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Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway

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ABSTRACT **Tomato plants overexpressing a prosystemin gene that encodes the precursor of a mobile wound signal called systemin have been shown previously to constitutively synthesize extraordinarily high levels of two defensive proteinase inhibitor proteins in leaves in the absence of wounding. We herein report that leaves of these transgenic plants possess enhanced levels of another defensive protein, polyphenol oxidase (PPO) at levels that are up to 70-fold higher than levels found in leaves of wild-type plants. Supplying young wild-type tomato plants with systemin through cut stems induced PPO activity in leaves, and wounding lower leaves of young tomato plants induced PPO activity in both wounded and unwounded leaves to levels equal to those induced by systemin. Exposing young tomato plants to methyl jasmonate vapor caused an increase in PPO activity equivalent to levels found in plants overexpressing the prosystemin gene. The data indicate that PPO and proteinase inhibitor genes are coactivated systemically by wounding via the octadecanoid signal transduction pathway and that systemin has a much broader role in signaling plant defensive genes than was previously known.**

Members of several plant families respond to herbivory and wounding by synthesizing proteinase inhibitor proteins that interfere with the digestive processes of insects (1). In tomato leaves, proteinase inhibitor I and II proteins are synthesized in response to injury by chewing insects, leading to their rapid accumulation in both wounded and unwounded leaves within a few hours of the initial damage (1, 2). The leading candidate for the mobile wound signal is an 18-amino acid polypeptide called systemin that is transported through the phloem (3) and is an active inducer of proteinase inhibitor synthesis when supplied to young tomato plants at femtomolar concentrations (4). Systemin is cleaved by proteolysis from a precursor protein of 200 amino acids called prosystemin either before or during injury. The gene encoding prosystemin has been isolated and characterized, and tomato plants that overexpress a prosystemin transgene in an antisense orientation have a reduced ability to systemically accumulate proteinase inhibitor proteins in response to herbivory (5). In contrast, tomato plants overexpressing the prosystemin transgene in the correct orientation express two proteinase inhibitor genes constitutively that are normally expressed in leaves only in response to wounding. These plants contain the proteinase inhibitors at >1 mg/ml in their leaf juice (6).

We now report that polyphenol oxidase (PPO), also known as catechol oxidase, is produced at high levels in leaves of tomato plants overexpressing the prosystemin transgene. We also report that PPO activity increases systemically in leaves of wild-type plants in response to wounding and is induced in tomato plants supplied with systemin or methyl jasmonate (MeJa), components of the wound-inducible octadecanoid-based signal transduction pathway. PPO is an inducible enzyme that oxidizes a wide range of plant phenolics (7–10). It is found throughout the plant kingdom and has been considered to have a possible defensive role against pathogens (11–13) and herbivores (14–17). The data presented here reveal that systemin has a much broader role in defense signaling than heretofore realized and that it likely acts as a systemic signal that activates a spectrum of inducible defense-related proteins in plants in response to pest and pathogen attacks.

MATERIALS AND METHODS

Plant Materials. Tomato plants (*Lycopersicon esculentum*, var. Better Boy) expressing a gene consisting of a prosystemin cDNA under control of the cauliflower mosaic virus 35S promoter were as described (6). The transgenic and untransformed control plants were grown in peat pots and maintained in environmental chambers under 17 h of light (300 $mE \cdot m^{-2} \cdot s^{-1}$) at 28°C and 7 h of dark at 18°C. Wild-type tomato plants (var. Castlemart) used for PPO induction experiments were similarly grown.

Wounding of Tomato Plants and Treatment with Systemin and MeJa. Two-week-old tomato plants (two-leaf stage) were used for all experiments. Plants were wounded by crushing the leaves across the main vein with a hemostat. All leaflets of each composite leaf were wounded once at the beginning of the experiment and then wounded again 3 h later on the same leaflet, basipetal to the original wound. Systemin (2.5 pmol) in 90 μl of 15 mM sodium phosphate (pH 6.5) was supplied to excised tomato plants through the cut stems over a 30-min period. The plants were transferred to water and incubated in sealed Plexiglas boxes as described (18). Plants were continuously exposed to MeJa vapor [100 nl as a 10% (wt/vol) solution in ethanol placed on a cotton wick] in sealed glass jars as described (19) and maintained in environmental chambers under constant light until assayed.

PPO Assays. Leaf tissue was ground in 5 vol of 100 mM sodium phosphate (pH 7.0) at 4°C and the extracts were clarified by centrifugation in a Microfuge. PPO activity was assayed spectrophotometrically as described (20) using chlorogenic acid (Sigma) as the substrate. The final assay mixture contained 2 mM chlorogenic acid, 50 mM 2-nitro-5-thiobenzoic acid, and 0.5–20 mg of total protein in 1 ml of 100 mM sodium phosphate/100 mM sodium citrate, pH 6.0. One unit of activity was defined as the amount of enzyme that converts 1 mmol of chlorogenic acid to 1 mmol of chlorogenoquinone per min at 20°C under the assay conditions. Protein concentrations were measured by the method of Bradford (21) using bovine serum albumin as the standard.

Gel Electrophoresis. Leaf issue was frozen in liquid nitrogen, ground to a fine powder with mortar and pestle, and

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Abbreviations: PPO, polyphenol oxidase; MeJa, methyl jasmonate.
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phenol-extracted essentially as described in Hurkman and Tanaka (22). Briefly, frozen leaf powder was thawed in 2 vol of ice-cold extraction buffer consisting of 0.7 M sucrose, 100 mM Tris·HCl (pH 6.8), 20 mM EDTA, 100 mM KCl, 2% (vol/vol) 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. The slurry was transferred to a polypropylene tube containing 1 vol of water-saturated phenol, vortex mixed, and centrifuged at 10,000 × *g* at room temperature. The protein-containing phenol phase was removed and the aqueous phase was extracted again with 1 vol of phenol. The phenol phases were pooled and washed with 1 vol of extraction buffer. Protein was precipitated overnight with 5 vol of 100 mM ammonium acetate in methanol at −20°C and pelleted by centrifugation at 10,000 × *g* for 15 min. Pellets were washed three times in 80% acetone before resuspending in two-dimensional lysis buffer consisting of 9.5 M urea, 2% (vol/vol) Nonidet detergent, 5% 2-mercaptoethanol, and 2% (vol/vol) ampholytes [pH 3–10 (Pharmacia)]. Protein concentrations were determined by the Bradford assay using bovine serum albumin as a standard. Isoelectric focusing and SDS/PAGE were carried out by the method of O'Farrell (23). Gels were stained with 0.1% Coomassie brilliant blue R-250 or were fixed for 30 min in 40% (vol/vol) methanol/10% (vol/vol) glacial acetic acid and silver-stained (24).

Purification and Sequencing of PPO. Leaf tissue (120 g) from transgenic tomato plants overexpressing the prosystemin transgene was frozen in liquid nitrogen and ground in a Waring blender with 3 vol of ice-cold buffer consisting of 20 mM Tris·HCl (pH 7.2), 10% (wt/vol) sucrose, 2 mM EDTA, 2 mM 2-mercaptoethanol, 2% (wt/vol) polyvinylpyrrolidone, and 100 mM phenylmethylsulfonyl fluoride. Homogenized tissue was filtered through four layers of cheesecloth, transferred to polypropylene tubes, and clarified by centrifuging at 10,000 × *g* for 20 min at 4°C. The pellet was resuspended in DEAE buffer consisting of 20 mM Tris·HCl (pH 7.5), 1 mM EDTA, 2 mM 2-mercaptoethanol, and 20 mM phenylmethylsulfonyl fluoride before adding ammonium sulfate to 55% saturation. After stirring for 1 h at 4°C, protein was pelleted by centrifugation at 10,000 × *g* for 15 min. The pellet was solubilized in DEAE buffer and dialyzed overnight at 4°C against 4 liters of the same buffer. After dialysis the protein concentration was determined and 300 mg of protein in 65 ml was loaded onto a 40 × 2.5 cm DEAE column equilibrated in DEAE buffer. Proteins were eluted using a linear NaCl gradient (0–700 mM) at 2 ml/min, and 4-ml fractions were collected. Eluted fractions were analyzed by SDS/PAGE, and those enriched with the 66-kDa protein were pooled and dialyzed against water. After protein quantitation, the pooled fractions were divided and lyophilized. SDS/PAGE was performed (25) except that gels were preelectrophoresed with 5 mM glutathione (Sigma) in the upper (anode) buffer chamber. After loading protein samples onto gels, fresh buffer containing 10 mM thioglycolate (Sigma) was added to the upper chamber. After electrophoresis, proteins were electroblotted onto poly(vinylidene difluoride) membranes (Millipore), briefly stained with Coomassie brilliant blue R-250, and destained. The portion of the filter containing the clearly distinguishable 66-kDa protein band was excised and subjected to amino acid sequencing with a model 475-A Applied Biosystems sequencer.

RESULTS

Transgenic tomato plants constitutively expressing a prosystemin transgene have been shown previously to constitutively accumulate high levels of proteinase inhibitors I and II in leaves (6) in contrast to wild-type plants that accumulate the inhibitors in leaves only in response to wounding. A characteristic of these transgenic plants is a marked browning of leaf homogenates that was much more pronounced than that found in homogenates from wild-type (untransformed) plants. Transgenic and wild-type plants were analyzed for both peroxidase and PPO activities, which are known to cause oxidative browning of plant tissues and extracts (26, 27). Extracts of leaves from transgenic and wild-type plants contained similar levels of peroxidase activity, but extracts from leaves of transgenic plants contained substantially more PPO activity than those of wild-type plants (Table 1). Kojic acid, a specific inhibitor of PPO (28), inhibited both the browning of the extracts and the PPO activity (data not shown). Leaves of transgenic plants showed high levels of PPO activity independently of leaf age, whereas in the control plants higher PPO activity was associated with the youngest leaves (Table 1).

Table 1. PPO activity in leaves of wild-type tomato plants and plants constitutively expressing a prosystemin transgene

Leaf position	PPO activity, units/mg of protein: Wild type plant	Transgenic plant
1	11.2	92.8
2	3.6	70.7
3	1.3	62.0
4	1.0	73.2
5	1.4	72.6
6	2.6	78.3

Leaves are numbered basipetally from the first expanding leaf just below the small apical leaf. The soluble protein extracts of leaves from 3-week-old plants were individually assayed for PPO activity.

Two-dimensional gel analysis revealed the presence of unusually large amounts of a 66-kDa protein in the transgenic plants compared to wild-type plants (Fig. 1). This 66-kDa protein consisted of at least three isoforms of apparently identical molecular mass, with slightly different isoelectric points (mean isoelectric point, 5.5). Both the molecular mass and the isoelectric point of this highly expressed protein were very similar to previously reported values for tomato PPO (14, 29). Purification and sequence analysis of the 66-kDa protein revealed that the N-terminal sequence PIPPPDLKSCGVVA is identical to the N-terminal sequence deduced from the tomato PPO-F gene sequence, one of seven known PPO genes identified in the tomato genome (29).

To confirm that the elevated level of PPO activity in the transgenic plants was indeed due to the inductive effects of systemin and not to secondary effects of prosystemin overexpression, the ability of synthetic systemin to induce PPO activity in leaves of young wild-type tomato plants was as-

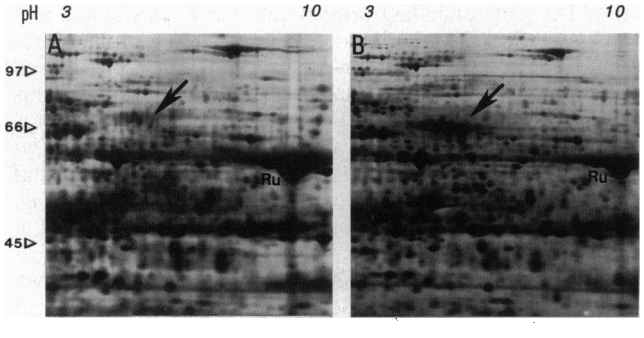

FIG. 1. Two-dimensional gel electrophoresis of leaf proteins from transgenic and wild-type tomato plants. (*A*) Proteins from leaves of wild-type untransformed plants. (*B*) Proteins from leaves of plants constitutively expressing a prosystemin transgene. Arrows point to a family of polypeptides specifically enhanced in the transgenic plants. Ru, ribulose-bisphosphate carboxylase. Numbers at the top indicate the pH gradient for the first dimension isoelectric focusing. Molecular mass markers in kDa are at the left.

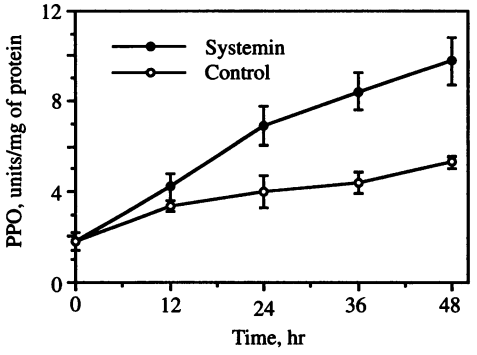

FIG. 2. Induction of PPO activity in tomato leaves by systemin. Excised 2-week-old tomato plants, having two expanding leaves and a small apical leaf, were supplied with 2.5 pmol of systemin or buffer through their cut stems over a 30-min period. The plants were then incubated for various time periods and leaf extracts were assayed for PPO activity. Each point represents the mean of results from five plants; bars represent the SEM.

sessed. Excised 2-week-old tomato plants were supplied with 2.5 pmol of systemin through the cut stem placed in water, and after various time periods, the leaves were assayed for their content of PPO activity. Systemin supplied to tomato plants by this method had been shown (4, 18) to induce the synthesis of proteinase inhibitors I and II in leaves. Fig. 2 shows that during a 48-h time course initiated by supplying young tomato plants with systemin, PPO activity increased 5-fold in the leaves. In control plants that had been supplied only buffer, PPO activity increased 2-fold. This increase in the controls was likely due to the inductive effects of excising the plants, i.e., wounding (see below).

The wound inducibility of PPO was examined by wounding the lowest leaf of wild-type tomato plants at the same leaf stage. Over the next 48 h, both the wounded leaf and the upper unwounded leaf were analyzed for their levels of PPO activity. An increase in PPO activity was detected in both the wounded leaves and the upper unwounded leaves as early as 12 h after wounding and it continued to increase during the next 36 h of incubation (Fig. 3). At the termination of the experiment (48 h), the systemically induced leaf had undergone a 5-fold increase in PPO with final levels comparable to the plants that had been excised and supplied with systemin (Fig. 2). The data clearly demonstrate that PPO is inducible by systemin and is locally and systemically inducible by wounding.

MeJa, a potent inducer of the proteinase inhibitors in tomato (19) and a component of the wound-inducible octadecanoid signal transduction pathway of tomato (30), was tested for its ability to induce PPO activity in tomato leaves.

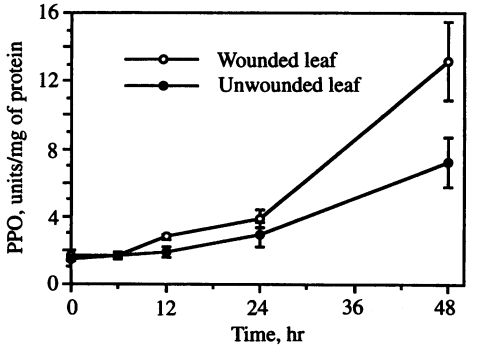

FIG. 3. Induction of PPO activity in tomato leaves after wounding. Two-week-old tomato plants were wounded twice on the lower terminal leaflet (3-h interval) and incubated in light for various time periods. Each point is the mean of results from wounded leaves or from the upper unwounded leaves of five plants; bars represent the SEM.

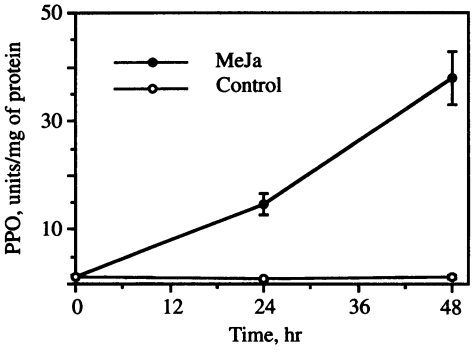

FIG. 4. Induction of PPO activity in tomato leaves by MeJa. Two-week-old tomato plants were exposed to vapors from 100 nl of MeJa (1:10 dilution in ethanol) placed on a cotton wick in sealed glass jars for 24 h or 48 h. Each point represents the mean of results of leaf extracts derived from five plants; bars indicate the SEM.

Wild-type tomato plants exposed to MeJa vapor exhibited a rapid and strong induction of PPO activity (Fig. 4). MeJa induced PPO activity to levels nearly 3 times higher than levels induced by wounding or systemin (Figs. 2 and 3), and the activity approached that found in the transgenic plants overexpressing the prosystemin transgene (Fig. 1). The high induction of PPO by MeJa is reminiscent of the strong induction of proteinase inhibitor mRNAs and proteins by MeJa that was observed in tomato (19), potato (31), and alfalfa (32).

The observed increase in PPO activity after wounding, systemin, and MeJa treatments was also accompanied by changes in the abundance of PPO protein. Two-dimensional gel analyses of leaf proteins, after wounding, systemin, and

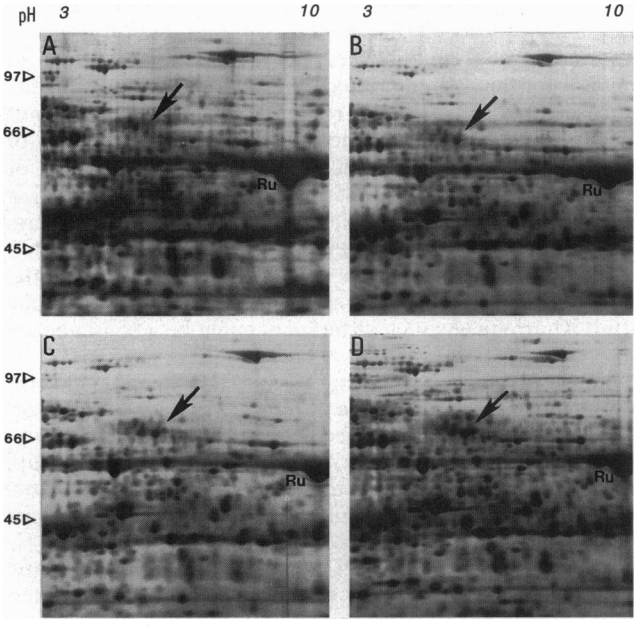

FIG. 5. Two-dimensional gel electrophoresis of tomato leaf proteins from excised young tomato plants. (*A*) Plants were supplied for 30 min with 100 μl of 0.01 M sodium phosphate (pH 7.0), through their cut stems and then incubated in constant light for 24 h. (*B*) Plants were supplied with 2.5 pmol of systemin in buffer and treated as in *A*. (*C*) Plants were wounded on their lower leaves and incubated in light for 24 hr before analyzing proteins from the upper unwounded leaves. (*D*) Plants were exposed to vapor from 100 nl of MeJa and light for 24 h. After incubation in light, the leaf proteins were extracted and subjected to isoelectric focusing and SDS/PAGE. Ru, ribulosebisphosphate carboxylase. Numbers at the top of the gels indicate the limits of the pH gradients for isoelectric focusing. The positions of molecular mass markers, in kDa, are shown at the left.

MeJa treatments of the wild-type plants, revealed that all three treatments led to an increase in the 66-kDa protein that corresponded with PPO (Fig. 5). Treatment with MeJa produced the most PPO protein. In all of the experiments, the most basic PPO isoform was induced most rapidly, suggesting that all of the isoforms do not accumulate similarly in response to the various treatments.

DISCUSSION

Evidence to date supports a role for the 18-amino acid polypeptide systemin as a mobile wound signal that induces the synthesis of proteinase inhibitors in tomato leaves (4, 6, 33) via the octadecanoid signaling pathway (30). Here we demonstrate that systemin induces the accumulation of an additional defense protein, PPO, whose activity, like the proteinase inhibitors, disrupts the normal digestive processes of herbivores. Similar to proteinase inhibitor genes, PPO was expressed at high levels in transgenic tomato plants constitutively overexpressing the prosystemin gene and was induced in wild-type plants by wounding or by supplying excised plants with systemin or MeJa. The absolute levels of PPO activity measured in the prosystemin-overexpressing plants were several times greater than could be induced by wounding or supplying excised plants with systemin, but comparable levels of activity were seen in plants treated with MeJa. Under all inducing conditions, increases in PPO activity were accompanied by increases in the 66-kDa (PPO) protein spots in the two-dimensional gel electrophoretic analyses (Figs. 1 and 5), suggesting that, like the proteinase inhibitors, the induction of PPO is likely regulated at the transcriptional level. Oligouronides and chitosan, known inducers of proteinase inhibitor synthesis in tomato leaves when supplied to tomato plants through their cut stems (34, 35), also caused an increase in PPO activity and in the 66-kDa protein region in two-dimensional gels (unpublished data), and salicylic acid, an inhibitor of the octadecanoid pathway (36), inhibited the increase in PPO activity in response to systemin (data not shown). The similarities in the characteristics of induction of proteinase inhibitors and PPO strongly suggest that both of these responses utilize the octadecanoid signal transduction pathway. The induction kinetics of PPO activity did not always correlate precisely with those of the proteinase inhibitors, so it is likely that some aspects of the individual steps of the induction process for PPO and proteinase inhibitors may differ. Alternatively, such differences might be a consequence of posttranslational events.

Seven PPO genes have been isolated from tomato, and five of the genes have considerable differences in their 5′ upstream regions (29). As judged by electrophoretic analysis of tomato leaf proteins, one PPO isoform, the most basic of the group, accumulated to higher levels than the others in tomato plants overexpressing the prosystemin gene (Fig. 1) and in plants induced by MeJa, systemin, or wounding (Fig. 5). The N-terminal sequence of the PPO purified from the transgenic tomato plants gave a perfect match with the deduced amino acid sequence of the PPO-F gene (29), but it is not yet clear whether it is this gene that is regulated by wounding, systemin, or MeJa.

PPO has long been known to be induced by wounding and by pathogens in many different plant species (11–13). In tomato, wounding leaves with carborundum or by insect feeding both caused increases in PPO activity (37, 38), and inoculation with the pathogen *Pseudomonas syringae* pv. *tomato* resulted in the systemic induction of PPO activity (37). Gentile *et al.* (39) also observed a systemic increase in tomato leaf PPO activity after treatment of roots with heat, chloroform, or a nonpathogenic strain of *Fusarium oxysporum*. Despite the well-documented pathogen and stress inducibility of PPO, there is no evidence for its direct involvement in pathogen defense (8, 10).

In contrast, Duffey and coworkers (16, 17) have provided strong evidence for a defensive role of PPO against herbivorous insects, specifically those with a slightly basic gut pH. PPO is compartmentalized in the chloroplast separate from its phenolic substrates (10), and therefore, only after disruption of the plant tissue by the insect can oxidation of plant phenolics by PPO begin. Under favorable conditions, the quinones produced by this reaction alkylate reactive lysine, histidine, and cysteine side chains of proteins (27), thereby reducing their nutritional quality. Felton *et al.* (16) found a strong correlation between PPO levels in tomato leaves and reduction in growth of the tomato fruit worm *Heliothis zea* feeding on this foliage. The reduced growth was also positively correlated with the amount of the plant phenolic compound, chlorogenic acid, that was bound to plant protein after passage through the insect gut, implying that PPO activity was responsible for the reduced growth. Interestingly, the amino acids most susceptible to attack by the quinones produced by PPO are those predicted to be most nutritionally limiting to herbivores (17). The proposal that PPO contributes to antiherbivore defense is consistent with our data showing that PPO in tomato plants is induced by the same wound signals as the proteinase inhibitors, proteins already known to be effective defenses against insects (40).

In summary, our analysis of transgenic tomato plants overexpressing the prosystemin gene has revealed that systemin not only regulates the systemic induction of proteinase inhibitors but also regulates PPO activity. PPO synthesis is further shown to be induced by wounding and MeJa, suggesting that the PPO gene(s) are regulated through the octadecanoid signaling pathway. Other genes in tomato and potato plants that are regulated by wounding and/or MeJa, including a cysteine proteinase inhibitor (41), the systemically wound-induced genes of potato that encode cysteine and aspartate proteinase inhibitors (42), a wound-inducible amino peptidase (43), and threonine deaminase (42), are also candidates for being systemin-regulated genes. We propose that in tomato plants, and likely in other plant species as well, systemin plays a major role in inducing an array of systemically inducible defensive genes in response to herbivore and pathogen attacks.

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