Isolation and Characterization of Hydroxyproline-Rich Glycopeptide Signals in Black Nightshade Leaves1[OA]

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A gene encoding a pro preprohydroxyproline-rich systemin, SnpreproHypSys, was identified from the leaves of black nightshade (Solanum nigrum), which is a member of a small gene family of at least three genes that have orthologs in tobacco (Nicotiana tabacum; NtpreproHypSys), tomato (Solanum lycopersicum; SlpreproHypSys), petunia (Petunia hybrida; PhpreproHypSys), potato (Solanum tuberosum; PhpreproHypSys), and sweet potato (Ipomoea batatas; IppreproHypSys). SnpreproHypSys was induced by wounding and by treatment with methyl jasmonate. The encoded precursor protein contained a signal sequence and was posttranslationally modified to produce three hydroxyproline-rich glycopeptide signals (HypSys peptides). The three HypSys peptides isolated from nightshade leaf extracts were called SnHypSys I (19 amino acids with six pentoses), SnHypSys II (20 amino acids with six pentoses), and SnHypSys III (20 amino acids with either six or nine pentoses) by their sequential appearance in SnpreproHypSys. The three SnHypSys peptides were synthesized and tested for their abilities to alkalinize suspension culture medium, with synthetic SnHypSys I demonstrating the highest activity. Synthetic SnHypSys I was capable of inducing alkalinization in other Solanaceae cell types (or species), indicating that structural conformations within the peptides are recognized by the different cells/species to initiate signal transduction pathways, apparently through recognition by homologous receptor(s). To further demonstrate the biological relevance of the SnHypSys peptides, the early defense gene lipoygenase D was shown to be induced by all three synthetic peptides when supplied to excised nightshade plants.

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preproHypSys is overexpressed, the plants are resistant to attack by Helicoverpa armigera larvae (Ren and Lu, 2006). The preproHypSys gene expression level was also found to increase in response to wounding, methyl jasmonate (MeJA), insect feeding, and treatment with abscisic acid in a manner that would suggest involvement in direct defenses (Rocha-Granados et al., 2005). However, in the native tobacco Nicotiana attenuate, it has been shown that silencing of the HypSys gene has very little effect on insect feeding or on the levels of defense-related compounds (Berger and Baldwin, 2007). Interestingly, in petunia, supplying young plants with HypSys was found to increase the expression of the defensin gene, known to be involved in protection from pathogen attack (Pearce et al., 2007). More recently, a HypSys glycopeptide was isolated from sweet potato that induced sporamin, a gene that codes for a major storage protein in the tuber with trypsin inhibitor activity that is induced upon wounding in the leaves (Chen et al., 2008). Other stresses, such as salinity, UV radiation, and pathogens, have yet to be tested.

Black nightshade (Solanum nigrum) was previously shown to express prosystemin, and when the deduced nightshade systemin peptide was fed to excised tomato plants, protease inhibitors were produced (Constabel et al., 1998). Nightshade plants accumulated both inhibitor I and II transcripts upon supplying systemin in an excised plant assay (Constabel et al., 1998), but whether this species expressed both systemin and Hyp-rich glycopeptides was not known. Recently, it was shown that silencing of SnproSys did not affect protease inhibitor synthesis and defenses against insects in nightshade. It was concluded that prosystemin does not play a role in the nightshade wound response and that other defense signals may be involved in initiating defense responses (Schmidt and Baldwin, 2006).

We report here the identification and isolation of three nightshade leaf Hyp-rich glycopeptides (SnHypSys) that are similar in size and activity to tobacco and tomato HypSys peptides. The three peptides are derived from a single gene, called SnpreproHypSys, that is an ortholog of the tobacco and tomato HypSys precursor genes within the Solanaceae family. We provide evidence that both nightshade systemin and Hyp-rich systemins are capable of inducing an early gene in the jasmonate signaling pathway, suggesting a cooperative action of these two signaling peptides.

RESULTS
cDNAs from Nightshade and Sequence Similarities to Tomato cDNA

Analysis of the cDNA sequences of Hyp-rich systemin precursors from tobacco, tomato, and petunia revealed a common 30-base sequence encoding the putative propeptidase splice site (Fig. 1). This region was utilized as a probe for northern-blot analysis of nightshade RNA, revealing a single, strong band at approximately 750 bases (data not shown). The conserved region was utilized as a primer in 3′ and 5′ RACE experiments for isolation of a cDNA from nightshade whose deduced amino acid sequence had Pro-rich regions, similar to sequences from members of the preproHypSys family of defense proteins (Fig. 2A). The cDNA has a coding region of 159 amino acids, containing the highly conserved propeptidase splice site, three Pro-rich regions, and a C-terminal SY motif, similar to other preproHypSys precursor proteins (Matsubayashi and Sakagami, 2006; Farrokhi et al., 2008). The cDNA coded for a precursor with high homology to SlpreproHypSys, with 63.4% amino acid sequence identity (Fig. 2B), and was designated SnpreproHypSys. Additionally, the locations and amino acid sequences of all three of the Pro-rich regions were similar to those of SlpreproHypSys.

SnpreproHypSys RNA Levels Are Induced by Wounding

SnpreproHypSys and nightshade prosystemin (SnproSys) cDNA-specific probes were employed in RNA gel-blot analysis to determine whether the respective mRNAs are wound inducible. A SnproSys-specific probe was used as a positive control. Three-week-old nightshade plants were repeatedly wounded across the midvein of the fifth and sixth leaves (from the apical top), and total RNA was extracted from the unwounded systemic upper leaves at different time intervals after wounding. Time-course analysis of RNA samples was performed using labeled probe specific to each of the two cDNAs (Fig. 3A). The HypSys precursor mRNA for SnpreproHypSys showed induction in the upper, unwounded systemic leaves due to wounding of lower leaves, reaching a maximum induction at 6 h. Accumulation of SnpreproHypSys
mRNA due to wounding maximized at 8 h. A higher level of the precursor transcript of SnpreproHypSys as well as SnproSys mRNA persisted even after 24 h in systemic leaves of wounded plants compared with leaves from unwounded plants (data not shown).

**SnpreproHypSys mRNA Levels Are Induced by MeJA**

MeJA is a powerful inducer of defense genes in plants (Farmer and Ryan, 1992; Wasternack, 2007). Three-week-old nightshade plants were sprayed with MeJA in a closed container, and total RNA was extracted at different time intervals. Northern-blot analysis revealed a strong induction of both SnpreproHypSys and SnproSys mRNA following MeJA treatment (Fig. 3B). The maximum level of SnpreproHypSys mRNA was attained at 2 h and remained high 4 h after treatment, whereas induction of SnproSys mRNA continued to increase through 8 h.

**SnpreproHypSys Is a Member of a Small Gene Family**

DNA gel blots were prepared by digesting total genomic DNA isolated from a single nightshade plant with three different restriction enzymes, BamHI, EcoRI, and XbaI. These enzymes do not have any internal site...
present within the SnpreproHypSys cDNAs, which were used as a probe in Southern-blot analysis; consequently, the number of bands in each lane of the autoradiogram would indicate the copy number of the SnpreproHypSys gene. Autoradiograms generated by hybridization of the DNA blot with a 32P-labeled SnpreproHypSys-specific probe showed the presence of three major bands and a minor band in each lane (Fig. 4), indicating that SnpreproHypSys is a member of a small gene family with at least two additional genes.

Isolation of Hyp-Rich Systemins from Nightshade Leaves

A search for mature, bioactive peptides from nightshade leaves was initiated by generating callus on solid medium and subsequently transferring to liquid medium for utilization in the alkalinization assay. We extracted the leaf preparation as described in “Materials and Methods” and separated the fractions by reverse-phase HPLC. The fractions were assayed for alkalinizing activity as described in “Materials and Methods” using tobacco, wild tomato (Solanum peruvianum), and nightshade suspension cells (Fig. 5). Interestingly, the first peak, designated peak 1, responded strongly in the alkalinization assay with tomato suspension cells, whereas peak 2 had a stronger response with tobacco suspension cells. Both of these peaks induced alkalinization equally with the nightshade cells. Peak 3 was nonresponsive in both the tobacco and tomato suspension cell assays but gave a similar response to peaks 1 and 2 with nightshade suspension cells. Upon further purification, peak 1 was separated into two fractions and designated peaks 1a (minor peak) and 1b (major peak; data not shown). The four activity peaks were further purified as described in “Materials and Methods”; however, only minute quantities of peak 1a were obtained, and this peak was directly subjected to mass spectral analysis. The final HPLC purifications of peaks 1b, 2, and 3, with their alkalinizing peaks (insets), are shown in Figure 6. Estimates of yield were determined from the peak areas as follows: peak 1b, 91 pmol; peak 2, 55 pmol; peak 3, 53 pmol. Amino acid sequence and mass
spectral data were obtained (Fig. 7A). The amino acid sequences for all three peptides were found within the isolated cDNA for SnpreproHypSys (Fig. 2A). Peaks 1a and 1b, designated SnHypSys IIIa and IIIb by location in SnpreproHypSys (Fig. 2A), were found to differ only in the number of pentoses attached to the peptide backbone, with SnHypSys Ia containing nine pentoses and SnHypSys Ib containing six pentoses. Peak 2, designated SnHypSys I, contained six pentoses, as did peak 3, designated SnHypSys II (Fig. 7A). The positions of the peptides within the cDNA are shown in Figure 7B.

Alkalization of Suspension-Cultured Cell Medium following Treatment with Synthetic HypSys Peptides

Because of the low yield of the SnHypSys peptides, synthetic peptides lacking the carbohydrate moieties were synthesized for activity experiments as done previously for other HypSys peptides (Pearce et al., 2007; Chen et al., 2008). Although some synthetic HypSys peptides have demonstrated little or no ability to alkalize suspension cell media, others have alkalizing activity in the high nanomolar to low micromolar range. Synthetic SnHypSys I, II, and III were compared with SIHypSys I for their abilities to induce medium alkalization in nightshade, tobacco, and tomato cells (Fig. 8). The SIHypSys I peptide induces
medium alkalinization in all solanaceous species tested at nanomolar concentrations (G. Pearce and C.A. Ryan, unpublished data). Of the three nightshade peptides, SnHypSys I was able to induce medium alkalinization in all three types of cell cultures, with a half-maximal response in nightshade cells of about 2.5 \( \mu M \), comparable to SlHypSys I. The native SnHypSys I also induced medium alkalinization in all three types of cell cultures (Fig. 5, peak 2), and its position in the cDNA matches SlHypSys I (Fig. 2B).

Lipoxygenase D Induction in Response to Synthetic SnHypSys Peptides

To study the biological relevance of the three HypSys peptides for defense in nightshade plants, the defense-related \textit{Lipoxygenase D} (\textit{LoxD}) gene was utilized in wounding and excised plant assays (Heitz et al., 1997) To establish that \textit{LoxD} was involved in defense responses in nightshade, young nightshade plants were wounded and the relative RNA expression levels of the gene were analyzed with real-time reverse transcription (RT)-PCR. The relative \textit{LoxD} expression levels increased at 0.5 h, peaking in wounded leaves after 1 h with a 170-fold increase in expression compared with unwounded control plants, before declining to a 25-fold increased expression at 4 h (Fig. 9A). The unwounded upper leaves of the wounded plant (systemic response) followed the same temporal pattern, but expression was much lower, peaking at a 10-fold increased expression after 1 h.

Since synthetic SnHypSys I was the most active of the three HypSys peptides in the alkalinization assay (Fig. 8), this peptide was utilized to establish a concentration range for peptide response in excised nightshade plant experiments. SnSys was also supplied at lower hormonal concentrations that had previously been established for a response in tomato plants (Pearce et al., 1991; Constabel et al., 1998). Merely excising and supplying water to the nightshade plants caused an 8-fold increase in \textit{LoxD} expression after 1 h (Fig. 9B). SnHypSys I increased \textit{LoxD} expression 26-fold at a concentration of 100 \( \mu M \), whereas SnSys produced a 38-fold increase at a concentration of 0.1 \( \mu M \).

The three synthetic SnHypSys peptides were assayed for their abilities to induce the expression of \textit{LoxD}. A synthetic sweet potato control peptide used in a previous study, IbHypSys IVA, was employed as a negative control at a concentration of 100 \( \mu M \) (Chen et al., 2008). A time course of expression of all three SnHypSys peptides and SnSys demonstrated increased expression of \textit{LoxD} at 0.5 h with maximal
induction at 1 h (Fig. 9C). Induction levels of *LoxD* by the three synthetic peptides were between 15- and 23-fold higher at 1 h than the 0-h control levels, whereas the control peptide displayed a 4-fold increase in expression at 1 h, similar to nightshade plants supplied with water (data not shown). The expression levels of *LoxD* induced by the three SnHypSys peptides at the 1-h maximal expression time are shown in Figure 9D. SnHypSys I produced the highest expression level of the three HypSys peptides, similar to the responses of the peptides in the alkalinization assay (Fig. 8). *LoxD* transcript levels increased dramatically for plants supplied with SnSys, with a relative expression increase of 32-fold.
In addition to testing LoxD expression levels, two other early genes, EEF53 (phospholipase) and F1L3.3 (jasmonate ZIM-domain protein), were evaluated for expression levels after supplying SnHypSys I for 1 h. Expression levels of EEF53 were induced 3.7-fold, while F1L3.3 expression levels were induced 3.9-fold over control levels (Fig. 9E).

The late genes for protease inhibitors 1 and 2 were also evaluated for expression levels after supplying SnHypSys I through the cut stem. After 4 h, the expression of inhibitor 1 was 1.4-fold, while that of inhibitor 2 was 2.1-fold, higher than control levels. Increasing the incubation time beyond 4 h caused a large increase in control values, and expression levels at later time intervals could not be evaluated.

DISCUSSION

The Hyp-rich systemin family of defense glycopeptides has recently expanded to include regulation of pathogenesis-related genes, with the discovery of the HypSys-induced up-regulation of defensin in petunia (Pearce et al., 2007). Also, a HypSys-coding gene was recently isolated from sweet potato, a member of the Convolvulaceae family (Chen et al., 2008). These two studies suggest a more universal and potentially more important role for this gene family in crop protection. The diversity of function(s) of the HypSys glycopeptides in the plants that are currently being investigated suggests that their roles may have evolved to assist species in coping with their unique environmental needs. In one instance, a strong systemic response may be required to insect attack, whereas in another species, defense to pathogen attack may be more important. Also, as the HypSys glycopeptide family is further characterized, other functions unrelated to defense are being revealed, such as involvement in self-pollination and flower morphology, related to jasmonate signaling in N. attenuate (Berger and Baldwin, 2009). We continue to characterize the HypSys family of defense genes to understand their functions and structural characteristics in this study of nightshade, a species closely related to the agriculturally important species tomato and potato.

A gene was identified from nightshade that encoded a precursor protein with identity to the Hyp-rich glycopeptide family of defense genes. Previously isolated members of this family were obtained by first isolating the glycopeptides. This method should be of use in isolating other members of the HypSys family of genes.

The isolated cDNA contained three sequences that were potential HypSys peptides (Fig. 2A). These sequences had a central region that was Pro rich, basic charges toward their N termini, and Gln or Glu at their C termini, similar to previously isolated Hyp Sys peptides (Matsubayashi and Sakagami, 2006; Farrokhi et al., 2008). The cDNA termination codon was preceded by a sequence coding for –QASY, similar or identical to homologous preproHypSys C-terminal sequences from other species. The SnpreproHypSys cDNA coded for a protein of 159 amino acids in length, similar in size to the homologous tomato cDNA (146 amino acids), and had 63% identity with the tomato sequence (Fig. 2B). The amino acid sequences around the proteolytic processing sites for the glycopeptide sequences were identical to the tomato cDNA, indicating a conservation of the processing machinery.

Wounding of the lower leaves of nightshade plants caused an increase in RNA levels of SnpreproHypSys in the upper unwounded leaves (Fig. 3A). The response was minimal at 4 h but was very strong between 6 and 10 h. In contrast, SlpreproHypSys and PphpreproHypSys have been shown to be strongly induced at 4 h in the upper leaves (Pearce and Ryan, 2003; Pearce et al., 2007). SnproSys RNA levels were slightly higher at 2 to 4 h than zero-time controls, but a maximal response was not observed until 6 to 8 h. Again, this is in contrast to SlproSys, which has been shown to obtain maximal RNA levels in the upper unwounded leaves of wounded plants by 3 h (McGurl et al., 1992). In general, the systemic wound response time of black nightshade appears to be somewhat slower than that of tomato and petunia. Recently, it was shown that there is a marked decrease in relative prosystemin levels 30 to 90 min after wounding and application of oral secretion from Manduca sexta (Schmidt and Baldwin, 2006). Although we did not test these early time points, the delay in prosystemin expression shown here may be due to an initial down-regulation.

Nightshade plants were incubated in the presence of MeJA vapors, and RNA was extracted and subjected to northern-blot analysis (Fig. 3B). An increase in SnpreproHypSys was detectable after 1 h, peaking at 4 h before decreasing. Early detection of increased preproHypSys RNA levels to MeJA has been found with both tomato and petunia (Pearce and Ryan, 2003; Pearce et al., 2007). proSys levels were detectable at 4 h and continued to increase with time through 8 h, similar to results found with tomato (Pearce and Ryan, 2003). These results indicate that SnpreproHypSys is both wound inducible and MeJA inducible in the same temporal pattern as other Hyp-rich systemin precursors.

An attempt to purify all three of the putative glycopeptides found within the cDNA by the same extraction methods utilized for HypSys peptides from
tomato and tobacco revealed only two alkalinizing peaks when tobacco or wild tomato cells were utilized in the alkalinization assay. It was only after nightshade cells were cultured that a third alkalinizing peak was found (Fig. 5). The three peaks were purified to near homogeneity (Fig. 6) and analyzed by N-terminal amino acid sequencing and by matrix-assisted laser-desorption ionization-tandem mass spectrometry (MALDI-MS/MS). The sequences of the three SnHypSys glycopeptides are shown in Figure 7A. The retention order of the glycopeptides on HPLC had previously been used for numerical designation of the HypSys peptides. With tobacco (Pearce et al., 2001a), the retention of the peptide coincided with the positioning of the HypSys in the gene. However, in tomato and petunia, the three HypSys peptides were named by HPLC retention time but did not coincide with their positioning in the gene (Pearce and Ryan, 2003; Pearce et al., 2007). Recently, a single HypSys glycopeptide was isolated from sweet potato, and when the cDNA was isolated it was found to be the fourth HypSys of six potential glycopeptide signals within the gene; it was designated IbHypSys IV (Chen et al., 2008). We propose that with the use of the 30-nucleotide homologous region as a primer, and with more knowledge about what constitutes a HypSys peptide, the more logical method for naming HypSys glycopeptides or putative HypSys glycopeptides is by position in the gene rather than by retention time, as shown in Figure 7B.

SnHypSys I (19 amino acids in length) has a different Hyp-rich motif than all other HypSys glycopeptides isolated to date. Instead of OOXO, where X is Ala, Ser, Thr, or Hyp, SnHypSys I contains OOOO. SnHypSys I contains six pentoses, as do all of the HypSys peptides isolated to date with a single OOXO motif.

SnHypSys II contains 20 amino acids with six pentoses. Interestingly, when SnHypSys II was purified and analyzed by MALDI-MS, it contained five more amino acids than the homologous HypSys in tomato. The tomato HypSys (SHypSys III) has an Asp at position 15 (instead of the Glu in SnHypSys II) followed by a Pro, a bond that is labile in weak acid. Previously, SHypSys III by MALDI-MS was thought to contain 10 pentoses with one unsaturated bond on a position in the gene rather than by retention time, as shown in Figure 7B.

SnHypSys II for the first 10 amino acids of the glycopeptide was isolated from sweet potato, and the cDNA was isolated to date with a single OOXO motif.

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The lack of carbohydrate moieties on the synthetic HypSys peptides may differentially affect the activities of the three peptides.

In addition to testing LoxD expression levels, two other early genes, EEF53 (phospholipase) and FIL3.3 (jasmonate ZIM-domain protein), involved in jasmonate production and jasmonate signaling, respectively, that had previously been shown to be up-regulated by both MeJA and insect feeding in nightshade (Schmidt and Baldwin, 2006) were evaluated for expression levels after supplying SnHypSys I for 1 h. Both of these genes had significantly higher expression levels, strongly suggesting a defense role for SnHypSys I. On the other hand, the expression levels of the late genes, inhibitors 1 and 2, were not significantly increased at 4 h, and longer incubation times after excision led to high control values. A definitive statement regarding late genes cannot be made for black nightshade from this study.

Gene silencing of prosystemin in tomato caused a marked increase in weight of M. sexta larvae feeding on the transgenic plants (Orozco-Cardenas et al., 1993). This demonstrated the importance of systemin in signaling defense responses against herbivorous insects in tomato. In tobacco, which lacks the prosystemin gene and has a weaker systemic wound response, it has been shown that overexpression of the preproHypSys gene enhanced the resistance against H. armigera (Ren and Lu, 2006). In contrast, recent evidence suggests that systemin in nightshade does not reduce damage by two herbivorous insect species (Schmidt and Baldwin, 2006), indicating that there are other primary signals involved in initiating defense responses. Perhaps the HypSys glycopeptides in nightshade plants provide enough protection against predator attack to explain a lack of difference between the plants silenced for prosystemin and control plants. Interestingly, in tomato, a strong systemic defense response requires the coordinated action of systemin and Hyp-rich glycopeptides, both being involved in amplification of the octadecanoid pathway to produce and Hyp-rich glycopeptides, both being involved in response requires the coordinated action of systemin and HypSys I. On the other hand, the expression levels of the late genes, inhibitors 1 and 2, were not significantly increased at 4 h, and longer incubation times after excision led to high control values. A definitive statement regarding late genes cannot be made for black nightshade from this study.

**Peptide Isolation**

Black nightshade plants were grown in peat pots under greenhouse conditions for approximately 4 weeks. The plants were sprayed with MeJA as described previously (Pearce et al., 2001a). After 15 h, the leaves were collected, ground in liquid nitrogen, and stored at –20°C until use. Three kilograms of wet weight material was collected for extraction. Frozen leaf material (approximately 1 kg) was homogenized in a 4-L blender for 2 min using 2.4 L of 1% trifluoroacetic acid (TFA) as the extraction solvent and then squeezed through four layers of cheesecloth and one layer of Miracloth (Calbiochem). The liquid was centrifuged at 10,000g for 20 min. The acidic supernatant was adjusted to pH 4.5 with 10 n NaOH and recentrifuged at 10,000g for 20 min. After readjusting the supernatant to a pH of 2.5 with TFA, the liquid was applied to a 40-μm, 3 × 25-cm C18 reverse-phase flash column (Bondesil; Varian Analytical Instruments) equilibrated with 0.1% TFA/water. Elution was performed at 8 psi with compressed nitrogen gas. After loading, the column was washed with 0.1% TFA/water and then with 250 mL of 60% methanol/0.1% TFA. The 60% methanol-eluting fraction was rotary evaporated to remove the methanol and then lyophilized to dryness. The yield from these extractions was 34 g. The dry powder was dissolved in 30 mL of 0.1% TFA/water, centrifuged at 10,000g for 30 min, and purified to a Sephadex G-25 column (4 × 40 cm) equilibrated with 0.1% TFA/water in two sequential runs. Eight-milliliter fractions were collected, and the alkalinizing activity was assayed as described above using 10 μL of each fraction per milliliter of nightshade cells. The activity was found at or near the void, and these fractions were pooled and lyophilized. The yield was 1.7 g. Seven hundred fifty milligrams was dissolved in 6 mL of 0.1% TFA/water for preparative reverse-phase C18-HPLC. After centrifugation and filtration, the sample was loaded onto a preparative column in three sequential runs (218TP102; 10 μm, 22 × 250 mm; Vydac) with a flow rate of 4 mL min⁻¹. After 2 min, a 25-g gradient was applied from 0% to 40% acetonitrile/0.1% TFA over 90 min. The absorbance was monitored at 225 nm. One-minute fractions were collected, and 10-μL aliquots were used with 1 mL of nightshade, tobacco, and tomato cells to determine alkalinizing activity. Three main activity peaks were detected along with the late-eluting RALF peptide peak (Pearce et al., 2001b). Fractions 42 to 44 (peak 1), fractions 47 to 49 (peak 2), and fractions 50 to 58 (peak 3) were pooled and lyophilized. The yields were 18.5, 21, and 18.4 mg, respectively. The alkalinizing activity peaks were subjected to strong cation-exchange chromatography on a poly-Sulphoethyl Aspartamide column (5 μm, 4.6 × 200 mm; Nest Group). The column was equilibrated in 5 mM potassium phosphate, pH 3, in 25% acetonitrile. Each peak was loaded onto the column in 1 mL of buffer, and after 2 min, a 90-min gradient was applied to 40% elution buffer (5 mM potassium phosphate, 1 mM potassium chloride, pH 3, in 25% acetonitrile) for peaks 1 and 2 and to 60% elution buffer for peak 3. Absorbance was monitored at 225 nm. A flow rate of 1 mL min⁻¹ was employed, and 1-min fractions were collected. Five-microliter aliquots were used to determine activity in the cell assay. Peak 1 eluted as a doublet in fractions 71 to 72 (peak 1a) and fractions 74 to 75 (peak 1b). Peak 2 activity eluted in fractions 61 to 63, and peak 3 activity eluted in fractions 64 to 65. Activity peaks were pooled, lyophilized, and further purified by reverse-phase C18 chromatography at pH 6 column (218TP54; 5 μm, 4.6 × 250 mm; Vydac). The samples were dissolved in 1 mL of column equilibration buffer and 10 mM potassium phosphate, pH 6, and after centrifugation, the supernatants were added to the column with a flow rate of 1 mL min⁻¹. After 2 min, a 90-min gradient was applied to 40% elution buffer consisting of 10 mM potassium phosphate, pH 6, in 50% acetonitrile, except in the case of peak 3, where 60% elution buffer was utilized. Absorbance was monitored at 220 nm, and 5-μL aliquots were used to determine alkalinizing activity. The peak 1a activity eluted in fractions 49 to 50, peak 1b in fractions 45 to 49, peak 2 in fractions 57 to 59, and peak 3 in fractions 55 to 58. The activity peaks were pooled and lyophilized. Further purification was carried out using a narrow-bore reverse-phase C18 HPLC column (218TP25; 5 μm, 2.1 × 250 mm; Vydac) with methanol/TFA as the elution solvent. The lyophilized peaks were dissolved in 1 mL of 0.1% TFA/water and centrifuged, and the supernatants were loaded onto the column at a flow rate of 0.25 mL min⁻¹. After 2 min, a 90-min gradient was applied from 0% to 40% methanol/0.05% TFA, except in the case of peak 3, where 60% methanol/0.05% TFA was utilized. One-minute fractions were collected, and the absorbance was monitored at 214 nm. After elution with 5-μL aliquots were used to determine alkalinizing activity. The peak 1a activity eluted in fractions 69 to 70, peak 1b in fractions 68 to 70, peak 2 in fractions 73 to 75, and peak 3 in fractions 72 to 76. Peak 1a appeared to be pure and had the smallest absorbance peak and activity. No further purification was performed, and mass analysis was performed. As a final purification step and for

**MATERIALS AND METHODS**

**Alkalination Assay**

Suspension cells were maintained in Murashige and Skoog medium as described previously (Scheer and Ryan, 1999), but excluding buffer. Instead, the medium was adjusted to pH 5.6 with KOH. Cultures were maintained by transferring 3 mL of cells to 45 mL of medium every 7 d and shaking at 160 rpm. Tobacco (Nicotiana tabacum) and black nightshade (Solanum nigrum) cells were used for assays 3 to 5 d after transfer. Tomato (Solanum lycopersicum) cells were used 4 to 7 d after transfer. One hour before assaying for alkalinizing activity, a flask of cells was divided into aliquots on 24-well cell culture plates (1 mL well⁻¹) and allowed to equilibrate at 160 rpm. Aliquots of HPLC fractions or purified peptide (1–10 μL) were added, and after 20 min the pH was recorded.
quantitation of peaks 1b, 2, and 3, an acetonitrile gradient was employed with the narrow-bore C18 column. After removal of the methanol, the pooled fractions were applied to the column and a 90-min gradient was performed, from 0% to 30% acetonitrile/0.1% TFA for each peak. The flow rate was 0.25 mL min⁻¹, and absorbance was monitored at 220 nm. Alkalizing activity was assayed using 5-µL aliquots of each. The activity eluted in fractions 45 to 46 for peak 1b, fractions 49 to 51 for peak 2, and fractions 64 to 65 for peak 3. The peaks were quantified by their areas relative to known quantities of synthetic SiHypSys 1 and 3. The yield for peak 1b was 91 pmol, that for peak 2 was 55 pmol, and that for peak 3 was 53 pmol.

Peptide Analysis and Synthesis

N-terminal sequencing was performed using Edman chemistry on an Applied Biosystems Procise model 492 protein sequencer. MALDI spectra were obtained using a PerSeptive Biosystems Voyager time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm). α-Cyano-4-hydroxycinnamic acid (Aldrich Chemical) was used as the matrix. Peptide synthesis was performed as described (Pearce et al., 2001b). Carbohydrate analysis was done by mild acid hydrolysis and MALDI-MS analysis as described previously (Pearce et al., 2001a).

Isolation of SnpreproHypSys cDNA

The cDNAs that were isolated from tobacco and tomato were found to contain a homologous region between the sequence coding for the signal peptide and the nucleotide sequence coding for the Hyp-rich systems. A degenerative 30-bp oligonucleotide primer was synthesized for amplification of potential defense signals in other solanaceous species. The tobacco nucleotide sequence was 5'-GGAGCCTGAACAACTTCTGATGAATA-3'; the designed primer was 5'-GGAGCCTNAAGAAGAACATTTCTGAGNAT-3'; the tomato nucleotide sequence 5'-GGAGCTCAAGCAAGAACATTCTACAGAAAT-3', where N = G, C, T/A, and R = G, A. The primer was used in 3' RACE PCR-PCR (Ambion) to amplify a product that was subsequently cloned by TOPO (Invitrogen), sequenced, and found to have homology to SnpreproHypSys. To obtain the complete sequence, 5' RACE PCR was performed using the reverse complement of an internal sequence that was subsequently cloned by TOPO (Invitrogen), sequenced, and found to have homology to SnpreproHypSys. To obtain the complete sequence, 5' RACE PCR was performed using the reverse complement of an internal sequence that overlapped the 3' RT-PCR product by 30 bp: 5'-CATGACGCTGCTTCACACAACTC-3' (nightshade inner). The overlapping sequence was homologous.

Mechanical Wounding of Plants

Three-week-old plants having six to eight expanded leaves were employed in the wound experiments, performed in the growth chamber. For each plant, the fifth and sixth leaves below the top four leaves were wounded repeatedly across the midvein using a hemostat. Time-course experiments were performed in which the unwounded systemic upper leaves were collected at 0, 2, 4, 6, 8, and 10 h following the mechanical injury. The corresponding leaves from the unwounded plants served as controls for each time point. The leaf samples were immediately frozen in liquid nitrogen and kept at −80°C until used.

MeJA Treatment

Three-week-old plants were treated by spraying the plants with solutions of 125 µL of MeJA in 500 mL of double distilled water containing 0.1% Triton X-100 or 0.1% Triton X-100/water. The leaf samples were collected for time-course experiments at 0, 1, 2, 4, 6, and 8 h after spraying, immediately frozen in liquid nitrogen, and kept at −80°C until used.

Northern-Blot Analysis

Leaves of treated and control plants (3 weeks old) were removed, immediately frozen in liquid nitrogen and stored at −80°C until extraction. Each sample consisted of approximately 500 mg of leaf material consisting of at least one leaf each from three independent plants. The leaf material was ground to a fine powder with a mortar and pestle with liquid N₂ and total RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was quantified, and 15 µg of each sample was fractionated by electrophoresis on 1.2% formaldehyde agarose gels, blotted on Hybond N membranes (Amersham Biosciences), and hybridized with [³²P]aCTP-labeled specific probes at 65°C. Ethidium bromide-stained RNA bands were used to monitor equal loading. Following hybridization, membranes were washed twice with 2× SSC/0.1% SDS for 10 min each at 55°C, followed by two washes each with 0.5× SSC/0.1% SDS for 10 min and two washes with 0.1× SSC/0.1% SDS for 5 min each at 65°C. Membranes were exposed to x-ray film at −80°C, from 4 to 24 h.

Southern-Blot Analysis

Genomic DNA was isolated from young leaves from a single plant of black nightshade according to the cetyl trimethyl ammonium bromide method described by Doyle and Doyle (1990). DNA samples were restriction digested with BamHI, EcoRI, HindIII, and XhoI, size fractionated on a 0.8% agarose gel, and Southern blotted onto a Hybond N membrane (Amersham Biosciences). The blots were hybridized to [³²P]aCTP-labeled specific probes.

Gene Expression Experiments

Black nightshade plants were grown in growth chambers under 18 h of light (300 µmol photons m⁻² s⁻¹) at 28°C and 6 h of dark at 18°C. Two-week-old (two expanded leaves) plants were wounded across the main vein of the lower leaf. At various intervals, the lower, wounded leaf and the upper, unwounded leaf with the small apical leaf were excised, frozen in liquid N₂, and stored at −80°C until used. Excised plants were supplied with peptide or water for 1 h, and material was collected for RNA extraction. Leaves from two plants were pooled per treatment. Synthetic peptides were supplied through a closed Plexiglas container to maintain high humidity to avoid wilting. The plants were supplied continuously with the synthetic peptides, and at various times, the two expanded leaves and the apical leaf were frozen in liquid N₂ and stored at −80°C.

For analysis of Nos gene expression, quantitative PCR was conducted using LuxD-F (5'-CATCCTCACACCCATCTATC-3') and LuxDR (5'-GGCACTGATTTGCTTGT-3') primers. Other primers used were EEF53-F (5'-TCTGTGGCTTACGTAGGAGG-3') and EEF53-R (5'-TTGTGCGTCACACATCCAGT-3'), FIL3.3-F (5'-AAGAAATACTCCTGATGCTA-3'), and FIL3.3-R (5'-CTGCAAATTCACCCAAAT-3'). PHI-F (5'-TTTCTCTGCAAATTCCTTTGCA-3') and PHI-R (5'-CAAAAAGCAGACACTGACAC-3') were primers used for Phi-2 (5'-CCAATTCAGGAAATTAGA-3'). Micrograms of total RNA isolated with Trizol reagent (Invitrogen) was treated with DNase I (New England Biolabs), and 2 µg was reverse transcribed using the DyNAmo cDNA Synthesis Kit (Finnzyme) with random hexamers. Quantitative PCR was performed using the DyNAmo HS SYBR Green qPCR Kit (Finnzyme) and Mx3000P PCR Systems (Stratagene). Elongation factor 1a (EF-1α) gene was also amplified as an internal control using EF1α-F (5'-ACACTGTGTTGTGTGGTG-3') and EF1α-R (5'-ACAGACACACCCGCGAT-3') primers.

The nucleotide sequence reported in this paper for SnpreproHypSys has been submitted to the GenBank/European Bioinformatics Institute Data Bank and assigned the accession number EU482410.

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