation, the signaling pathway is hypothesized to proceed via stimulation of a phospholipase, resulting in intracellular release of linolenic acid, metabolism of linole-

nic acid to jasmonate and methyl jasmonate, and transcriptional activation by jasmonate of proteinase inhibitor genes and other resistance mechanisms (Farmer and Ryan, 1992; Mueller et al., 1993).

Plants are also able to mount defense responses against disease-causing microbes. After recognition of the invading pathogen, the responsive plant cell may attempt to limit fungal/bacterial ingress by promoting a variety of disease-resistance strategies, including cell wall stabilization (Bradley et al., 1992), stomatal closure (Hammond-Kossack et al., 1996), phytoalexin biosynthesis (Nicholson, 1992; Davis et al., 1993), expression of pathogenesis-related proteins and other toxic peptides (Bol et al., 1990), induction of localized cell death leading to a hypersensitive response (Doke, 1983a; Greenberg et al., 1994), and generation of active oxygen species, primarily O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> (Doke et al., 1996; Low and Merida, 1996). The latter process, frequently termed the oxidative burst, may be the most rapid, arising within minutes of elicitor addition and extending for various lengths of time, depending on the compatibility of the host plant-pathogen interaction (Baker et al., 1993; Chandra et al., 1996). Because it is thought to be required for many subsequent defense responses (Doke et al., 1996; Low and Merida, 1996), and because it is probably expressed in most if not all plant species (Doke, 1983b; Baker et al., 1993; Chandra and Low, 1995; Kauss and Jeblick, 1995; Chandra et al., 1996; Fauth et al., 1996), the oxidative burst has often been used as a crude gauge of a plant's ability to recognize and respond to a disease-causing microbe (Doke et al., 1996).

Although these two defense mechanisms are directed against very different enemies, they nevertheless share several common features. Both responses are initiated at the site of attack but eventually involve mobile messengers that communicate a localized defense alert throughout naive regions of the plant (Malamy et al., 1990; Pearce et al., 1991; Ryals et al., 1995). Both responses involve the biosynthesis of toxic compounds such as phytoalexins, which presumably retard the invasion of microbes and macroscopic herbivores (Nicholson, 1992; Davis et al., 1993; Nojiri et al., 1996). Finally, in both responses, similar pathogenesis-related genes are induced that are thought to increase the probability of plant survival against subsequent attempts at invasion/colonization (Wasternack and

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\* Corresponding author; e-mail lowps@omni.cc.purdue.edu; fax 1-765-494-0239.

Abbreviation: OGA, oligogalacturonic acid.

Parthier, 1997). Thus, it is not surprising that several second messengers such as ethylene and jasmonic acid can participate in both defense responses and possibly even activate shared resistance responses (O'Donnell et al., 1996; Creelman and Mullet, 1997).

In view of the commonalities between pathogen- and herbivore-resistance strategies, the question naturally arises whether successful defense against onslaught from one prepares the plant to resist attack by the other. In an initial attempt to address this issue, we have examined whether pretreatment of tomato cells with systemin affects the cells' ability to respond to pathogen-generated elicitors. We report here that systemin has no immediate effect on the capacity of tomato cells to generate an elicitor-induced oxidative burst. However, after a several-hour pretreatment that involves systemin-induced gene expression, we observed tomato cells responding to elicitation by generating H<sub>2</sub>O<sub>2</sub> at a rate at least 1 order of magnitude greater than cells not previously exposed to systemin. We propose that this cross-talk between herbivore- and pathogen-directed defense strategies may help protect the plant against opportunistic microbes.

## MATERIALS AND METHODS

Pyranine was obtained from Molecular Probes (Eugene, OR). Systemin and A-17, an inactive analog of systemin, were synthesized as described elsewhere (Pearce et al., 1993). All other chemicals were obtained from major suppliers.

### Tomato Cell-Suspension Cultures

Rio Grande-PtoR (*Pto/Pto*, *Fen/Fen*) is a variety of tomato (*Lycopersicon esculentum* L.) that is both resistant to bacterial speck disease caused by *avrPto*-expressing *Pseudomonas syringae* pv *tomato* and sensitive to the organic pesticide fenthion (Martin et al., 1993). This tomato variety was used to generate cell-suspension cultures that were used in all experiments. Tomato stems were surface sterilized and placed on agar plates (R-3 medium [Schnapp et al., 1991] with 5% agar) to allow callus formation. The calli were fragmented, transferred to liquid R-3 medium, and cultured by rotary shaking at 19°C as described previously (Bressan et al., 1981). The resulting cell cultures were maintained by transferring 3 mL of culture to 25 mL of fresh medium every 14 d. Cells were most responsive to elicitor stimulation up to 18 h after transfer to fresh medium and were used for oxidative-burst evaluations during this period.

### Elicitors

An OGA fraction that elicits H<sub>2</sub>O<sub>2</sub> production in a variety of cultured plant species was purified as described previously (Legendre et al., 1993). The OGA stock preparation used in this study contained approximately 500 µg/mL, as determined by the method of Blumenkrantz and Asboe-Hansen (1981).

### Spectrofluorimetric Determination of H<sub>2</sub>O<sub>2</sub> Production

H<sub>2</sub>O<sub>2</sub> production by cultured tomato cells was detected by monitoring the oxidative quenching of the fluorescent peroxidase substrate pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt; 405 nm excitation/512 nm emission) as described previously (Apostol et al., 1989a; Legendre et al., 1993). Cells (1.5 mL) were mixed with 5 µL of pyranine (0.2 mg/mL stock solution) in a fluorimetric cuvette and maintained in suspension by mild stirring. After addition of elicitor, the lost dye fluorescence was continuously monitored, and the initial slope of the quenching curve was used to determine the rate of H<sub>2</sub>O<sub>2</sub> production.

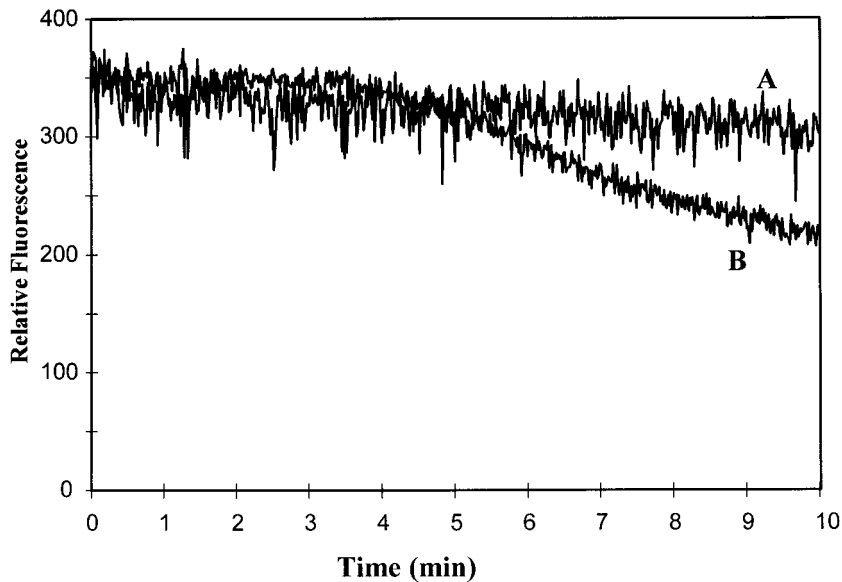
## RESULTS

### Systemin Is Not an Elicitor or Immediate Potentiator of the Oxidative Burst

To determine whether systemin might serve as an elicitor of pathogen defense responses, we investigated systemin's ability to directly stimulate or augment an elicitor-stimulated oxidative burst in cultured tomato cells. As shown in Figure 1 (trace A), with the addition of systemin at concentrations (1 µM) above those known to induce maximal wounding responses such as proteinase inhibitor biosynthesis (Pearce et al., 1991), the peptide promoted no detectable H<sub>2</sub>O<sub>2</sub> production in tomato suspension cultures. Furthermore, the addition of systemin directly before elicitor stimulation of the same cells (trace B) caused no further increase in H<sub>2</sub>O<sub>2</sub> biosynthesis compared with the treatment of the cells with OGA elicitor alone. As anticipated, A-17, an inactive analog of systemin differing only by a single Ala substitution at position 17, also had no effect on oxidant biosynthesis, regardless of whether it was introduced alone or in conjunction with OGA. These data indicate that any effect systemin might have on the oxidative burst is not manifested immediately after systemin addition.

### Preincubation with Systemin Enhances Both the OGA-Induced and the Osmotically Induced Oxidative Bursts

Previous research has established that the systemin-induced accumulation of proteinase inhibitor mRNAs typically begins approximately 2 to 4 h after the addition of systemin to plant leaves and continues to increase for more than 12 h (Farmer and Ryan, 1990; Pearce et al., 1991). Therefore, we investigated whether an induction period might also be required before systemin could independently induce or potentiate an elicitor-induced oxidative burst. As noted above, OGA readily stimulated the synthesis of H<sub>2</sub>O<sub>2</sub> within minutes of its addition to cultured tomato cells (Fig. 1). For the cells used here, which had been transferred to fresh medium 12 h before elicitation, the low concentration of OGA used was observed to promote a mild oxidative burst within approximately 6 min of addition to the suspension culture (Fig. 2, trace B). Importantly, transfer of another aliquot of the same cell suspension to systemin-containing medium 12 h before examina-

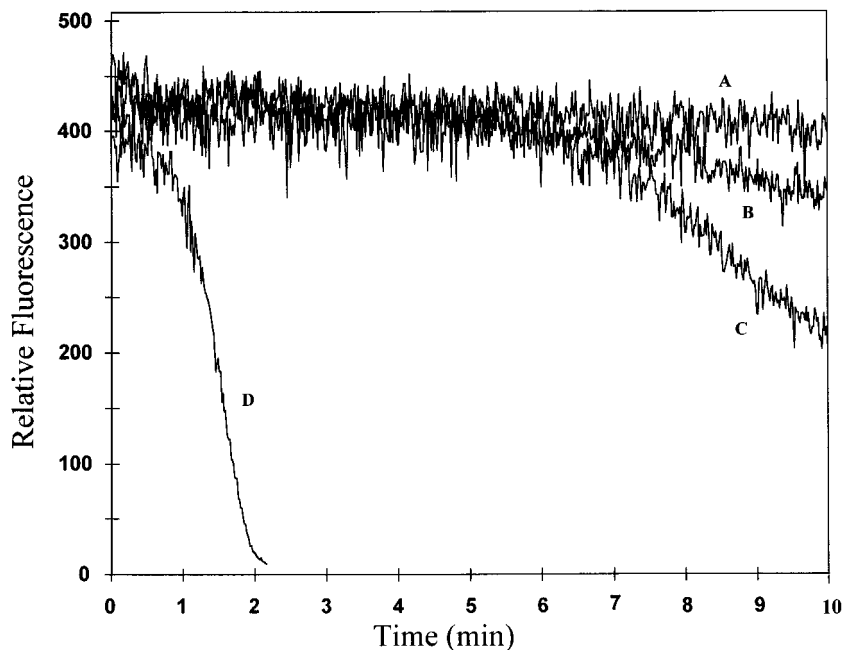


**Figure 1.** Effect of systemin and A-17 on the generation of  $\text{H}_2\text{O}_2$  by unstimulated and OGA-elicited tomato cell-suspension cultures. Twelve-hour-old tomato cell-suspension cultures were treated with buffer (control),  $1 \mu\text{M}$  systemin, or  $1 \mu\text{M}$  A-17, the inactive systemin analog, and assayed directly for  $\text{H}_2\text{O}_2$  production (trace A). Alternatively, a separate flask of cells was treated as above and then stimulated with a low concentration of OGA elicitor ( $20 \mu\text{L}$  of a  $500 \mu\text{g}/\text{mL}$  stock solution added to  $1.5 \text{ mL}$  of cell suspension) and assayed for  $\text{H}_2\text{O}_2$  production (trace B). Because the tomato cells that were treated with buffer alone, systemin, or A-17 generated no measurable  $\text{H}_2\text{O}_2$ , they are collectively represented by a single trace (A). Similarly, because the buffer-, systemin-, and A-17-treated cells all yielded an identical, slowly developing oxidative burst after OGA elicitation, they are also displayed as a single representative trace (B). Each experiment was conducted at least four times with similar results.

tion caused no unelicited synthesis of active oxygen species (Fig. 2, trace A), indicating that even extended exposure to systemin induces no autologous generation of an oxidative burst. In contrast, stimulation of the 12-h systemin-pretreated cell suspension with OGA not only shortened the delay before onset of  $\text{H}_2\text{O}_2$  production, but also promoted oxidant biosynthesis at approximately 16 times the rate of OGA-stimulated cells that were not pretreated with systemin (Fig. 2, compare traces B and D). In more than 10 replicates of this series of experiments on many different batches of cells, the systemin-promoted enhancement of  $\text{H}_2\text{O}_2$  generation ranged from 10 to 20 times that of non-pretreated but OGA-stimulated cells. Because similar pretreatment with A-17 exerted no effect, we conclude that

pretreatment with systemin augments the tomato cell's ability to activate  $\text{H}_2\text{O}_2$  production in response to elicitor stimulation.

The effects of systemin and A-17 on oxidant biosynthesis by tomato cells were also assayed at much lower ( $10^{-9} \text{ M}$ ) peptide concentrations. As above, no unelicited production of  $\text{H}_2\text{O}_2$  was observed, regardless of the duration of exposure to either peptide (data not shown). Unfortunately, the enhancement of OGA-stimulated  $\text{H}_2\text{O}_2$  generation subsequent to the usual 12-h preincubation with systemin was more variable than at higher systemin concentrations. Although we suspect that different rates of systemin breakdown during the 12-h preincubation may be the cause of the observed variability, all further incubations were still



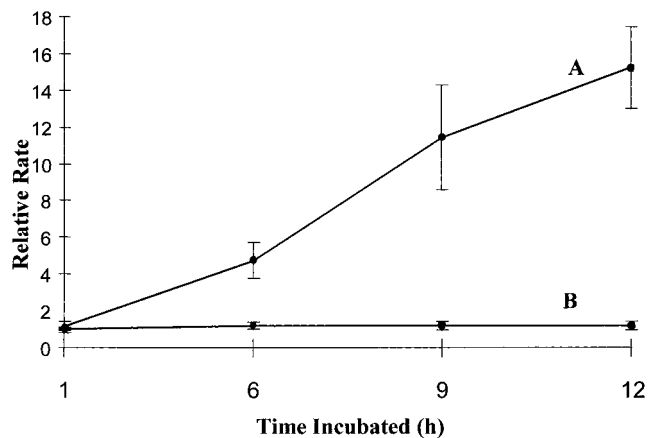
**Figure 2.** Effect of preincubation with systemin on  $\text{H}_2\text{O}_2$  production by unstimulated and OGA-elicited tomato cells. Suspension-cultured tomato cells were transferred to fresh growth medium containing  $1 \mu\text{M}$  A-17 or no additive (traces B and C), or  $1 \mu\text{M}$  systemin (trace D). Twelve hours later, the cells were stimulated with a low amount ( $20 \mu\text{L}$ , traces B and D) or a saturating amount ( $100 \mu\text{L}$ , trace C) of OGA, and the rate of  $\text{H}_2\text{O}_2$  biosynthesis was monitored by following the quenching of pyranine fluorescence. Alternatively, an aliquot of the A-17-, systemin-, or buffer (control)-treated cell suspension was incubated for 12 h, as above, and then assayed for  $\text{H}_2\text{O}_2$  generation in the absence of OGA elicitation (trace A). Because none of the three unelicited cultures (A-17-, systemin-, or buffer-pretreated cells not elicited with OGA) generated detectable  $\text{H}_2\text{O}_2$ , they are all represented by a common trace (A). Each study was conducted at least five times with similar results.

conducted at  $10^{-6}$  M systemin to avoid the variability problem altogether.

In addition to chemical elicitation, pathogen ingress can generate mechanical signals that are sensed by the plant and used to activate defense responses (Yahraus et al., 1995). However, because mechanical stimuli may be perceived very differently by a host plant than chemical stimuli, it seemed important to also explore whether pretreatment with systemin might enhance a mechanically induced oxidative burst. As described previously (Yahraus et al., 1995), mechanical initiation of the burst reaction can be accomplished in suspension-cultured cells either by direct physical pressure or by a decrease in osmotic pressure. Figure 3 shows that 12-h-old tomato cells produced a mild oxidative burst after dilution with an equal volume of water (trace B). A similar response was also measured in cells exposed for the entire 12-h incubation period to A-17, the inactive analog of systemin (trace B). In contrast, tomato cells cultured for the same 12-h period with  $1 \mu\text{M}$  systemin generated a measurably ( $>10$  times) enhanced oxidative burst after the same 1:1 dilution with water (trace D). As with elicitor stimulation, the lag period between application of the mechanical signal and generation of reactive oxidants was also measurably shortened in the systemin-pretreated cells (compare traces B and D).

#### Time Course of Systemin-Induced Enhancement of the OGA-Stimulated Burst

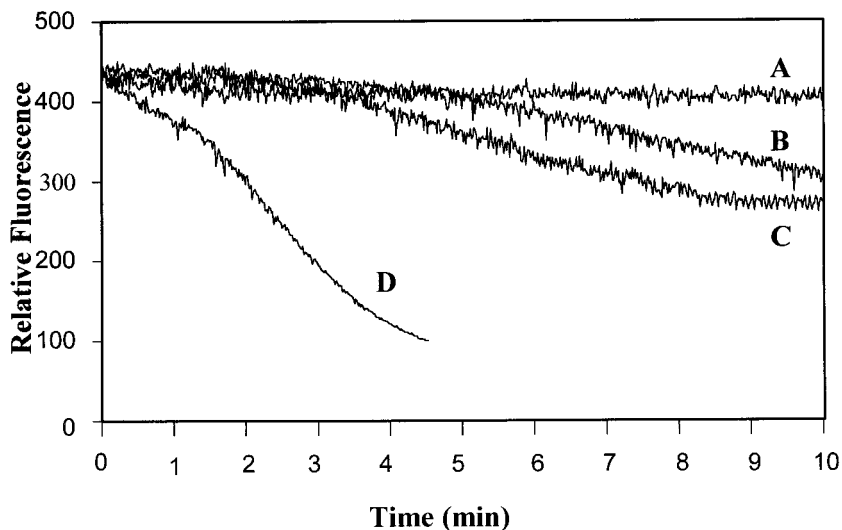
To more accurately evaluate the time required for systemin to exert its effects on the OGA-stimulated oxidative burst, we preincubated suspension-cultured cells with  $1 \mu\text{M}$  systemin for varying periods of time for up to 12 h and then determined the magnitude of the OGA-activated burst. As shown in Figure 4, systemin enhancement of oxidant biosynthesis was seen as early as 6 h after its addition, and this augmentation of the burst continued to increase with longer incubation times. By 12 h after addition, the average rate enhancement reached approximately



**Figure 4.** Effect of preincubation time on the magnitude of systemin enhancement of the OGA-stimulated oxidative burst. Tomato cells were transferred to fresh medium containing  $1 \mu\text{M}$  systemin (A),  $1 \mu\text{M}$  A-17 (B), or no additive. At various times after transfer, cells were stimulated with OGA and the rate of  $\text{H}_2\text{O}_2$  biosynthesis was assayed, as described in "Materials and Methods." The relative rate plotted on the y axis refers to the rate of oxidant generation of the systemin-/A-17-pretreated sample divided by the rate of oxidant production of the nonpretreated sample, each stimulated by OGA at the same time point. The data represent the average  $\pm$  SD, where  $n = 3$ .

16-fold. Unfortunately, the experiment could not generally be continued beyond 12 h because the untreated cells gradually entered the usual elicitor-refractory period (Apostol et al., 1989b) that prohibited further analyses. However, it should be noted that the systemin-treated cells invariably continued to be responsive to elicitors such as OGA many hours beyond the time when untreated cells became inactive. This prolongation of sensitivity to elicitors adds support to the hypothesis that systemin promotes a fundamental change in the tomato cells that renders them more capable of elicited oxidant production. Figure 4 also shows that A-17-pretreated cells were elicited by OGA similarly to control cells, confirming that the effect of systemin was

**Figure 3.** Effect of preincubation with systemin on  $\text{H}_2\text{O}_2$  production by unstimulated and osmotically stressed tomato cells. Suspension-cultured cells were transferred to fresh growth medium containing  $1 \mu\text{M}$  A-17 or no additive (traces B and C), or  $1 \mu\text{M}$  systemin (trace D), and assayed 12 h later for  $\text{H}_2\text{O}_2$  production after 1:1 dilution (B and D) or 1:2 dilution (C) with water. Concurrently, the unstimulated rate of  $\text{H}_2\text{O}_2$  biosynthesis in each of the three samples (A-17, systemin, and buffer [control]-treated suspensions) was measured at the same 12-h time point. Because none of the three unstimulated cell cultures generated any  $\text{H}_2\text{O}_2$ , they are collectively represented by a common curve (trace A). Similarly, because A-17 addition had no effect on the osmotically stimulated oxidative burst, it is shown together with a nonpretreated but osmotically stimulated control in trace B. Each study was conducted at least three times with similar results.



sequence dependent and was not a consequence of non-specific effects.

### Systemin Enhancement of the OGA-Induced Oxidative Burst Is Dependent on Protein Synthesis

Systemin-mediated activation of the octadecanoid pathway culminates in the transcription of proteinase inhibitor genes that are thought to contribute to herbivore resistance (Farmer and Ryan, 1992; Mueller et al., 1993; Schaller and Ryan, 1995). Consequently, it seemed prudent to investigate whether systemin-mediated enhancement of the oxidative burst might also require *de novo* protein synthesis. Cycloheximide, a well-known protein translation inhibitor, was administered to cells concurrently with systemin (i.e. at the time of transfer to fresh medium) and its effects were monitored 12 h later. As seen in Figure 5, cycloheximide abolished the systemin-induced enhancement of the OGA burst. In contrast, cycloheximide had little or no influence on the OGA-stimulated burst in nonpretreated or A-17-pretreated cells, indicating that cycloheximide had no effect on the oxidase complex itself but, rather, prevented its enhancement by systemin. Taken together, these data suggest that the major mechanism by which systemin enhances elicitation of the oxidative burst pathway involves the translation of new polypeptides.

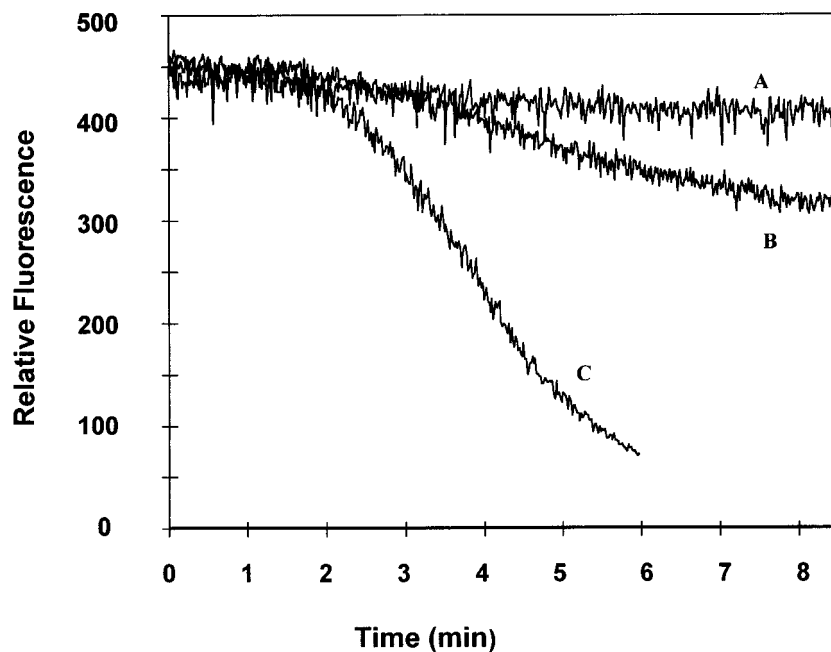
### DISCUSSION

We have demonstrated that pretreatment of tomato cells with a wounding-inducible peptide hormone, systemin, strongly augments both an elicitor-induced and an osmotically induced oxidative burst. Not only is the delay between stimulation and appearance of the burst greatly shortened after prolonged exposure to systemin, but the rate of oxidant production is enhanced, and the length of

time during which cell cultures remain responsive to elicitation is significantly extended. Because A-17, an inactive peptide analog that differs from systemin in only 1 of 18 amino acids, has no significant effect, we suggest that systemin exerts its influence on the oxidative burst through its normal cell-surface receptor.

Based on functional characteristics, the oxidative burst is thought to serve a defensive function directed primarily at disease-causing microbes (Chandra et al., 1996; Doke et al., 1996; Low and Merida, 1996). The currently acknowledged properties of systemin, in contrast, indicate that it is generated during herbivore feeding and acts to induce systemic protection against herbivores (Farmer and Ryan, 1990; Schaller and Ryan, 1995). Therefore, augmentation of the oxidative burst by pretreatment with systemin implies that a wounded tomato may have evolved strategies to prophylactically counteract opportunistic pathogens. Although no constitutive biosynthesis of H<sub>2</sub>O<sub>2</sub> can be detected in systemin-pretreated cells, once properly initiated by an elicitor, the rate of oxidant production is enhanced by a factor of 16. It would seem, therefore, that an insect-damaged plant not only undertakes to prevent further feeding by insects, but may simultaneously mobilize to thwart adventitious pathogen attack.

The effects of systemin on the tomato-cell oxidative burst were not immediate. Only several hours after its addition were the changes promoted by systemin clearly measurable. That this extended incubation period probably involves the synthesis of new proteins was shown by the inhibition of enhancement of oxidant biosynthesis by cycloheximide. Although no information was obtained regarding the identities of the newly synthesized polypeptides, it is tempting to speculate that more than one biochemical complex might be involved. Thus, not only were the timing of initiation and the rate of oxidant synthesis altered, but the duration of the period during which



**Figure 5.** Effect of cycloheximide on systemin-induced enhancement of the OGA-elicited oxidative burst. Tomato cells were transferred into fresh medium containing or lacking the following components: 1  $\mu\text{M}$  systemin, 1  $\mu\text{M}$  A-17, and/or 2  $\mu\text{M}$  cycloheximide. After 12 h of incubation, the cell suspensions were examined for basal (trace A) and OGA-elicited (traces B and C) H<sub>2</sub>O<sub>2</sub> production. Because many of the responses were similar, they are grouped into the following representative traces: A, nonpretreated and unelicited cells, systemin-pretreated cells (no OGA), A-17-pretreated cells (no OGA), and cycloheximide-pretreated cells (no OGA); B, nonpretreated cells elicited with OGA, A-17-pretreated cells elicited with OGA, A-17- plus cycloheximide-pretreated cells elicited with OGA, systemin- plus cycloheximide-pretreated cells elicited with OGA, and cycloheximide-pretreated cells elicited with OGA; and C, systemin-induced cells elicited with OGA. Each study was repeated at least three times with similar results.

the cells remained responsive to elicitation was also extended. It will be important to identify the genes whose products are involved in these regulatory events, because they likely encode proteins that control the oxidant-generating capacity of the cell.

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