The Plant Cell, Vol. 11, 1525–1535, August 1999, www.plantcell.org © 1999 American Society of Plant Physiologists

A 160-kD Systemin Receptor on the Surface of *Lycopersicon peruvianum* Suspension-Cultured Cells

Justin M. Scheer and Clarence A. Ryan[1]

Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164-6340

Systemin, an 18–amino acid polypeptide wound signal, activates defense genes in leaves of young tomato plants and induces rapid alkalinization of media containing suspension-cultured *Lycopersicon peruvianum* cells. A monoiodinated form of a systemin analog synthesized with Tyr-2 and Ala-15 (Tyr-2,Ala-15–systemin) likewise exhibits similar biological activities. ^{125}I—Tyr-2,Ala-15–systemin rapidly, reversibly, and saturably bound to suspension-cultured *L. peruvianum* cells with a K_d of 0.17 nM and a Hill coefficient of 0.92. The specificity of binding was assessed with alanine-substituted systemin analogs and was found to correlate with their respective biological activities. Treatment of suspension-cultured cells with methyl jasmonate increased the total binding of ^{125}I—Tyr-2,Ala-15–systemin more than threefold, suggesting that methyl jasmonate was activating transcription of the gene encoding the binding protein. Treatment of cells with cycloheximide markedly decreased binding of iodinated systemin to the cells, indicating that the binding protein was constantly being synthesized and degraded. A photoaffinity systemin analog, *N*-(4-[*p*-azidosalicylamido]butyl)-3′(2′—Cys-3,Ala-15–systemindithiol)propionamide, specifically labeled a 160-kD cell surface protein, and the labeling was completely inhibited by a 20-fold excess of unlabeled systemin. These data indicate that a 160-kD protein may be the physiological receptor for systemin in suspension-cultured cells.

INTRODUCTION

The 18–amino acid polypeptide hormone systemin has been shown to play an essential role in systemically signaling the expression of defense genes in tomato plants in response to herbivore attack and other mechanical wounding (Pearce et al., 1991; Ryan and Pearce, 1998). The polypeptide is derived from a 200–amino acid precursor, prosystemin, by proteolytic cleavage (McGurl et al., 1992). The critical importance of prosystemin in the signaling pathway was shown by transforming tomato plants with a prosystemin antisense gene that is regulated by the cauliflower mosaic virus 35S promoter (McGurl et al., 1992). The transgenic plants produced high levels of prosystemin antisense mRNA, resulting in a severely compromised wound response (McGurl et al., 1992). *Manduca sexta* larvae readily consumed the transgenic plants, whereas wild-type plants exhibited a normal defense response by inhibiting larval growth (McGurl et al., 1992).

By contrast, plants transformed with a cauliflower mosaic virus 35S–prosystemin gene, with the prosystemin mRNA in the sense orientation, produced high levels of prosystemin mRNA in plants (McGurl et al., 1994). These transgenic plants exhibited constitutive expression of >20 systemic wound response proteins in leaves (Bergey et al., 1996; Ryan and Pearce, 1998), and they seemed to be in a permanently wounded state (McGurl et al., 1994). Because prosystemin is synthesized in the vascular bundles of wild-type plants (Jacinto et al., 1997), other cell types constitutively expressing prosystemin apparently produced abnormal amounts of active systemin polypeptides. Systemin appears to be a master signal that reprograms leaf cells for defense against predators (Ryan and Pearce, 1998).

Systemin activates a signaling cascade that produces intracellular alterations in ion transport (Schaller and Oecking, 1999); intracellular increases in th activities of mitogen-activated protein kinase (Stratmann and Ryan, 1997) and phospholipase A_2 (Lee et al., 1997; J. Narvaez-Vasquez and C.A. Ryan, submitted manuscript); the induction of calmodulin (Bergey and Ryan, 1999); and increases in intracellular Ca^{2+} (Moyen et al., 1998). A model has been proposed (Farmer and Ryan, 1992) in which these early events result in the release from membranes of linolenic acid (Conconi et al., 1996), its conversion to jasmonic acid by way of the octadecanoid pathway, and the activation of defense genes (Farmer and Ryan, 1992; O'Donnell et al., 1996).

A key step in the initiation of these early events is the interaction of systemin with a putative receptor. A systemin binding site has been reported to be present in isolated membranes of a wild tomato species, *Lycopersicon peruvianum* (Meindl et al., 1998). The interaction exhibited a K_d of 1 nM that was reversible and competitively displaced by

[1] To whom correspondence should be addressed. E-mail maertens @wsu.edu; fax 509-335-7643.

systemin and Ala-17–systemin, an inactive derivative in which the threonine at position 17 is substituted with an alanine, and by other biologically active derivatives.

We have constructed a ^{125}I-labeled systemin that exhibits nearly the same potency as does systemin in inducing proteinase inhibitor synthesis in intact tomato plants. Application of this systemin derivative causes a rapid alkalinization of media that contain suspension-cultured *L. peruvianum* cells. Radiolabeled systemin binds to the surface of the suspension-cultured cells with the characteristics of a systemin–receptor interaction. The kinetics and reversibility of the binding are described, including the synthesis of binding sites by methyl jasmonate (MeJA). Using a photoaffinity label, we identified the binding protein by both photoaffinity labeling and electrophoretic analysis.

RESULTS

Bioactivity and Radioiodination of Systemin Analogs

A biologically active synthetic systemin analog with Val-2 →Tyr-2 and Met-15→Ala-15 substitutions that could be readily iodinated was synthesized (Figure 1). These substitutions were made because native systemin does not contain tyrosine, and the methionine at position 15 is susceptible to oxidations due to the iodination conditions. Substitutions of alanine at positions 2 and 15 were previously shown not to affect biological activity substantially (Pearce et al., 1993).

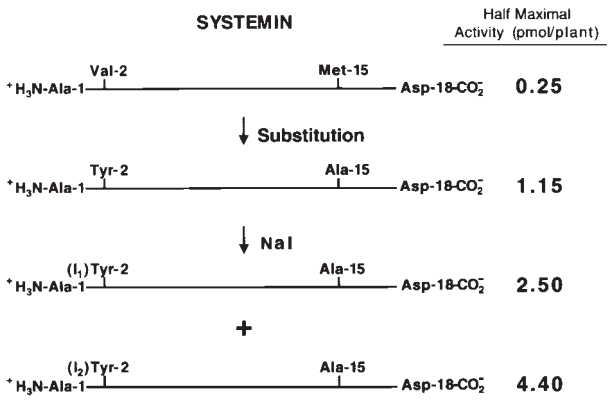

Figure 1. Systemin and Analogs.

Analogs of systemin were synthesized with a tyrosine residue at position 2 and an alanine residue at position 15. Tyr-2,Ala-15–systemin was iodinated, and the monoiodinated and diiodinated products were separated by HPLC. Systemin, Tyr-2,Ala-15–systemin, and iodinated derivatives were assayed for proteinase inhibitor–inducing activity in tomato plants, as described in Methods.

To assess the biological activity of the noniodinated and iodinated forms of Tyr-2,Ala-15–systemin, the analogs were purified by HPLC and assayed for proteinase inhibitor induction in tomato plants and for their ability to cause alkalinization of the medium of suspension-cultured cells. The monoiodinated and diiodinated systemins were found to be an order of magnitude less active than systemin in the proteinase inhibitor–inducing assay (Figure 1), with the monoiodinated form being slightly more active than is the diiodinated form.

Both iodinated forms were fully active in causing alkalinization of the suspension-cultured cell medium (Figure 2). Half-maximal medium alkalization occurred between 0.25 and 2.5 nM, which is the concentration range for half-maximal activity of systemin as well as for Tyr-2,Ala-15–systemin.

Radioiodination of 11 μg (5.6 nmol) of Tyr-2,Ala-15–systemin with 1 mCi of Na-^{125}I (approximately a 5 to 1 stoichiometry) resulted in a much higher yield (∼95%) of the monoiodinated form of systemin than the diiodinated form when separated by HPLC. The monoiodinated derivative yielded $\sim 4 \times 10^8$ cpm of ^{125}I—Tyr-2,Ala-15–systemin having a specific activity of ∼1000 Ci/mmol. This derivative was used in all of the following kinetic experiments.

The kinetics of association of radiolabeled systemin with the binding site(s) were similar to the kinetics of the alkalinization response (Figure 3). The binding of radiolabeled systemin to cells was detected as early as 1 min after addition and continued to increase until 7 min, when it began to diminish. Nonspecific binding remained constant throughout the time course of the experiments, indicating that uptake by the cells was not occurring. The medium alkalization began at 4 min after the addition of systemin and continued for ∼8 min, with a maximum pH change of ∼1 unit (data not shown). Because of these results, all of the following binding studies were performed at 7 min after the addition of systemin and systemin derivatives.

Binding Kinetics of Iodinated Systemin to Suspension-Cultured Cells

The binding of ^{125}I—Tyr-2,Ala-15–systemin to suspension-cultured cells was diminished by the addition of native, unlabeled systemin (Figure 4A). The competition by systemin, as well as by two systemin analogs, Ala-17–systemin and Ala-5–systemin, for the ^{125}I—Tyr-2,Ala-15–systemin binding was investigated. Both analogs at an ∼200-fold molar excess strongly competed with the iodinated systemin for >75% of the total binding to the cells, whereas a random 18–amino acid synthetic polypeptide containing the same charge and relative hydrophobicity of systemin was not able to compete.

The competition by systemin and systemin analogs was assessed in experiments in which increasing amounts of nonradioactive systemin and systemin antagonists com-

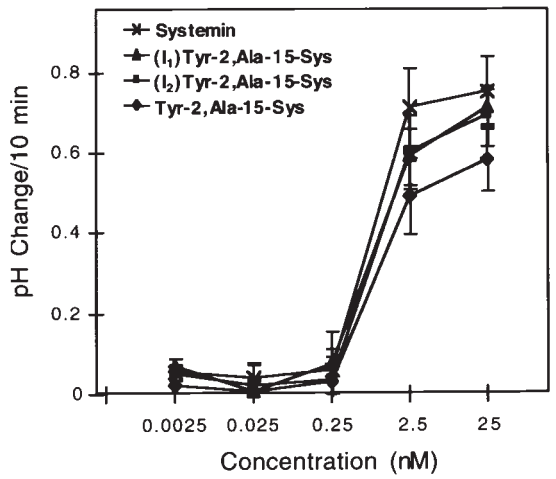

Figure 2. Medium Alkalinization of Suspension-Cultured *L. peruvianum* Cells by Systemin, Tyr-2,Ala-15–Systemin, and Iodinated Derivatives.

Alkalinization of the medium of suspension-cultured cells was measured using 2 mL of cultured cells after equilibration on an orbital shaker for 1 hr at room temperature, as described in Methods. Solutions of the polypeptides were added and the changes in pH were monitored using a pH meter. Error bars indicate standard deviation. I_1, monoiodinated systemin; I_2, diiodinated systemin; Sys, systemin.

peted with radioiodinated systemin for binding by the cells. Native systemin, Ala-17–systemin, Ala-5–systemin, and another analog, Ala-13–systemin, all competed for the binding sites with half-maximal inhibitory concentrations (IC_{50}s) of ~1, ~1.8, ~1.1, and ~140 nM, respectively (Figure 4B). Ala-13-systemin was previously shown to induce proteinase inhibitor synthesis in tomato plants, but its half-maximal effective concentration (EC_{50}) was ~900-fold lower than that of systemin (Pearce et al., 1993). A similar decrease in competitor ability was observed. The random 18–amino acid polypeptide did not compete for ^{125}I—Tyr-2,Ala-15–systemin binding at concentrations up to 6 mM. A comparison of the EC_{50} values of systemin, Ala-5–systemin, and Ala-13–systemin for alkalinization of culture media and the IC_{50} values for competitive displacement of ^{125}I—Tyr-2,Ala-15–systemin by systemin and systemin analogs is provided in Table 1. These values are closely correlated.

To determine whether the binding was saturable, as would be expected for a receptor–ligand interaction, cells were incubated with ^{125}I—Tyr-2,Ala-15–systemin in the presence or absence of an excess of unlabeled systemin. A plot of the specific binding as a function of increasing ^{125}I—Tyr-2,Ala-15–systemin indicated that the binding was saturable at a level of ~1 nM ^{125}I—Tyr-2,Ala-15–systemin.

The affinity (K_d) and maximal binding (B_{max}) were calculated by Scatchard analysis (Figure 5B), using the saturation data from Figure 5A. By taking the negative inverse of the slope, the K_d for binding of ^{125}I—Tyr-2,Ala-15–systemin to the cells was calculated to be 0.17 nM. This is in agreement with the concentration of systemin required for half-maximal medium alkalinization. The B_{max} is determined by the x-intercept and indicates a maximal binding of 5 fmol/10^6 cells. Assuming one ligand per receptor, this estimates to ~3000 receptor sites per cell. The cooperativity of the binding was analyzed in a Hill plot. The slope generated by this plot, a measure of the cooperativity of the binding, gave a Hill coefficient of $n = 0.92$ (Figure 5C).

The reversibility of the systemin–receptor interaction is shown in Figure 6. The addition of native systemin to cultured cells 4 min after the addition of radiolabeled systemin caused a reversal of binding that equilibrated within ~2 min. The bound radioactivity after equilibration was ~50% of the total originally bound. The binding that could be competed in this experiment was equivalent to the specific binding found previously (e.g., Figure 3).

MeJA-Mediated Induction of Binding Sites

MeJA is a potent inducer of systemic wound response proteins in tomato plants (including enzymes involved in signal transduction); its effect on systemin binding to tomato suspension-cultured cells was assayed over time. Figure 7

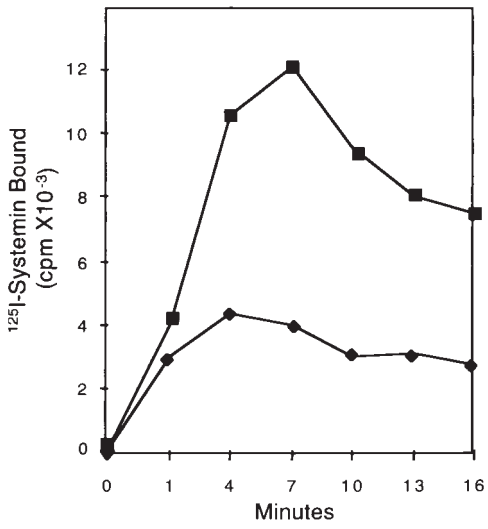

Figure 3. Time Course of Binding of ^{125}I—Tyr-2,Ala-15–Systemin to Suspension-Cultured *L. peruvianum* Cells.

Binding of ^{125}I—Tyr-2,Ala-15–systemin in the absence (squares, total binding) or presence (diamonds, nonspecific binding) of a 200-fold excess of unlabeled systemin was determined over time. At the indicated times, the cells were collected, washed, and analyzed for radioactivity.

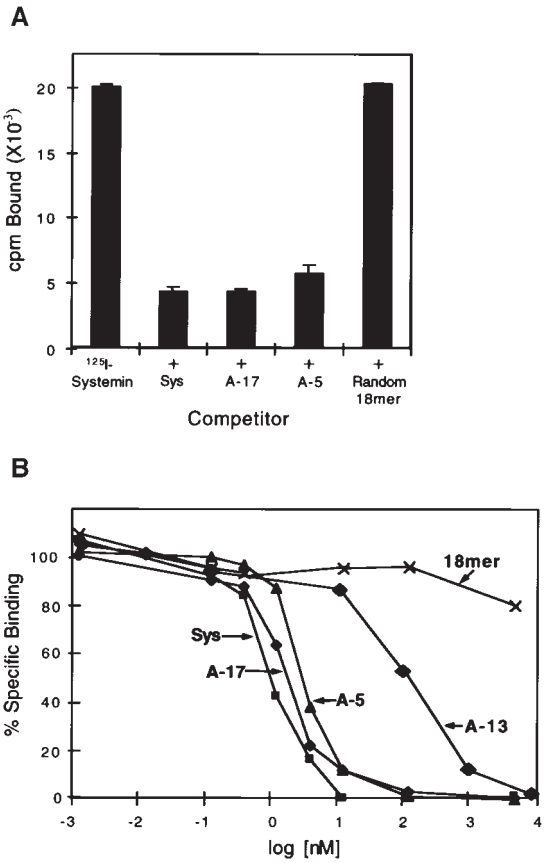

Figure 4. Competition of Unlabeled Systemin and Systemin Analogs with ^{125}I—Tyr-2,Ala-15–Systemin for Binding Sites on the Surface of *L. peruvianum* Suspension-Cultured Cells.

(A) Competition of the binding of ^{125}I—Tyr-2,Ala-15–systemin to suspension-cultured *L. peruvanium* cells by native systemin (Sys), Ala-17–systemin (A-17), Ala-5–systemin (A-5), and a random 18–amino acid polypeptide (Random 18mer). The competing polypeptides were added (+) at a 200-fold molar excess over ^{125}I—Tyr-2,Ala-15–systemin. Error bars indicate standard deviation.

(B) Competition analysis of ^{125}I—Tyr-2,Ala-15–systemin binding by systemin and systemin analogs was determined by treating the cells with increasing concentrations of unlabeled, competing peptides and by calculating the percentage of specific binding as a ratio of specific binding at the indicated concentration to maximal specific binding found in the presence of a 200-fold excess of unlabeled systemin (Sys; squares); Ala-17–systemin (A-17; small diamonds); Ala-5–systemin (A-5; triangles); Ala-13–systemin (A-13; large diamonds); and a random 18–amino acid polypeptide (18mer; crosses).

shows that the total binding per 10^6 cells increased within 6 hr after adding MeJA to suspension-cultured cells and peaked at 18 hr. Neither the volume of packed cells nor the dry weight of the cells increased over the course of the experiments (data not shown). The K_d estimated by half-maximal saturation was found to be the same for control and MeJA-induced cells (data not shown).

To determine whether the increase in binding sites in response to MeJA was a result of de novo protein synthesis, cells were treated with cycloheximide, a protein translation inhibitor, and compared with control cells incubated without cycloheximide. Binding of radiolabeled systemin was assayed 15 hr after treatment with either MeJA or water (control). The binding to cells treated with cycloheximide in the absence of MeJA decreased approximately fourfold when compared with untreated cells (Table 2), whereas binding to cells treated with both cycloheximide and MeJA was decreased nearly 10-fold with respect to cells treated with MeJA only. These experiments indicate that cycloheximide caused the inhibition of synthesis of the binding protein(s), which was likely being degraded over the course of the experiments as a result of normal protein turnover. Thus, in both MeJA-treated and MeJA-untreated cells, the difference in levels of binding protein between the two conditions was likely due to a modulation of synthesis coupled to a steady state rate of degradation.

Photoaffinity Labeling

A photoaffinity analog, *N*-(4-[*p*-azidosalicylamido]butyl)-3′(2′–Cys-3, Ala-15–systemindithiol)propionamide, herein called ^{125}I—azido-Cys-3,Ala-15–systemin, was constructed as shown in Figure 8. The analog was determined in the alkalinization assay with suspension-cultured cells to be as biologically active as native systemin (Figure 9). When UV-B irradiated, the reagent was shown to label efficiently a single protein species in the cell membranes (Figure 10). This protein, with a molecular mass of ~160 kD, was the only protein labeled among all of the proteins in the cells, suggesting that the azido moiety of the photoaffinity label was poised to react with a nucleophile of the receptor and was inaccessible to other proteins. The labeling of this protein could be fully competed by a 20-fold excess of unlabeled systemin (Figure 10). The concentration-dependent competition of systemin for the azido-labeled systemin correlates well with the competition of ^{125}I—Tyr-2,Ala-15–systemin by systemin (Figure 4B).

Table 1. IC_{50} Values for Competition of Systemin Analogs with ^{125}I—Tyr-2,Ala-15–Systemin Binding to *L. peruvianum* Suspension-Cultured Cells When Compared with the EC_{50} Values of Their Biological Activities in Inducing Medium Alkalinization in the Cells

Analog	IC_{50} (nM)	EC_{50} (nM)
Systemin	1.0	0.8
Ala-5–systemin	1.1	0.9
Ala-13–systemin	139.0	125.0

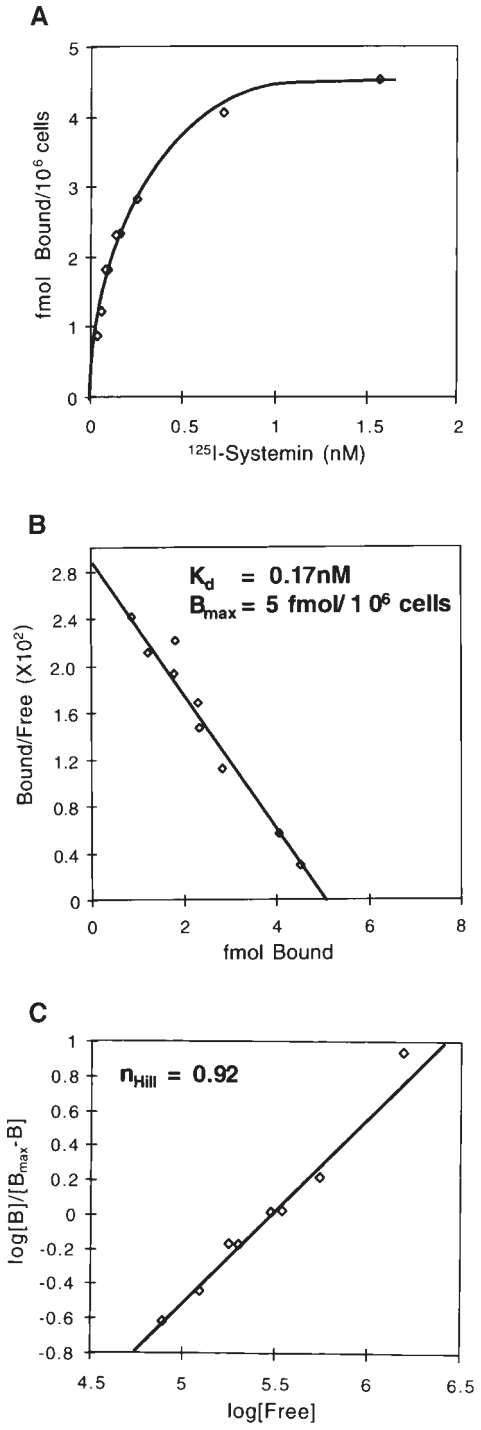

Figure 5. Saturation Analysis of ^{125}I—Tyr-2,Ala-15–Systemin Binding to Suspension-Cultured *L. peruvianum* Cells.

(A) Suspension-cultured cells were treated with increasing concentrations of ^{125}I—Tyr-2,Ala-15–systemin. The specific binding (total binding minus nonspecific binding) is shown. The specific activity of ^{125}I—Tyr-2,Ala-15–systemin was ~1000 Ci mmol^{-1} (2200 cpm fmol^{-1}).

(B) Scatchard analysis of ^{125}I—Tyr-2,Ala-15–systemin binding to cells from the data shown in **(A)**. The K_d is calculated as the negative inverse of the slope of the plot, and B_{max} is given by the x-axis intercept.

(C) Hill plot of ^{125}I—Tyr-2,Ala-15–systemin binding to cells derived from the data shown in **(A)**. The slope of the plot is the Hill coefficient (n_{Hill}).

DISCUSSION

Wound signaling of defense genes in tomato leaves by systemin is modulated by intracellular events that involve the activation of a 48-kD mitogen-activated protein kinase, a phospholipase A_2, the induction of calmodulin, and the mobilization of $Ca^{+}2$ and H^{+} (Felix and Boller, 1995; Stratmann and Ryan, 1997; Moyen et al., 1998; Bergey and Ryan, 1999). These early responses together may result in the activation of the phospholipase A_2, resulting in the release of linolenic acid from membranes and its subsequent conversion to phytodienoic acid and jasmonic acid. In this regard, levels of free linolenic acid have been shown to increase in tomato leaf cells in response to wounding (Conconi et al., 1996). The extremely low levels of systemin required to activate defense genes (picomolar concentrations per plant) suggested that the polypeptide, like animal polypeptide hormones, must be perceived by a specific receptor (Farmer and Ryan, 1992; Schaller and Ryan, 1995). Schaller and Ryan (1994) had shown previously that a systemin binding protein, SBP50, resided in purified plasma membranes of tomato plants, but this protein had characteristics of a Kex2-like protease rather than a receptor.

In 1995, Felix and Boller demonstrated that a rapid alkalinization of the culture medium occurred when suspension-cultured *L. peruvianum* cells were supplied with systemin. This alkalinization response was maximal with subnanomolar concentrations of systemin and could be inhibited by the systemin antagonist Ala-17–systemin. Because of the sensitivity, specificity, and rapidity of the response, it was thought that the response was likely to be mediated by a cell surface systemin receptor. By using a systemin analog that had been iodinated at a tyrosine residue attached to its C terminus, Meindl et al. (1998) demonstrated that a systemin binding site that was present in microsomal membranes was saturable and reversible and represented a single class of binding sites having a K_d of ~1 nM. In our laboratory, we prepared a systemin analog that was almost fully biologically active in inducing proteinase inhibitors in young tomato plants and in causing alkalinization in cultured cells (cf. Figures 1 and 2). The monoiodinated form of this analog, ^{125}I—Tyr-2,Ala-15–systemin, retained high biological activity and was used to investigate the perception of systemin by intact suspension-cultured cells.

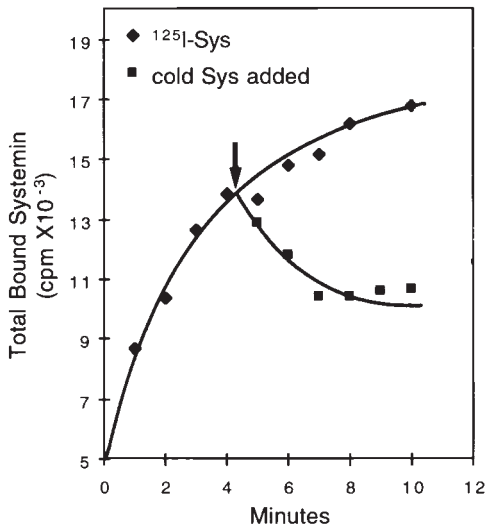

Figure 6. Reversibility of ^{125}I—Tyr-2,Ala-15–Systemin Binding to Suspension-Cultured *L. peruvianum* Cells.

Two sets of cells were treated with saturating levels of ^{125}I—Tyr-2,Ala-15–systemin, and the total radioactivity associated with the cells was determined at the indicated time. A 200-fold excess of unlabeled systemin (Sys) was added to one set of cells 4 min after the addition of ^{125}I—Tyr-2,Ala-15–systemin (^{125}I-Sys). The total radioactivity associated with both sets of cells was determined at the indicated times.

Monoiodinated ^{125}I—Tyr-2,Ala-15–systemin was shown to associate rapidly with the suspension-cultured *L. peruvianum* cells within 1 min (Figure 3). After reaching maximal binding at 7 min, a rapid decrease in the specific binding due to a decrease in the total binding was observed. This indicates that with time, ^{125}I—Tyr-2,Ala-15–systemin loses affinity for its binding site, which could result from various possibilities, including proteolytic inactivation of the ligand or conformational changes in the binding sites. When the kinetics of binding and the kinetics of the alkalinization response are compared, there is a direct correlation. Whereas binding begins within 1 min and peaks at 7 min, the alkalinization response under the same conditions begins at 4 min and reaches a maximum by 10 min (data not shown) (Felix and Boller, 1995).

A further correlation between binding and biological activity was observed in experiments in which three different Ala-substituted systemin analogs were analyzed for competitive displacement of ^{125}I—Tyr-2,Ala-15–systemin bound to cells (IC_{50} values) and for their biological activities (EC_{50} values) (Table 1). These three analogs have been described previously for their proteinase inhibitor–inducing activities in tomato plants (Pearce et al., 1993). Ala-17–systemin is a totally inactive inducer of defense genes, but it is a potent antagonist of systemin induction of proteinase inhibitors by systemin. Ala-17–systemin activates neither the 48-kD mitogen-activated kinase nor the alkalinization response (Pearce et al., 1993; Felix and Boller, 1995; Stratmann and Ryan, 1997). However, this analog was able to compete with systemin for binding to the cells at the same concentrations as native systemin, as shown in Figures 4A and 4B. This is in contrast to the results obtained by Meindl et al. (1998) in which Ala-17–systemin had nearly a 1000-fold decreased competitive ability with the ^{125}I—Tyr-19–systemin used in assessing systemin binding to isolated microsomal membranes. This discrepancy can be explained by either a change in the binding site as a result of the membrane preparation or a difference in binding due to a difference in the structures of the radiolabeled analogs.

The second analog, Ala-5–systemin, had been shown previously to be incapable of competing for binding to the membrane-associated systemin-binding protein SBP50 (Schaller and Ryan, 1994). In our study, Ala-5–systemin was able to compete for binding to the cells at concentrations near those of native systemin (Figure 4B). Furthermore, *p*-chloromercuribenzene sulfonic acid was a potent inhibitor of SBP50 binding (Schaller and Ryan, 1994), but this sulfhydryl reagent was unable to compete for binding of radiolabeled systemin at concentrations up to 1.25 mM (data not shown). Taken together, these data indicate that the binding protein is not SBP50.

The final analog tested was Ala-13–systemin. This was used to bring specific pharmacological relevance to the

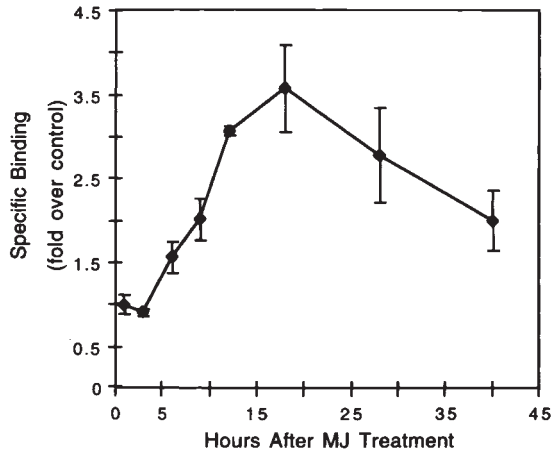

Figure 7. Time Course of MeJA Induction of the Total Binding of ^{125}I—Tyr-2,Ala-15–Systemin to Suspension-Cultured *L. peruvianum* Cells.

Saturating levels of ^{125}I—Tyr-2,Ala-15–systemin were used to determine the total level of binding at the indicated times after treatment with MeJA (MJ). Specific binding was determined in MeJA-treated and water-treated (control) cells, and the fold increase is expressed as MeJA-specific binding to control specific binding. Error bars indicate standard deviation.

Table 2. Effect of Cycloheximide on the Binding of ^{125}I–Tyr-2,Ala-15–Systemin to *L. peruvianum* Suspension-Cultured Cells

	Specific Binding (cpm)	
Culture Medium	With H_2O	With Cycloheximide
With H_2O	11,581	3121
With MeJA	25,726	2148

binding. Ala-13–systemin imparts a 900-fold decrease in proteinase inhibitor–inducing activity in the plants as compared with systemin (Pearce et al., 1993), and it is 100-fold less active in causing alkalinization of suspension cell culture medium (Figure 4B). As a competitor for radioiodinated systemin with the suspension cells, it exhibited a 100-fold reduction in competitive binding ability. This correlation between biological activity and binding is an important criterion in assessing true receptor binding (Hahn, 1996).

Saturation analysis with ^{125}I—Tyr-2,Ala-15–systemin showed that systemin binding to the cultured cells was saturable above 1 nM (Figure 5A). In the alkalinization bioassay, a maximal pH change was reached at ~2.5 nM (Figure 2), which illustrates a correlation between concentrations giving maximal binding and maximal biological activity. The level of binding observed was calculated to be 5 fmol/10^6 cells (~3000 sites per cell; assuming one ligand bound per binding site). This value is an estimate based on cell counts but does not include accurate counts in clumped cells or cells that may have been broken. Similar numbers for binding sites per cell have been observed for other plant defense elicitors: for example, β-glucan binding to soybean protoplasts is 3800 per cell (Cosio et al., 1988), and the elicitor peptide Pep13 binding to parsley is 2900 per cell (Nurnberger et al., 1994).

The saturation data were analyzed by Scatchard and Hill plots. The K_d observed in the Scatchard plot was calculated to be 0.17 nM (Figure 5B). This is a high-affinity association and reflects the ability of low concentrations of systemin to be biologically active. The binding site observed for the systemin receptor in microsomal membranes (Meindl et al., 1998) had a K_d of ~1 nM. The Hill plot for the systemin receptor revealed a coefficient of $n = 0.92$, which indicates that the binding is not cooperative (Figure 5C). This also in agreement with the data of Meindl et al. (1998). From the cumulative data, it is concluded that the systemin receptor is a single class of high-affinity binding sites that do not bind cooperatively.

The binding of radiolabeled systemin was readily reversible, as determined by the rapid decrease in binding within 2 min of the addition of a 200-fold molar excess of native unlabeled systemin. Approximately 50% of the total binding could be competed, which we found consistently among our experiments. When the cells were exposed to radiolabeled systemin for 7 min, treated with 0.1% trifluoroacetic acid for ~5 min, and then recovered, all radioactivity associated with the cells was lost, further indicating that the peptide had not been internalized (data not shown).

In tomato plants, several signaling pathway enzymes and proteins that are induced by wounding are also induced by MeJA, including lipoxygenase (Heitz et al., 1997; Royo et al., 1997), allene oxide synthase (Laudert and Weiler, 1998), the systemin precursor prosystemin (Bergey et al., 1996), and a polygalacturonase and its regulatory subunit (Bergey et al., 1999). This suggested that perhaps the receptor also might be induced to amplify signaling in response to herbivore grazing or pathogen attack. The effect of MeJA on the binding of radiolabeled systemin was assessed at increasing times after treatment of the cultured cells with MeJA. A time-dependent increase in binding was observed that maximized at 18 hr and then declined, suggesting that a transcriptional upregulation of the receptor was occurring. The induction of binding sites on the cell surfaces suggests that the receptor may be transcriptionally upregulated along with other signal pathway components to enhance the mechanism for perception of the systemin signal.

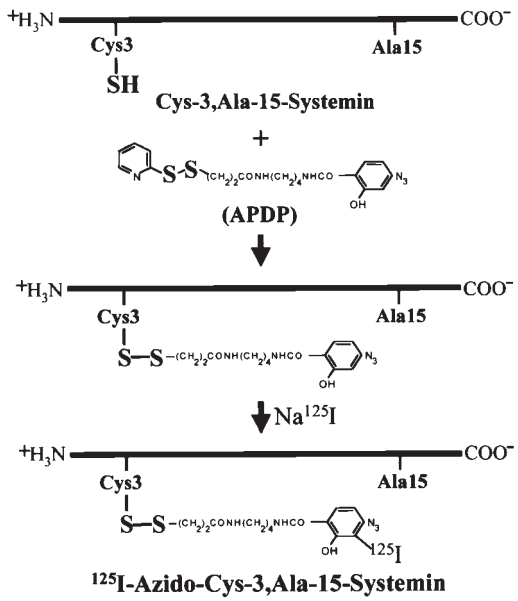

Figure 8. Construction of the Photoaffinity Analog ^{125}I—Azido-Cys-3,Ala-15–Systemin.

Systemin was coupled to the photoaffinity cross-linker *N*-(4-[*p*-azidosalicylamido]butyl)-3′(2′-pyridyldithio)propionamide (APDP) by the formation of a disulfide bond between the cysteine of Cys-3,Ala-15–systemin and the sulfhydryl reactive end of APDP. After purification of the reaction product, the photoaffinity analog was radioiodinated by substitution into the hydroxyl-containing ring of APDP.

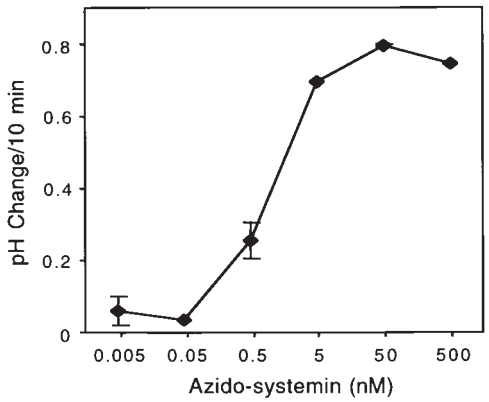

Figure 9. Alkalinization of *L. peruvianum* Suspension Cell Culture Medium by Azido-Cys-3,Ala-15–Systemin.

Alkalinization of the cell suspension culture medium in response to azido-Cys-3,Ala-15–systemin (Azido-systemin) was monitored as described in the legend to Figure 2. Error bars indicate standard deviation.

Treatment of the suspension-cultured cells with cycloheximide caused a severe decrease in binding of radiolabeled systemin to the cells. The total bound radioactivity in both water- and MeJA-treated cells decreased markedly after 18 hr of treatment with cycloheximide. This result also indicated that the systemin receptor is in constant turnover, and the loss of synthesis resulted in a loss of receptor protein. Investigations are in progress currently to quantify the rates of synthesis and degradation of the receptor protein.

Photoaffinity labeling was performed using the photoaffinity systemin analog shown in Figure 8. As shown in Figure 9, the ligand was fully biologically active when assayed using alkalinization of suspension-cultured cells as a criterion. The binding of this ligand to the receptor was highly efficient and occurred when systemin maximally binds to the cells. SDS-PAGE of the labeled proteins in the crude extract after photoaffinity cross-linking revealed only a single labeled band with a molecular mass of ~160 kD (Figure 10). This protein, called systemin receptor-160 (SR-160), was not observed with proteins obtained from cells that had been treated with a 20-fold excess of unlabeled, competing systemin (Figure 10). The concentrations of competing systemin were equivalent to concentrations that competed for binding by ^{125}I—Tyr-2,Ala-15–systemin (Figure 4B). The data indicate that the SR-160 protein is a biologically relevant systemin receptor in these cells. Single subunit receptors of this size have been characterized for animal epidermal growth factors (Adamson and Rees, 1981; Cadena et al., 1994) but not for plant hormones. The evidence at hand supports a role for the systemin binding protein as a cell surface receptor, and the purification of the protein and the molecular cloning of the cDNA and gene are in progress. Ultimately, understanding the mechanism of systemin perception and its role in initiation of early signaling events will depend on the isolation and structural and functional characterization of SR-160.

METHODS

Plant Growth

Tomato (*Lycopersicon esculentum* cv Castlemart) plants used for proteinase inhibitor assays were grown for 14 to 16 days under a 17-hr-light and 7-hr-dark cycle (28°C at >300 μE m^{-2} sec^{-1} and 17 and 21°C [in the dark]).

Cell Suspension Cultures

A tomato (*L. peruvianum*) suspension cell line Msk8 (Koornneef et al., 1987) was maintained in 125-mL Ehrlenmeyer flasks on orbital shakers (160 rpm) under constant light. Cells (3 mL) were subcultured every 7 days into 45 mL of sterile media (unbuffered; pH 5.5, adjusted with 0.1 M KOH) containing 3% sucrose, 4.3 g L^{-1} Murashige and Skoog salt mixture (Gibco, Gaithersburg, MD), 5 mg L^{-1} 1-napthylacetic acid (Sigma), 2 mg L^{-1} 6-benzylaminopurine (Sigma), 110 mg L^{-1} Nitsch and Nitsch vitamin powder (Sigma), 1 mg L^{-1} thiamine, 100 mg L^{-1} *myo*-inositol, and 1 mM EDTA. Cells were used for experiments 4 to 8 days after subculturing.

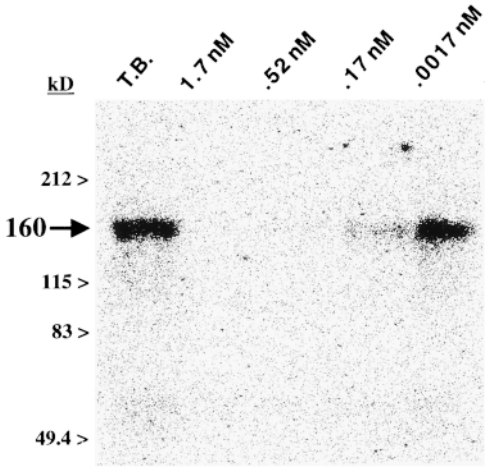

Figure 10. Electrophoresis of Proteins from Photoaffinity-Labeled Suspension-Cultured *L. peruvianum* Cells.

Cells were treated with ~5.0 × 10^5 cpm of ^{125}I—azido-Cys-3,Ala-15–systemin in the absence (T.B.) or presence (1.7, 0.52, 0.17, and 0.0017 nM) of unlabeled systemin. After 7 min of incubation, the cells were filtered, washed, and irradiated for 10 min with UV-B light. After irradiation, the cells were collected in SDS-PAGE sample buffer (containing 5% β-mercaptoethanol), and the extracted proteins were separated on a 10% SDS–polyacrylamide gel. The labeled protein of 160 kD was visualized using a PhosphorImager. Numbers at left indicate molecular mass markers given in kilodaltons.

Systemin and Substituted Systemins

Systemin, Ala-17–systemin, Ala-5–systemin, Ala-13–systemin, Tyr-2,Ala-15–systemin, and Cys-3,Ala-15–systemin were synthesized by the solid-phase technique, using a synthesizer (model 431A; Applied Biosystems, Foster City, CA). After synthesis, the synthetic polypeptides were purified by C18 reverse-phase, high-pressure liquid chromatography (RP-HPLC), as previously described (Pearce et al., 1993). Stock solutions of the peptides were prepared in water and stored at −20°C.

Biological Assays of Systemin and Its Derivatives

Biological activities of unlabeled analogs and monoiodinated and diiodinated Tyr-2,Ala-15–systemins were assessed in tomato plants for proteinase inhibitor–inducing activity and in cell suspension cultures for their ability to cause alkalinization of the medium. Proteinase inhibitor–inducing activities of systemin and its derivatives were assayed in 14-day-old tomato plants by supplying solutions of the polypeptides through the cut stems for 30 min and then with water for 24 hr in the light (30 μE m^{-2} sec^{-1}). Proteinase inhibitors were assayed in leaf juice by radial immunodiffusion, as previously described (Ryan, 1967; Trautman et al., 1971). Alkalinization assays of the cell culture medium by the addition of systemin or its derivatives were performed by aliquoting 2 mL of suspension into 12-well culture plates and allowing them to equilibrate on an orbital shaker (150 rpm) at room temperature for ~1 hr. After equilibration, the cells were monitored for biological activity by directly measuring the alkalinization of the extracellular medium with a pH meter (model EA940; Orion, Beverly, MA) and a semimicrocombination pH electrode (model 8103BN; Orion) before and after challenge with systemin or systemin analogs.

Iodination of Tyr-2,Ala-15–Systemin

Tyr-2,Ala-15–systemin was iodinated with both nonradioactive and radioactive iodine. Nonradioactive iodinations were performed by reacting 90 μL of Tyr-2,Ala-15–systemin (2.8 mM), 10 μL of NaI (500 mM), and three iodobeads (Pierce, Rockford, IL) (prerinsed in the same buffer) in an Eppendorf tube containing 400 μL of 100 mM sodium phosphate buffer, pH 7.5. The reaction was terminated after 10 min by removing the iodobeads and adding 1 mL of 0.1% trifluoroacetic acid. The reaction products were separated by analytical C18 RP-HPLC with a 0 to 40% CH_3CN/60-min gradient in a base solvent of 0.1% trifluoroacetic acid/water at a flow rate of 1 mL min^{-1}. The unreacted, monoiodinated, and diiodinated systemin derivatives eluted as well-separated peaks and were identified by electrospray mass spectrometry. The purified derivatives were quantified by using the bicinchoninic acid method (Pierce) with systemin as a standard and stored in Eppendorf tubes at 4°C.

Radioactive iodinations were performed in a similar manner. Twenty μL of Tyr-2,Ala-15–systemin (280 μM), 10 μL of Na-^{125}I (1 mCi, 17 Ci mg^{-1}; Du Pont), and one iodobead (prerinsed) were reacted for 8 min without mixing in an Eppendorf tube containing 103 μL of 100 mM sodium phosphate buffer, pH 7.5. The iodobead was removed, and the iodinated systemin derivatives were separated by analytical C18 RP-HPLC, as previously described. The two iodinated products were recovered, with the monoiodinated derivative being in approximately a 20 to 1 excess over the diiodinated product. A typical iodination yielded ~4×10^8 cpm of monoiodinated ^{125}I—Tyr-2,Ala-15–systemin, which was stored in plastic screw-cap tubes at 4°C and used for experimentation within 10 days after iodination. The specific activity of monoiodinated systemin was typically ~1000 Ci $mmol^{-1}$ and was used in all of the following binding studies.

Binding Assays

Radioligand binding assays were performed by aliquoting 1.5 mL of suspension-cultured cells into wells of 12-well culture plates and allowing the cells to shake on an orbital shaker (150 rpm) at room temperature. The binding was initiated by adding monoiodinated ^{125}I—Tyr-2,Ala-15–systemin directly to the cells. In competition assays, unlabeled native systemin or its derivatives were added just before the addition of radiolabeled systemin. Both ^{125}I—Tyr-2,Ala-15–systemin and the competing peptides were diluted with 4°C culture media, after which 50 μL was added to the cells. Binding was allowed to take place in the wells of the plates for precisely 7 min, except when measuring the time course and reversibility of binding. The reaction was terminated by removing 1.0 mL of cells and filtering them through two layers of 2.5 cm Whatman No. 1 filter paper housed in a 12-well vacuum filtration manifold (Millipore, Bedford, MA). The cells were then washed twice with 5 mL of 4°C culture media, and the top filter that retained the cells with the bound radioligand was analyzed for total radioactivity in a γ-ray counter (Isodata 2020; Isodata Inc., Palatine, IL). Specific binding was calculated by subtracting nonspecific binding (binding in the presence of excess nonradioactive native systemin) from total binding. All values were determined as an average of duplicates in each experiment. Unless otherwise indicated, ^{125}I—Tyr-2,Ala-15–systemin was added at saturating levels, and competing peptides were added at a 200-fold excess. The percentage of specific binding used in displacement experiments was calculated by dividing the specific binding at the indicated concentrations of competing peptides by the maximum specific binding determined in the presence of an excess of native systemin.

Induction of Binding Activity by Methyl Jasmonate

A 50 mM stock solution of methyl jasmonate (MeJA; Bedoukian, Danbury, CT) was prepared in water, and 50 μL was added to culture flasks containing 50 mL of cells to give a final concentration of 50 μM MeJA. Control cells were treated with an equal volume of water without MeJA, and both cultures were incubated under normal growth conditions. At various time points, aliquots of cells were removed from the flasks and assayed for binding, as previously described. Specific binding was determined in both the water-treated and MeJA-treated cells and expressed as a fold increase over controls by dividing the MeJA-treated specific binding by the water-treated specific binding.

Experiments for cycloheximide inhibition of MeJA induction were performed by treating cells as described above in the presence or absence of 2 μM cycloheximide (Sigma).

Photoaffinity Labeling with ^{125}I—Azido-Cys-3,Ala-15–Systemin

Cys-3,Ala-15–systemin was first coupled to the photoaffinity crosslinker. A 10 mM stock solution of the heterobifunctional crosslinker *N*-(4-[*p*-azidosalicylamido]butyl)-3′(2′-pyridyldithio)propionamide (APDP; Pierce, Rockford, IL) was prepared in dimethylformamide, and 50 μL of 10 mM APDP was coupled to Cys-3,Ala-15–systemin in a reaction solution containing 200 μL of 2.8 mM Cys-3,Ala-15–systemin and 300 μL of 100 mM sodium phosphate

buffer, pH 8.0, in the dark for 2.5 hr at room temperature. After coupling, the reaction was terminated by the addition of 0.5 mL of 0.1% trifluoroacetic acid and purified by C18 RP-HPLC, as described for iodination of Tyr-2,Ala-15–systemin (data not shown). The single product, azido-Cys-3,Ala-15–systemin, represented a complete coupling reaction in which all of the peptide was converted to the photoaffinity analog. The fractions containing the purified azido-Cys-3,Ala-15–systemin were lyophilized and resuspended in water, resulting in a stock solution of ~280 μM which was stored at −80°C.

Radioiodination was performed by reacting 20 μL of 280 μM Cys-3,Ala-15–systemin—APDP in 100 μL of 100 mM sodium phosphate buffer, pH 7.5, with 10 μL Na-^{125}I (1 mCi, 17 Ci mg^{-1}; Du Pont) and one prerinsed iodobead. The mixture was reacted for 8 min, separated by C18 RP-HPLC, and stored at 4°C in screw-cap Eppendorf vials in the dark. The specific activity of the photoaffinity label was ~2000 Ci mMol^{-1}.

Photoaffinity labeling was performed under red light conditions in which 1.5 mL of suspension cells on an orbital shaker were incubated with ~5 × 10^5 cpm of ^{125}I—azido-Cys-3,Ala-15–systemin in the presence or absence of unlabeled systemin. After 7 min, 1 mL of cells was filtered on three layers of 2.5-cm Whatman No.1 filter paper by vacuum filtration and washed with 5 mL of culture media maintained at 4°C. The filters containing the cells were removed and irradiated for 10 min with UV-B lamps (model F15T8.UV-B, 15 W; UVP Inc., Upland, CA) at a distance of 2 cm. After UV-B activation of the cross-linker, the cells were added to the sample buffer and frozen in liquid nitrogen.

The samples were boiled for 10 min and centrifuged at 12,000*g* for 5 min, and the supernatant was removed. The extracted proteins were separated by discontinuous SDS-PAGE (10% SDS–polyacrylamide gel). After electrophoresis, the gels were dried and analyzed using a PhosphorImager (molecular imager with Molecular Analyst software; Bio-Rad) after 50 hr of exposure.

ACKNOWLEDGMENTS

We thank Dr. Andreas Schaller for providing the suspension-cultured cell line and Amanda Vaughn for maintaining the cells. We also thank Dr. Gerhart Munske for peptide synthesis and helpful discussions, Dr. Jerry Reeves and David de' Avila for the generous use of laboratory space and equipment and for helpful advice, and Greg Pearce for advice. This research was supported in part by Washington State University College of Agriculture and Home Economics, Project No. 1791, and by Grant No. IBN 9601099 from the National Science Foundation (to C.A.R.) and a Graduate Research Fellowship from the Department of Energy/National Science Foundation/U.S. Department of Agriculture Plant Biochemistry Research and Training Grant (to J.M.S.). We thank Sue Vogtman and Thom Koehler for growing and maintaining the plants.

Received April 9, 1999; accepted May 10, 1999.

REFERENCES

Adamson, E.D., and Rees, A.R. (1981). Epidermal growth factor receptors. Mol. Cell. Biochem. **34,** 129–152.

Bergey, D., and Ryan, C.A. (1999). Wound- and systemin-inducible calmodulin gene expression in tomato leaves. Plant Mol. Biol., in press.

Bergey, D., Howe, G., and Ryan, C.A. (1996). Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. Proc. Natl. Acad. Sci. USA **93,** 12053–12058.

Bergey, D.R., Orozco-Cardenas, M.L., de Moura, D.S., and Ryan, C.A. (1999). A wound- and systemin-inducible polygalacturonase in tomato leaves. Proc. Natl. Acad. Sci. USA **96,** 1756–1760.

Cadena, D.L., Chan, C., and Gill, G.N. (1994). The intracellular tyrosine kinase domain of the epidermal growth factor receptor undergoes a conformational change upon autophosphorylation. J. Biol. Chem. **269,** 260–265.

Conconi, A., Miquel, M., and Ryan, C.A. (1996). Changes in the intracellular lipid composition and free fatty acids of tomato leaves in response to wounding. Plant Physiol. **111,** 797–803.

Cosio, E.G., Popperl, H., Schmidt, W.E., and Ebel, J. (1988). High-affinity binding of fungal β-glucan fragments to soybean (*Glycine max* L.) microsomal fractions and protoplasts. Eur. J. Biochem. **175,** 309–315.

Farmer, E.E., and Ryan, C.A. (1992). Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell **4,** 129–134.

Felix, G., and Boller, T. (1995). Systemin induces rapid ion fluxes and ethylene biosynthesis in *Lycopersicon peruvianum* cells. Plant J. **7,** 381–389.

Hahn, M.G. (1996). Microbial elicitors and their receptors in plants. Annu. Rev. Phytopathol. **34,** 387–412.

Heitz, T., Bergey, D., and Ryan, C.A. (1997). A gene encoding a chloroplast lipoxygenase is transiently induced by wounding, systemin, and methyl jasmonate in tomato leaves. Plant Physiol. **114,** 1805–1809.

Jacinto, T., McGurl, B., Franceschi, V., Delano-Freier, J., and Ryan, C.A. (1997). Tomato prosystemin confers wound-inducible, vascular bundle–specific expression of the β-glucuronidase gene in transgenic tomato and tobacco plants. Planta **203,** 406–411.

Koornneef, M., Hanhart, C.J., and Martinelli, L. (1987). A genetic analysis of cell culture traits in tomato. Theor. Appl. Genet. **74,** 633–641.

Laudert, D., and Weiler, E.W. (1998). Allene oxide synthase: A major control point in *Arabidopsis thaliana* octadecanoid signaling. Plant J. **15,** 675–684.

Lee, L., Suh, S., Kim, S., Crain, R.C., Dwak, J.M., Nam, G.G., and Lee, Y. (1997). Systemic elevation of phosphatidic acid and lysophospholipid levels in wounded plants. Plant J. **12,** 547–556.

McGurl, B., Pearce, G., Orozco-Cardenas, M., and Ryan, C.A. (1992). Structure, expression, and antisense inhibition of the systemin precursor gene. Science **255,** 1570–1573.

McGurl, B., Orozco-Cardenas, M., Pearce, G., and Ryan, C.A. (1994). Overexpression of a CaMV-prosystemin gene in transgenic tomato plants generates a systemic signal that induces proteinase inhibitor synthesis. Proc. Natl. Acad. Sci. USA **91,** 9799–9802.

Meindl, T., Boller, T., and Felix, G. (1998). The plant wound hormone systemin binds with the N-terminal part to its receptor but needs the C-terminal part to activate it. Plant Cell **10,** 1561–1570.

Moyen, C., Hammond-Kosack, K.E., Jones, J., Knight, M.R., and Johannes, E. (1998). Systemin triggers an increase of cytoplasmic calcium in tomato mesophyll cells: Ca^{2+} mobilization from intra- and extracellular compartments. Plant Cell Environ. **21,** 1101–1112.

Nurnberger, T., Nennstiel, D., Jabs, T., Sacks, W.R., Hahlbrock, K., and Scheel, D. (1994). High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. Cell **78,** 449–460.

O'Donnell, P.J., Calvert, C.M., Atzorn, R., Wasternack, C., Leyser, H.M.O., and Bowles, D.J. (1996). Ethylene as a signal mediating the wound response of tomato plants. Science **274,** 1914–1917.

Pearce, G., Strydom, D., Johnson, S., and Ryan, C.A. (1991). A polypeptide from tomato leaves activates the expression of proteinase inhibitor genes. Science **253,** 895–898.

Pearce, G., Johnson, S., and Ryan, C.A. (1993). Structure–activity of deleted and substituted systemin, an eighteen–amino acid polypeptide inducer of plant defensive genes. J. Biol. Chem. **268,** 212–216.

Royo, J., Vancanney, G., Perez, A.G., Sanz, C., Stormann, K., Rosahl, S., and Sanchez-Serrano, J.J. (1997). Characterization of three potato lipoxygenases with distinct enzymatic activities and different organ-specific and wound-regulated expression patterns. J. Biol. Chem. **271,** 21012–21019.

Ryan, C.A. (1967). The quantitative determination of soluble cellular proteins by radial diffusion in agar gels containing antibodies. Anal. Biochem. **19,** 434–440.

Ryan, C.A., and Pearce, G. (1998). Systemin, a polypeptide signal for plant defensive genes. Annu. Rev. Cell. Dev. Biol. **14,** 1–17.

Schaller, A., and Oecking, C. (1999). Modulation of plasma membrane H^+-ATPase activity differentially activates wound and pathogen defense responses in tomato plants. Plant Cell **11,** 263–272.

Schaller, A., and Ryan, C.A. (1994). Identification of a 50-kDa systemin-binding protein in tomato plasma membranes having Kex2p-like properties. Proc. Natl. Acad. Sci. USA **91,** 11802–11806.

Schaller, A., and Ryan, C.A. (1995). Systemin—A polypeptide signal in plants. Bioessays **18,** 27–33.

Stratmann, J.W., and Ryan, C.A. (1997). Myelin basic protein kinase activity in tomato leaves is induced systemically by wounding and increases in response to systemin and oligosaccharide elicitors. Proc. Natl. Acad. Sci. USA **94,** 11085–11089.

Trautman, R., Cowan, K.M., and Wagner, G.G. (1971). Data for processing for radial immunodiffusion. Immunochemistry **8,** 901–916.