The primary structure-activity relationships of systemin, an 18-amino acid polypeptide from tomato leaves that regulates the expression of two wound-inducible proteinase inhibitor genes in tomato and potato plants, were investigated. Analogs of systemin, the only example of a polypeptide signal from plants, were synthesized with progressive deletions of amino acids from both the NH₂ terminus and COOH terminus and assayed in young excised tomato plants. All of the analogs exhibited severely decreased proteinase inhibitor-inducing activities, indicating that the entire 18-amino acid sequence is necessary for maximal activity. Deletion of the COOH-terminal Asp abolished inducing activity. Progressive replacement of each amino acid of the entire polypeptide with Ala revealed two regions, near residues Pro³⁸, where Ala substitution reduced activity to less than 0.2%, and Thr¹⁷, which totally inactivated the analog. Other replacements with Ala had little or only moderate effects on activity. The two inactive analogs, des-Asp¹⁸ systemin and Ala¹⁷ systemin, were potent inhibitors of the inducing activity of the native systemin. These analogs, therefore, contain structural conformations sufficient for competition with systemin, but they are not competent for proteinase inhibitor gene induction. A synthetic COOH-terminal tetrapeptide, Met–Gln–Thr–Asp, retained low proteinase inhibitor inducing activity, but virtually any replacements with other amino acids either eliminated activity or reduced the activity to very low or nearly undetectable levels. These results indicate that residues near the COOH terminus of systemin are necessary for activity, possibly involving a phosphorylation at Thr¹⁷, and that other regions of the systemin sequence are important for interacting with a receptor(s) but are not sufficient to activate proteinase inhibitor gene expression.

An 18-amino acid polypeptide, called systemin, was recently isolated from tomato leaves that is the most powerful inducer of proteinase inhibitor genes reported from plants and is the only plant polypeptide hormone-like signaling molecule presently known (1, 2). Systemin, when supplied to young tomato or potato plants through cut stems, activates in leaves the expression of genes coding for two well characterized wound-inducible proteinase inhibitor proteins, called Inhibitor I (Mr 8100) and Inhibitor II (Mr 12,300) (3-6). These genes are systemically induced in tomato and potato leaves in response to attacking insects and pathogens (7, 8) and are considered to be part of the inducible defensive chemicals of the plants. ¹⁴C-Labeled systemin placed in wounds on tomato leaves was shown to be rapidly transported throughout plants (1) and is therefore a primary candidate for being a systemic signal, released in response to pest or pathogen attacks.

Systemin is proteolytically processed from an internal sequence of a 200-amino acid precursor called prosystemin (2). The cDNA and gene coding for prosystemin have been isolated and characterized (2). An antisense construct driven by the CaMV promoter was stably integrated into the genome of tobacco and expressed, where it severely inhibited the systemic wound induction of the two proteinase inhibitors (2). This directly demonstrated that the synthesis of prosystemin is necessary for the systemic wound induction to occur in tomato plants.

We have now investigated the amino acid residues in the systemin structure that are necessary for induction of proteinase inhibitor genes. We have found that all deletions within the 18-amino acid structure of systemin severely decrease or eliminate its inducing activity. Substitutions of individual residues in systemin with other amino acid residues have revealed regions that can be modified without loss of activity and other regions that severely decrease or eliminate inducing activity. Two totally inactive systemin analogs, substituted or deleted in the penultimate and terminal amino acids at the COOH terminus, are powerful inhibitors of the proteinase inhibitor-inducing activity of native systemin.

MATERIALS AND METHODS
Systemin and systemin analogs were synthesized using Fmoc¹ chemistry by solid phase techniques with an Applied Biosystems Inc. model 431A synthesizer on p-methylbenzhydrylamine resin, using the manufacturer's protocol. Amino-terminal modifications were performed manually as follows. 0.1 mM of each Fmoc amino acid (Fmoc amino acids were purchased from Bachem Bioscience Inc., Switzerland) was dissolved in 250 µl of N-methylpyrrolidone (NMP) in an Eppendorf tube. To this was added 100 µl of 1 M N,N-dicyclohexylcarbodimide/NMP and 100 µl of 1-hydroxybenzotriazole/NMP. All reagents were purchased from Applied Biosystems Inc. After incubating for 2 h with stirring at room temperature, the samples were centrifuged at 5 min and 10 mg of p-methylbenzhydrylamine resin containing a synthesized systemin fragment from residues 2 to 18 was added to the supernatant. After stirring for 2.5 h, the resin was washed with NMP, twice with MeOH:methylene chloride (1:1), twice with methylene chloride. The solvents were removed from the resin, which flocculated to the top of the tube. The resin was lyophilized and dispersed with stirring in 1 ml of 20% ¹ The abbreviations used are: Fmoc, N-(9-fluorenylmethoxy carbonyl); NMP, N-methylpyrrolidone; HPLC, high performance liquid chromatography.

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RESULTS AND DISCUSSION

Systemin had been shown previously to be a powerful inducer of the expression of genes coding for proteinase Inhibitor I and Inhibitor II in leaves of young excised tomato plants, when supplied to the plants through their cut stems (1). The maximum levels of accumulation of Inhibitor I protein in the leaves of plants supplied with systemin in the femtomole to picomole range was about two times that of Inhibitor II protein (1). In Fig. 2 is shown the accumulation of Inhibitor II protein in leaves of young tomato plants in response to increasing concentrations of systemin. The induction of Inhibitor II in leaves of excised tomato plants has therefore provided a convenient and quantitative assay to examine the structure-activity relationships of several synthetic derivatives of systemin in order to identify individual amino acids, or sequences of amino acids, that may by important to the biological activity of the native molecule.

Effects of Deletions and Substitutions in the NH2- and COOH-terminal Regions on the Biological Activity of Systemin—Polypeptides were synthesized that represented stepwise deletions of systemin from both the COOH terminus and the NH2 terminus. The Inhibitor I-inducing activity of each polypeptide was determined in the detached tomato plant bioassay. The quantity of each polypeptide that induces 50% maximal Inhibitor I accumulation was determined. In Table I are shown the results of the comparisons of the activities of all of the deleted polypeptides with that of systemin. These results indicate that none of the deletions exhibit the full activity of systemin. Deletions of the NH2-terminal alanine reduces the activity about 300-fold, and progressive deletions cause increasing losses in activity. However, the COOH-terminal tetrapeptide, Met-Gln-Thr-Asp, still retains some activity. This activity, while about 3 x 10⁻⁶ less than that of systemin, is approximately the same as that found with oligouronides, which are considered localized signals for defense in plants against predators and pathogens (12, 13). Thus, although the entire 18-amino acid polypeptide is necessary for full inducing activity, the NH2-terminal 14 amino acids can be eliminated while maintaining some activity. Deletion of the Met or the Asp from the tetrapeptide completely eliminated activity. Deletion of the Asp from the COOH terminus of native systemin completely eliminated inducing activity.

![Fig. 1. Elution profiles of systemin and selected synthetic polypeptide fragments of systemin from HPLC.](image)

![Fig. 2. Induction of inhibitor I synthesis in leaves of young tomato plants in response to systemin.](image)
activity from the remaining 17-amino acid polypeptide. The cumulative evidence indicates that the tetrapeptide Met-Gln-Thr-Asp is sufficient for biological activity but not for the full activity found in native systemin. A recent analysis of the solution structure of systemin by proton NMR (14) did not reveal any persistent common secondary or tertiary structural elements in the polypeptide, but two weak distinct structural features were detected at the carboxyl terminus that may be due to internal hydrogen bonding. Whether these conformations are related to the activity found associated with this region of systemin remains to be established.

To further investigate the role of alanine as the NH₂-terminal amino acid in systemin, analogs of systemin were synthesized that contained several different single amino acid substitutions at the NH₂-terminal amino acid. Substitutions of Ala with D-Ala or Ser had little effect on the inducing activity of the modified systemins, whereas substitutions with β-Ala, acetyl-L-Ala, Tyr, Leu, Asp, and Gly severely reduced the activities below that of native systemin. The activities of these latter derivatives were similar to systemin in which Ala has been deleted (Tables I and II). The effects of the different substitutions on activity could be due to conformational constraints necessary for the interaction of systemin with a receptor. The data can be interpreted to mean that the free NH₂-terminal α-amino positive charge, i.e. β-Ala or acetyl-L-Ala, or that introduce large bulky R groups, i.e. Tyr, Leu, or Asp, or no R group, i.e. Gly, severely reduce the activity. On the other hand, the activities of the analogs may reflect the half-lives of the different polypeptides when supplied to the plants during assay. The pattern of activities generally fits the pattern expected by the N-end rule of protein degradation (15), with NH₂-terminal Ala and Ser being about equal in activity but more active than the other substitutions. In plants, however, the degradation of polypeptides has not been studied in detail.

The smallest fragment of systemin that retained some inducing activity was the Met-Gln-Thr-Asp at the COOH terminus (Table I). Several tetrapeptide analogs of this motif were synthesized and assayed for inducing activities. In Table III is summarized the analogs and their activities when supplied to plants at the level at which the wild type polypeptide

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### Table I

**Structure and Activity of Systemin Analogs**

| Systemin fragments | fmol/plant (50% maximal activity)* |
|--------------------|-------------------------------------|
| AVQSKPSKRDPKMQTD  | 12                                  |
| VQSKPSKRDPKMQTD    | 3,600                               |
| QVSKPSKRDPKMQTD    | 4,000                               |
| AVQSKPSKRDPKMQTD   | 76,000                              |
| AVQSKPSKRDPKMQTD   | 231,000                             |
| AVQSKPSKRDPKMQTD   | 273,000                             |
| AVQSKPSKRDPKMQTD   | 10,900,000                          |
| AVQSKPSKRDPKMQTD   | 4,000,000                           |
| AVQSKPSKRDPKMQTD   | Inactive                            |
| AVQSKPSKRDPKMQTD   | Inactive                            |
| AVQSKPSKRDPKMQTD   | Inactive                            |
| AVQSKPSKRDPKMQTD   | Inactive                            |

*Indicates were determined in bioassays of proteinase Inhibitor I induction in young excised tomato plants. Values represent the increase Eighteen plants were assayed per Standard deviation. Supplied to young excised tomato plants at

### Table II

**Induction of proteinase Inhibitor I in leaves of young tomato plants by systemin, substituted at its N-terminal amino acid**

| N-terminal amino acid | Inhibitor I induced/tissue (μg/tissue) |
|-----------------------|----------------------------------------|
| L-Ala (wild type)     | 112                                    |
| D-Ala                 | 95                                     |
| Ser                   | 100                                    |
| Acetyl-L-Ala          | 75                                     |
| β-Ala                 | 63                                     |
| Gly                   | 79                                     |
| Tyr                   | 69                                     |
| Leu                   | 83                                     |
| Asp                   | 75                                     |
| NQTD                  | 0                                      |
| MQTD                  | 157                                    |
| MQT                   | 0                                      |
| MQTN                  | 21                                     |
| MQTE                  | 26                                     |
| MQSD                  | 23                                     |
| MFTD                  | 8                                      |
| MNMT                  | 0                                      |
| LQTD                  | 31                                     |
| TQTD                  | 30                                     |
| QQT                  | 23                                     |
| DQTD                  | 0                                      |
| NQTD                  | 8                                      |
| YQTD                  | 18                                     |

*Assayed by immunodiffusion analyses; approximately ±20% standard error. Average of 18 plants/treatment.

### Table III

**Induction of proteinase Inhibitor I by the C-terminal tetrapeptide of systemin and its analogs**

| Peptide* | Inhibitor I induced* (μg/g of tissue) |
|----------|---------------------------------------|
| MQTD     | 157                                    |
| MQT      | 0                                      |
| MQT      | 157                                    |
| MQTN     | 21                                     |
| MQTE     | 26                                     |
| MQSD     | 23                                     |
| MFTD     | 8                                      |
| MNMT     | 0                                      |
| LQTD     | 31                                     |
| TQTD     | 30                                     |
| QQT      | 23                                     |
| DQTD     | 0                                      |
| NQTD     | 8                                      |
| YQTD     | 18                                     |

*Supplied to young excised tomato plants at 100 nmol/plant. Eighteen plants were assayed per peptide. Standard deviation approximately ±20%.

*Values are the increase over controls of 45 ± 8 μg/g of tissue.
produces maximal activity. Deletion of the COOH-terminal Asp completely abolished activity, similar to the COOH-terminal deletion in the native systemin. All substitutions severely reduced the activity of the tetrapeptide, but substitutions of Asp for Met completely abolished activity. Interestingly, several substitutions for Met other than Asp did not completely eliminate activity.

Effects of Scanning the Systemin Sequence with Alanine Substitutions on Biological Activity—The powerful proteinase inhibitor-inducing activity of systemin suggested that the polypeptide may initially interact with a receptor on or in tomato leaf cells. Such an interaction would likely involve conformational changes that would result in the positioning of systemin at the receptor site. In seeking such a receptor through the use of affinity chromatography or photoaffinity labels derived from systemin, identifying regions of systemin that can be modified without affecting biological activity were of greatest interest. In order to determine whether the modification of individual amino acids within systemin would affect inducing activity, analogs of systemin were synthesized that were singly substituted with Ala at each of the positions of the polypeptide, and the biological activities of each were determined. The biological activity of the native systemin with Ala as its NH₂ terminus was used as the standard for comparing the activities of the seventeen analogs containing Ala at each position in the sequence. In Fig. 3 is shown these comparisons, reported as the ratio of the activity (femtomoles required to produce 50% maximal activity) of the wild type (NH₂-terminal alanine) over the activities of the various analogs. Substitution of Ala for residues at positions 2–6, 8, 9, 10, 14, and 15 produced only modest changes in activity, indicating that these residues appear to be relatively unimportant to the activity of systemin. Ala substitutions at positions 7, 11, 12, 16, and 18 produced moderate changes in activity; substitutions at residue 13 (Pro) caused a major reduction in activity; and Ala at position 17 (Thr) totally eliminated biological activity. Thus, the activities of the Ala-substituted analogs indicate that the amino acid sequences of at least two regions of systemin are vitally important for biological activity, i.e. residues 11–13 and 16–18.

Substitution of the penultimate Thr in systemin with Ala totally abolished activity. The substitution of Thr with Ser in the tetrapeptide Met-Gln-Thr-Asp severely reduced activity, inviting speculation that Thr (or Ser) is phosphorylated as part of the signaling process and that any other substitution at this residue totally inactivates the signaling pathway, although no direct evidence for a phosphorylated form of systemin has yet been detected.

Assays of the proteinase inhibitor activity of systemin analogs Ala¹³ and Ala¹⁷ are shown in Fig. 4, compared with activity of wild type systemin. Ala¹³ activity requires about 3 orders of magnitudes more polypeptide to achieve 50% maximal activity as the native systemin, whereas Ala¹⁷ is totally inactive, even at nanomole levels. Since the possibility had been proposed that the Thr at position 17 might be the substrate for a kinase as part of the signaling process, it was considered that the two analogs of systemin that were totally inactive, i.e. the Ala¹³ and the deleted COOH-terminal aspartic acid derivative (cf. Table I), might still bind to a receptor and might compete with the activity of native systemin. Fig. 5 shows that both inactive analogs are potent inhibitors of the proteinase inhibitor inducing activity of wild type systemin. The Ala¹³ analog (SYS-MQAD) inhibits wild type systemin much more potently than the analog with the deleted COOH-terminal Asp, which is itself a strong inhibitor. This indicates that although the modifications in the COOH-terminal sequence at positions 17 and 18 totally inhibit inducing activity, they retain the property to strongly inhibit the native systemin, presumably by interacting with the binding sites of the systemin receptor.

**CONCLUSIONS**

The evidence presented here indicates that the entire 18-amino acid sequence of systemin is important for maximal proteinase inhibitor-inducing activity. Although many of the individual amino acids could be substituted with negligible or moderate changes in activity, substitutions of Ala at Pro¹³ and Thr¹⁷ caused major reductions in activity. The regions of the systemin primary structure that can be modified without seriously impairing activity are obvious candidates for modification to generate affinity probes to identify and isolate the putative systemin receptor. The total loss of biological activity of systemin analogs that are modified at the COOH-terminal Thr¹⁷ or deleted at the adjacent COOH-terminal Asp position...
 Structure and Activity of Systemin Analogs

Wild Type: Analog

![Graph](image)

FIG. 5. Inhibition of systemin activity in leaves of young excised tomato plants by the inactive analogs Ala_17 systemin (SYS MQAD), and des-Asp systemin (SYS MQT). The plants were supplied with 250 pm of each analog and then supplied with systemin at the concentrations indicated on the abscissa. The values at the top of the figure indicate the ratios of analog/systemin at each concentration of systemin.

suggests these residues are crucial for activating the expression of the proteinase inhibitor genes, perhaps via phosphorylation of systemin. This possibility is somewhat supported by the evidence that the biologically inactive Thr_17 and des-Asp analogs are powerful inhibitors of the activity of native systemin, interacting with the signaling process initiated by systemin but not capable of activating the genes. The inactive analogs with potent inhibitory activities should be valuable tools for the continuing studies of the mode of action of systemin and for the search for the components of the signaling cascade with which systemin interacts in tomato and potato cells.

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