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ACTION OF PROTEOLYSIS-RESISTANT SYSTEMIN ANALOGUES IN WOUND SIGNALLING

ANDREAS SCHALLER

Institute of Plant Sciences, ETH-Zürich Universitätsstrasse 2, CH-8092 Zürich, Switzerland

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Key Word Index—*Lycopersicon peruvianum*; *Lycopersicon esculentum*; Solanaceae; tomato; systemin; plant defense; wound response; protease; alkalinization; signal transduction.

Abstract—In cultured cells of *Lycopersicon peruvianum*, the oligopeptide systemin which mediates systemic signalling in the tomato wound response is rapidly inactivated by proteolytic cleavage of the bond carboxy-terminal to Lys^{14}. A systemin derivative in which this peptide bond had been modified by *N*-methylation was resistant to proteolytic inactivation. Systemin elicits a rapid, transient alkalinization of the growth medium in *L. peruvianum* cells. Consistent with its metabolic stability, the response elicited by the *N*-methylated peptide was found to be more sustained than that caused by systemin. In differentiated tomato plants, the stabilized peptide was found to be 3 times more active than systemin with respect to the induction of proteinase inhibitors I and II. This result indicates the possible physiological significance of the observed proteolytic degradation for systemin inactivation *in planta*. The activity of a protease capable of processing systemin carboxy-terminal of Lys^{14} was detected in tomato plasma membranes and may be responsible for the inactivation process. Two further peptides, *N*-methylated at the bonds carboxy-terminal of Gln^{3} and Arg^{10} had proteinase inhibitor inducing activities lower by a factor of 8 and 80, respectively, as compared to systemin. Correspondingly, the alkalinization response elicited by these two peptides in cultured cells was found to be more transient than the systemin response. The correlation between the duration of the alkalinization response and the proteinase inhibitor inducing activities of systemin analogues may be indicative of a casual relationship between ion fluxes and defense gene induction. © 1997 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

Plants respond to local injury inflicted by herbivores with the systemic induction of defense genes and the accumulation of the corresponding proteins [1]. These proteins are collectively called systemic wound-response proteins (SWRPs) and include, in tomato plants, proteins that are actively involved in plant defense, such as various protease inhibitors and polyphenol oxidase, as well as exoproteases and endoproteases, and, furthermore, proteins that seem to be involved in wound signal transduction processes [2, 3]. Possible mechanims of long distance signal transmission which include electrical signalling as well as phloem transport or hydraulic dispersal of chemical signal molecules, are still being discussed controversially (reviewed in [4]). On the other hand, protein kinases as well as a number of potential signalling molecules including oligosaccharides, octadecanoate derived molecules, ethylene, abscisic acid, and the oligopeptide systemin have clearly been implicated in the wound response [5–11]. Among these, systemin is the most likely candidate for both a systemically transmissible signal molecule and a mediator of cellular responses (reviewed in [3] and [12]).

Like animal peptide hormones, systemin is synthesized *in planta* as a larger precursor protein which is called prosystemin [13]. Thus, proteases must exist that release systemin from prosystemin. Such enzymes have remained obscure, however, and no proteases have been identified that either inactivate or degrade systemin.

In tomato plants, a number of proteases are induced upon wounding, including leucine aminopeptidase [14, 15], carboxypeptidase [16], an aspartic protease [17], and a cysteine proteinase [3]. A possible role of these proteases in the wound response is to increase protein turnover and thus to facilitate synthesis of active defense proteins as well as salvage of C- and N-resources from the injured organ. Alternatively, such proteases may be involved in systemin processing or degradation. The latter function has been proposed for a kex2-like protease identified in tomato plasma membranes [18]. The presence of a protease in plasma membranes—the presumed site of systemin perception [12, 19]—that specifically interacts with sys-

temin [18], prompted the present investigation. Here, the ability of membrane associated proteases to cleave systemin has been analysed. Various sites of proteolytic processing were detected within the systemin sequence. *N*-methylated systemin analogues were found to be resistant to proteolysis at the modified peptide bonds and were employed in intact plants and cultured cells to assess the relevance of systemin processing for wound signal transduction.

RESULTS AND DISCUSSION

Systemin processing by plasma membrane proteases

Proteases within tomato plasma membrane preparations capable of processing systemin were detected by using systemin, radioactively labelled with [^{35}S]Met at residue 15, as a substrate. Labelled systemin was incubated with plasma membrane vesicles purified by aqueous two phase partitioning from *Lycopersicon esculentum* Mill. leaves (cf. Experimental, and [18]). Peptide fragments generated by membrane associated proteases were analysed by radiosequencing: The amount of radioactivity released in each cycle of sequencing was determined and thus the position of [^{35}S]Met relative to the amino terminus of the peptide fragments was identified. From this information the identity of the corresponding peptides could be deduced (Fig. 1). Under the conditions of the assay, cleavage at the carboxy side of Gln^{3} was by far the most frequent event, followed by cleavage carboxy-terminal of Lys^{14}. Minor events were processing carboxy-terminal of Ser^{8}, Arg^{10}, or Asp^{11}. As was reported previously, most of these proteolytic activities were inhibited by $ZnCl_2$ ([18]; Fig 1). The two main processing events (behind Lys^{14} and Gln^{3}), as well as cleavage behind Arg^{10}, which is the site of processing by a putative kex2-like protease [18], were chosen for further analysis.

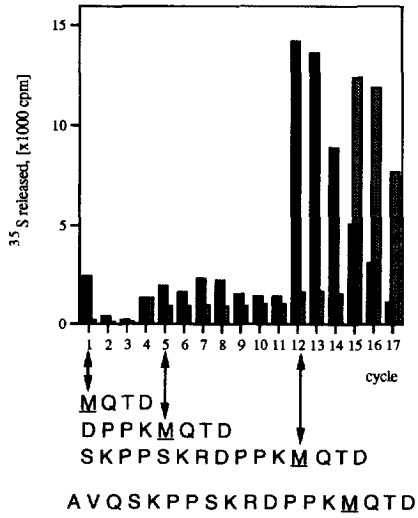

Fig. 1. Radiosequencing of systemin fragments. [^{35}S]Met-systemin was incubated with tomato plasma membrane vesicles and the mixture of peptide fragments generated was then subjected to automated Edman-degradation. The [^{35}S]Met released in each cycle was determined in a scintillation counter (black bars). The same experiment was performed in the presence of 5 mM $ZnCl_2$ as inhibitor of proteases (shaded bars). An increase in radioactivity as compared to the preceeding sequencing cycle indicates a peptide fragment with [^{35}S]Met in the respective position. The data represent one of two independent experiments with essentially the same result. Two-headed arrows indicate processing events that were further analysed in this study; the corresponding peptide fragments are shown, with 'M' indicating the [^{35}S]Met residue. For comparison, the full systemin structure is shown below.

Peptide bond stabilization by N-*methylation*

Initial studies of the structure/activity-relationship in peptide hormones usually focus on the relevance of the amino acid side chains. Systematic replacement of amino acid residues by alanine or glycine (alanine- or glycine-scans) is often performed to assess the relative importance of individual amino acid side chains for the biological activity of the respective peptide. Such an analysis has revealed that Ala^{17} and Pro^{13} are the two single most important residues in systemin for the elicitation of the wound response [20]. A more refined analysis of the structure/activity-relationship would involve the substitution or elimination of certain elements of the peptide backbone and of the amide bond itself. This can cause either the loss or the induction of secondary structural elements, eliminate hydrogen bonds, or increase the stability of the peptide against proteolytic degradation [21]. *N*-alkylation is among the most frequent modifications used and has been shown to increase the metabolic half-life of peptide hormones in numerous studies ([21–23], and references therein). *N*-methylation of certain peptide bonds is easily achieved by incorporation of protected *N*-methylated (*N*-Me) amino acids during solid phase synthesis and was therefore used in attempts to inhibit systemin processing. We used commercially available proteases, to test *in vitro* if *N*-methylation had a stabilizing effect. *N*-MeAsp^{11}-systemin was incubated with the endoprotease ArgC, which specifically cleaves the peptide bond carboxy-terminal of arginine residues (i.e. the Arg^{10}/Asp^{11}-bond in systemin). Under conditions allowing efficient processing of systemin, cleavage of the respective bond in *N*-MeAsp^{11}-systemin was not detectable (Fig. 2). Likewise, the protected bonds in *N*-MeAla^{4}-systemin and *N*-MeAla^{15}-systemin showed enhanced resistance to proteolytic cleavage by leucine aminopeptidase and trypsin, respectively (data not shown).

Effect of N-*methylation on the biological activity of systemin*

Two bioassays have been described which allow the assessment of the biological activity of systemin and its analogues. The first one employs young tomato plants, cut at the base of their stem. Elicitors are

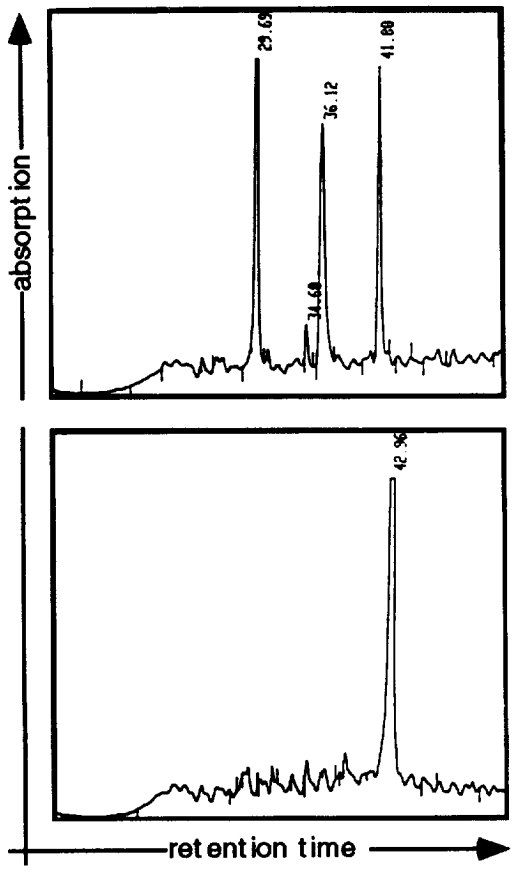

Fig. 2. ArgC-digest of systemin and *N*-MeAsp[11]-systemin. HPLC-traces are shown of endoprotease-ArgC digests of systemin (top panel) and *N*-MeAsp[11]-systemin (bottom panel). 50 μg of peptide were incubated with 0.4 μg of protease for 2 hr at 37°. Reaction products were separated by a linear gradient of acetonitrile (0–30% in 45 min) during RP-HPLC on a semi-preparative C_{18}-column (Vydac 218TP510, 5μ, 10 × 250 mm), with detection at 215. The three peptides generated in the systemin-digest were identified by analysis of amino acid composition as the amino-, and carboxy-terminal cleavage products, and intact systemin, respectively.

supplied to the plant via the transpiration stream and the induction of proteinase inhibitors in the leaves of the plant is analysed 24 hr later. The conditions of this bioassay, which was used during the initial purification of systemin [11] as well as for the analysis of its structure/activity-relationship [20], closely resemble the *in vivo* situation. More recently, systemin has been reported to elicit H^+-influx/K^+-efflux as well as the induction of phenylalanine ammonia-lyase activity and ethylene biosynthesis in cultured cells of *Lycopersicon peruvianum* (L.) Mill. [19]. Systemin caused a marked pH change of the culture medium at subnanomolar concentrations. The ΔpH after 10 min was found to be a steady function of systemin concentration and was employed as a simple bioassay to quantitate systemin activity [19]. In cultured *L. peruvianum* cells, the Ala[17]-analogue of systemin was found to act as an antagonist of systemin activity, which resembles its behaviour in intact plants. Also, in both systems the C-terminus of systemin was found to be the part of the molecule most relevant for its biological activity [19, 20]. This strongly indicates that the response in *L. peruvianum* cells is mediated by a perception system homologous to that in intact *L. esculentum* plants.

The biological activities of *N*-methylated systemin analogues were compared to the activity of the parent compound in both assay systems. In intact plants, the accumulation of proteinase inhibitor I in response to increasing doses of systemin and its *N*-methylated analogues was analysed [Fig. 3(A); comparable results were obtained for the induction of proteinase inhibitor II, data not shown]. Proteinase inhibitor I was half maximally induced by 80 fmols of systemin per plant. The activities of the *N*-MeAsp[11] and *N*-MeAla[4] derivatives were lower by a factor of 8 and 80, respectively. *N*-MeAla[15]-systemin, on the other hand, was more active than wild type systemin by a factor of 3. The small increase in biological activity may be explained by an increased half life of the modified peptide *in planta*, due to stabilization of the respective peptide bond.

It has previously been shown that systemin fragments resulting from processing at residues 3/4, 10/11, and 14/15 are essentially inactive [20]. Nevertheless, cleavage at sites 3/4 and 10/11 does not appear to contribute to systemin inactivation, since *N*-methylation of the respective peptide bonds did not result in an enhanced biological activity. On the contrary, the reduction in biological activity observed in *N*-MeAsp[11]- and *N*-MeAla[4]-systemin may indicate that proteolysis at these sites contributes to signal transduction. Alternatively, modification of the peptide bond at these sites either could induce a shift to a less active conformation of the modified peptides, or else, could interfere with the binding to a systemin receptor.

When analysed in cultured cells of *L. peruvianum*, all of the *N*-methylated analogues showed some reduction in activity as compared to systemin. The concentration of peptide required to elicit the same amplitude in ΔpH was greater by a factor of 3, 5, and 7 for *N*-MeAsp[11], *N*-MeAla[4], and *N*-MeAla[15], respectively [Fig. 3(B)]. This result was unexpected, because the mechanism of systemin perception is believed to be the same in *L. esculentum* plants and *L. peruvianum* cells [19]. It seems as if the ultimate biological response, i.e. the accumulation of defense proteins in the leaves, is correlated not only to the amplitude of the initial pH shift but also to the duration of alkalinization [Fig. 3(C)]. The alkalinization elicited by *N*-MeAla[15]-systemin was much more sustained than that brought about by any of the other peptides tested. The response to *N*-MeAla[4]-systemin, on the other hand, was much more transient in nature than that produced by systemin [Fig. 3(C)]. The variation in the duration of alkalinization correlated with the capacity of the peptides to induce proteinase inhibitor I in the whole plant bioassay. Duration of

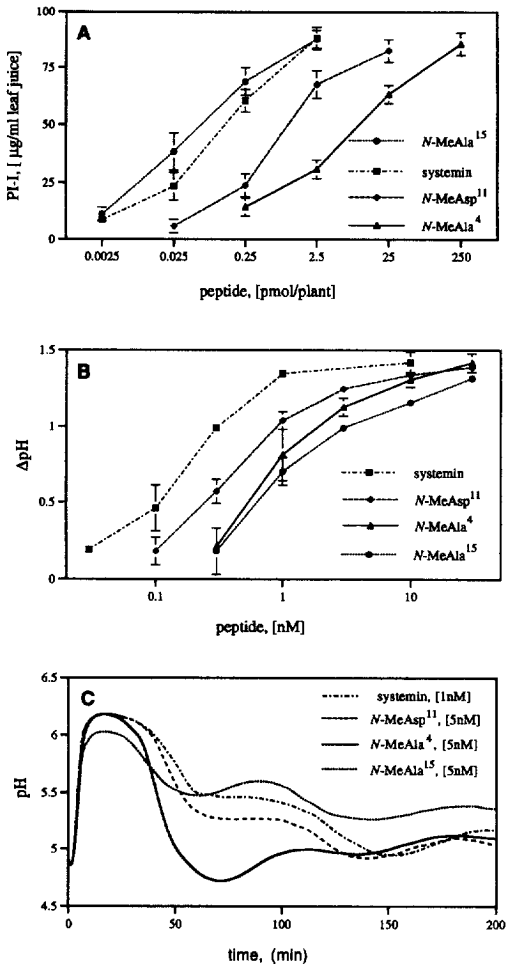

Fig. 3. Biological activities of systemin and its *N*-methylated analogues. In panel **A**, the proteinase inhibitor I (PI-I) inducing activity of systemin and its derivatives was assayed in excised tomato plants. For each data point at lest 24 plants were assayed in 4 independent experiments. Error bars indicate the same standard error of the mean. Panel **B** shows the pH shift observed 15 min after addition of increasing concentrations of systemin or its analogues to a cell culture (5 ml) of *L. peruvianum*. Each data point represents three independent experiments with different batches of cells. Error bars indicate the standard error of the mean. In panel **C**, changes in the pH of the growth medium of cultured *L. peruvianum* cells were monitored in response to peptide elicitors over 200 min. The result is representative of three independent experiments with different batches of cells.

the response, rather than amplitude of the initial pH shift, is likely to be affected by the metabolic half life of the peptides.

Re-acidification of the growth medium depends on systemin inactivation

The alkalinization of the culture medium by systemin is transient in nature. Forty min after initial alkalinization, the pH of the medium rapidly declines and after 150 min it is back close to the starting value [Fig. 3(C)]. Felix and Boller [19] could show that the transient character of the alkalinization response is not due to desensitization but rather to enzyme-dependent inactivation of systemin: Systemin was rapidly inactivated in the growth medium by a heat labile mechanism. Figure 4 reveals that after an initial alkalinization, the cells are able to respond to consecutive doses of systemin with repeated alkalinization of the medium. A slight desensitization was observed when the second dose of systemin was given very early (40–60 min) after the initial elicitation. Full capacity to respond was regained after about 100 min (Fig. 4). These data also point to inactivation of systemin in the cell culture. For *N*-MeAsp^{11}-systemin, the initial response as well as the response to subsequent doses of systemin were very similar to that produced by wild type systemin.

The alkalinization caused by *N*-MeAla^{4}-systemin was more transient than the response to systemin and the capacity to respond to consecutive doses of systemin was regained faster than with any of the other peptides (Fig. 4). Apparently, despite the stabilization of the bond 3/4, *N*-MeAla^{4}-systemin is inactivated more rapidly than systemin. Obviously, inactivation of systemin does not depend on cleavage of bond 3/4. *N*-methylation of this bond may result in an altered conformation facilitating inactivation at a different site.

The response to *N*-MeAla^{15}-systemin differed from that of systemin in that the pH remained at an elevated level for hours after initial elicitation (Fig. 4). The persistent activity of this peptide points to metabolic stabilization of *N*-MeAla^{15}-systemin vs wild type systemin. Subsequent doses of systemin were not able to induce further alkalinization for up to 2.5 hr (Fig. 4). Possibly, *N*-MeAla^{15}-systemin remains bound to its receptor thereby inducing a sustained H^+ influx. Only after cleavage of *N*-MeAla^{15}-systemin, binding and activity of the peptide are lost, the H^+ gradient is re-established by action of the plasma membrane H^+-ATPase [24] and cells become susceptible to further elicitation.

Inactivation of systemin by cleavage carboxy-terminal to Lys^{14}

In order to identify the mechanism of systemin inactivation in the *L. peruvianum* cell culture, systemin was incubated with cultured cells, recovered from the growth medium and analysed. To facilitate the recovery of the peptide after inactivation, a radioactively labelled analogue was used rather than systemin itself. The Cys^{8}-derivative of systemin was labelled by covalent modification of the thiol group with iodo [^{14}C]acetamide to yield Cys^{8}-[^{14}C]-carboxymethylated systemin (C*-systemin). Labelled systemin (10 nM) was incubated with cultured cells (100 ml). After 90 min the medium was harvested, peptides were purified and analysed by reversed-phase HPLC in comparison to a C*-systemin standard (Fig. 5). The peak of radioactivity was shifted to smaller retention volumes and

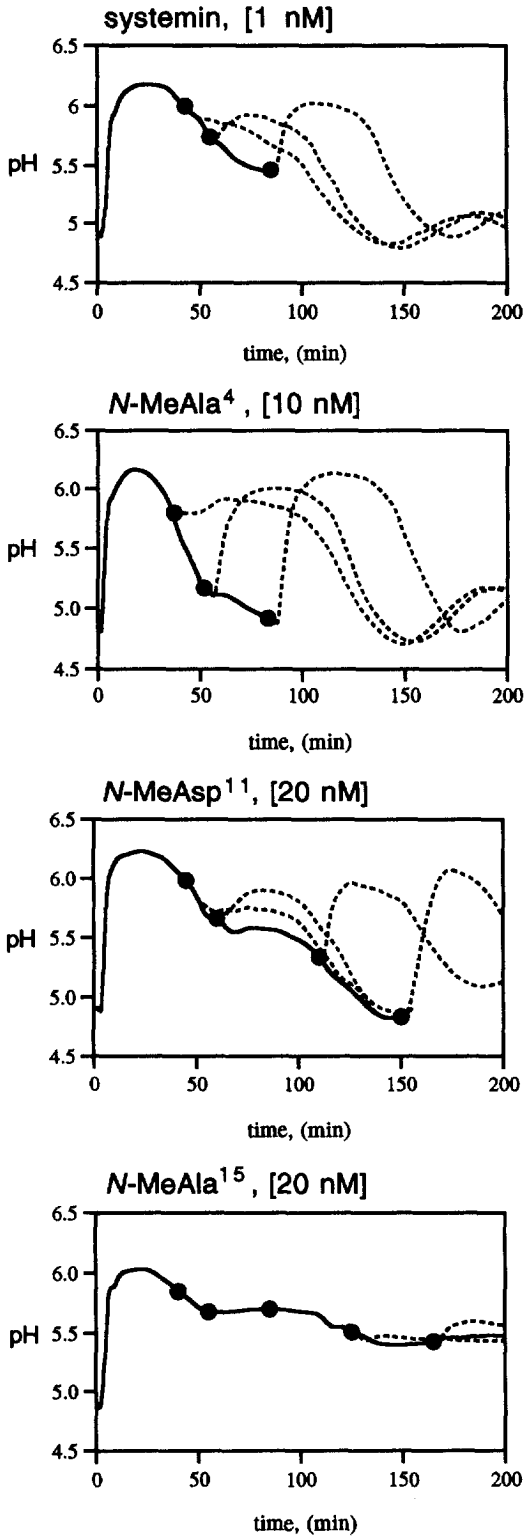

Fig. 4. pH-responses elicited by consecutive doses of systemin. The pH of the growth medium of cultured *L. peruvianum* cells was monitored over time. At the beginning of each experiment, the culture was elicited as indicated on top of each panel. Dots indicate time points of subsequent elicitation by 1 nM systemin. Each experiment was repeated at least three times with different batches of cells and comparable results were obtained.

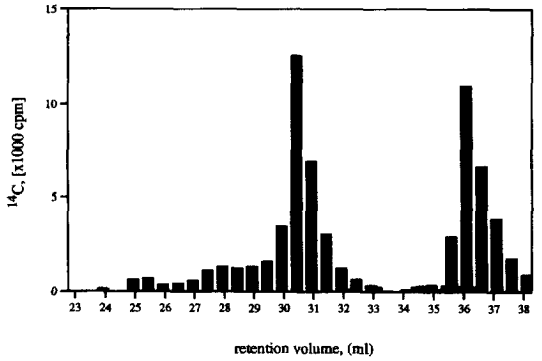

Fig. 5. Processing of labelled systemin in a *L. peruvianum* cell culture. Systemin (10 nM), [^{14}C]-carboxymethylated at residue 8, was incubated with 100 ml (*ca* 10 g fr. wt) of cultured *L. peruvianum* cells. After 90 min the medium was harvested, peptides were purified on a C_{18}-cartridge, and were subsequently analysed by RP-HPLC (linear gradient of 0–20% MeCN during 45 min on a C_{18}-column). Fractions were collected (0.5 ml) and the radioactivity was determined in a liquid scintillation counter (black bars). Shaded bars show a standard of unprocessed [^{14}C]-labelled systemin. The peptides eluted between 30 and 31 min were subjected to mass spectral analysis (MALDI-MS).

intact systemin was no longer detectable. Systemin degradation products were subjected to mass spectral analysis and two peptides were identified (AVQSK PPC*KRDPPK, and AVQSKPPC*KRDPP). Most likely, these peptides were generated by initial cleavage of the peptide bond carboxy-terminal to Lys^{14} and the subsequent action of a carboxypeptidase. This view is supported by the observation that with longer incubation times, the relative amounts of the degradation products were shifted toward the shorter peptide (data not shown). More importantly, *N*-MeAla^{15}-systemin was found to be stable under conditions that led to a complete degradation of C*-systemin (cf. Fig. 5). Greater than 90% of the peptide were recovered intact from the growth medium as verified by mass spectral analysis (not shown). This is consistent with initial cleavage between amino acids 14 and 15, as well as with the observation that *N*-MeAla^{15}-systemin caused a more sustained alkalinization response than any of the other peptides under investigation.

Conclusions

N-methylation increases the resistance of a peptide bond to cleavage by proteolytic enzymes [21, 23]. This was found to be true for *N*-methylation of the peptide bonds carboxy-terminal of Gln^3, Arg^{10}, and Lys^{14} in systemin. *N*-methylation of the bonds carboxy-terminal of Gln^3 and Lys^{14} resulted in profound changes in the biological activities of the respective peptides. This implies that the proteases responsible for the hydrolysis of these bonds may be involved in the regulation of systemin activity. The biological activities of *N*-methylated analogues of systemin as compared to

Table 1. Biological activities of *N*-methylated analogues as compared to systemin activity

	N-Me Ala[4] -systemin	*N*-MeAsp[11] -systemin	*N*-MeAla[15] -systemin
Elicitation of ΔpH*	1/5	1/3	1/7
Duration of alkalinization†	severely reduced	reduced	increased
Induction of proteinase inhibitors*	1/80	1/8	3/1

*The amount of systemin necessary for the elicitation of the half maximal response was set as 1. The amounts of *N*-methylated analogues needed to elicit the same response is given as fractions thereof.

†The duration of medium alkalinization elicited by systemin analogues is compared to the systemin response.

the parent compound are summarized in Table 1. Complex changes were observed that can be explained in part by an increased metabolic half life of the modified peptides. However, it cannot be excluded that conformational changes induced by modification of the peptide backbone contribute to the differences in biological activities.

A correlation was observed between the duration of the elicited alkalinization response and the proteinase inhibitor-inducing activities of systemin and its *N*-methylated derivatives. *N*-MeAla[15]-systemin caused an extended alkalinization of the growth medium and had an enhanced proteinase inhibitor-inducing activity as compared to native systemin. In contrast, the alkalinization elicited by *N*-MeAsp[11]-systemin and *N*-MeAla[4]-systemin is more transient and at the same time their proteinase inhibitor-inducing activities are reduced in comparison to the systemin response (Table 1). This points to a possible causal link between systemin induced ion fluxes and defense gene induction. Alterations in ion fluxes (namely H^+, K^+, Ca^{++}, and Cl^-) have been observed in response to many different elicitors and have been implicated in the induction of defense responses [25–32]. Two studies directly implicate altered ion fluxes across the plasma membrane in the induction of tomato proteinase inhibitors in response to oligosaccharides and systemin [33, 34]: The induction of proteinase inhibitors was found to be inhibited by the fungal toxin fusicoccin which counteracts the extracellular alkalinization by activating the plasma membrane H^+-ATPase [24]. These data support a direct role of systemin mediated changes in H^+-transport across the plasma membrane in wound signalling.

Systemin was rapidly degraded in cell cultures of *L. peruvianum* by cleavage of the bond carboxy-terminal to Lys[14]. Such a proteolytic activity was also detected in plasma membranes of *L. esculentum* leaves. Modification of this bond by *N*-methylation in *N*-MeAla[15]-systemin resulted in increased stability of the peptide in cell cultures. At the same time, the alkalinization of the growth medium elicited by the stabilized peptide was extended in comparison to the systemin response and cells were unable to respond to consecutive doses of systemin. Apparently, re-acidification of the growth medium as well as the ability to respond to later stimuli of systemin depend on proteolytic cleavage carboxy-terminal of Lys[14]. When assayed in *L. esculentum* plants, the *N*-methylated peptide analogue exhibited enhanced proteinase inhibitor-inducing activity in comparison to systemin. The data indicate that cleavage of systemin carboxy-terminal of Lys[14] is involved in systemin inactivation *in vivo*.

EXPERIMENTAL

Growth of plants and bioassay. Tomato plants (*L. esculentum* Mill. cv. Castlemart II) were grown as described [20]. The proteinase inhibitor inducing activity of systemin and its derivatives was analysed by immuno-detection of the inhibitor proteins in leaves of tomato plants 12 to 14 days after planting [20]. A suspension culture of *L. peruvianum* (L.) Mill. (kindly provided by T. Boller, Basel, Switzerland) was maintained as described [19]. Continuous measurements of extracellular pH were done in 5 ml of cultured cells 6 to 8 days after subculture [19]. Peptide elicitors were added from 100-fold concn, aq. stock sols as indicated.

Peptide synthesis. Solid phase peptide synthesis was performed with an Applied Biosystems model 431A synthesizer using fluoren-9-ylmethyoxycarbonyl (Fmoc)-protected amino acids whenever possible [20].

Cys[8]-[^{14}C]-carboxymethylated systemin was synthesized by modification of the Cys[8]-thiol with iodo-[^{14}C]acetamide (Amersham) as described [35]. The sp. act. of the labelled peptide was 60 m Ci mmol^{-1}.

The synthesis of [^{35}S]Met[15]-systemin has been described previously [18]. Briefly, the Fmoc-derivative of [^{35}S]Met was synthesized and coupled to the immobilized C-terminal tripeptide of systemin (QTD). A peptide corresponding to residues 1 to 14 of systemin was synthesized on Sasrin resin (Bachem). The protected Fmoc-14mer was cleaved from the resin and reacted with the C-terminal [^{35}S]MQTD. [^{35}S]Met[15]-systemin exhibited a sp. act. of 1 Ci mmol^{-1}.

Peptides *N*-methylated at bonds, 3, 10, and 14, respectively, were synthesized by solid phase incorporation of Fmoc-*N*-MeAsp or Fmoc-*N*-MeAla. The amino acid sequence of *N*-MeAsp[11]-systemin is identical to that of native systemin. In *N*-MeAla[4]-systemin and *N*-MeAla[15]-systemin, Ala replaces the amino acids Gln and Met in positions 4 and 15 of native systemin respectively. These substitutions do not affect the proteinase inhibitor inducing activities of the peptides [20].

All peptides were purified by reversed-phase HPLC and their identity was confirmed by mass spectral analyses.

Radiosequencing of systemin fragments. Plasma membrane vesicles were isolated from *L. esculentum* leaves by aq. two phase partitioning as has been described [18]. [^{35}S]Met^{15}-systemin was incubated with plasma membrane vesicles (0.4 mg of protein) in a total vol. of 100 μl, containing 375 mM sorbitol, 5 mM dithiothreitol, and 7.5 mM potassium phosphate (pH 7.6). Membranes were washed with 1 ml of glycine–HCL, pH 2.3. Peptides in the wash soln were purified on a C_{18}-cartridge (Waters) and were then subjected to sequence analysis. The total activity released in each cycle of Edman degradation was analysed in a scintillation counter (Packard). The number of sequencing cycles required to release [^{35}S]Met indicated the position of the Met-residue relative to the N-terminus of the sequenced peptides and allowed the identification of proteolytic cleavage sites within systemin.

Processing of systemin in a L. peruvianum *cell culture.* Cys^8-[^{14}C]-carboxymethylated systemin (10–50 nM) was incubated with 100 ml of a *L. peruvianum* cell suspension, 7 days after subculture (*ca* 10 g fr. wt of cells). After 20–90 min, the medium was harvested and peptides were purified on a C_{18}-cartridge (Waters). Peptides were then analysed by reversed-phase HPLC on a C_{18}-column (250 mm × 4 mm, Merck), using a linear gradient of 0–20% of MeCN during 45 min, and were compared to a standard of Cys^8-[^{14}C]-carboxymethylated systemin. Peaks of radioactivity were collected in 0.5 ml frs and peptides were identified by mass spectral analyses (MALDI MS).

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