Three Hydroxyproline-rich Glycopeptides Derived from a Single Petunia Polyprotein Precursor Activate defensin 1, a Pathogen Defense Response Gene*

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Hydroxyproline-rich glycopeptides (HypSys peptides) are recently discovered 16–20-amino acid defense signals in tobacco and tomato leaves that are derived from cell wall-associated precursors. The peptides are powerful wound signals that activate the expression of defensive genes in tobacco and tomato leaves in response to herbivore attacks. We have isolated a cDNA from petunia (Petunia hybrida) leaves encoding a putative protein of 214 amino acids that is a homolog of tobacco and tomato HypSys peptide precursors and is inducible by wounding and MeJA. The deduced protein contains a leader sequence and four predicted proline-rich peptides of 18–21 amino acids. Three of the four peptides were isolated from leaves, and each peptide contained hydroxylated prolines and glycosyl residues. Each of the peptides has a -GR- motif at its N terminus, indicating that it may be the substrate site for a processing enzyme. The peptides were active in a petunia suspension culture bioassay at nanomolar concentrations, but they did not induce the expression of defense genes that are directed against herbivores, as found in tobacco and tomato leaves. They did, however, activate expression of defensin 1, a gene associated with inducible defense responses against pathogens.

Peptide signals that activate receptor-mediated defense signaling pathways in plants are derived from either pathogens (1–3) or from host plants (4–7) in response to wounding or infection. The peptides interact with cell surface receptors to signal innate immune responses (1–3, 7). Peptide signals derived from host plants include systemin (8), hydroxyproline-rich glycopeptides (5, 6), and AtPep1 (7). Systemin and AtPep1 are processed from larger precursor proteins that lack leader peptide sequences, and systemin is known to be synthesized and stored in the cytoplasm (9). Both systemin and AtPep1 have been shown to interact with cell surface leucine-rich repeat receptor kinases in plasma membranes (10, 11), indicating the presence of a mechanism of peptide transport of either the precursors or processed peptides from the cytoplasm to the apoplas. In contrast, hydroxyproline-rich glycopeptides in tobacco and tomato leaves (5, 6) are processed from precursors that are post-translationally modified and are sequestered in cell walls (12). The rapid medium alkalization responses of the hydroxyproline-rich glycopeptides in suspension-cultured cells at low nanomolar concentrations (5, 6), a response common in response to peptide ligands, suggest that the activities of the peptides are receptor-mediated. Because of the similarities to systemins in size, proline/hydroxyproline-rich compositions, and their powerful biological activities in activating expression of defensive genes, the hydroxyproline-rich glycopeptides have been included in a functionally defined systemin subfamily called HypSys peptides (4).

HypSys defense peptides were initially discovered when investigating systemic wound signaling in tobacco plants. Tobacco plants lack a prosystemin gene, and therefore systemin, but still can systemically activate a tobacco trypsin inhibitor gene in leaves in response to wounding (13). In seeking the systemic signal, two hydroxyproline-rich glycopeptides of 18 amino acids were isolated from tobacco leaf extracts and shown to be inducers of tobacco trypsin inhibitor activity when supplied to tobacco leaves through their cut petioles at a concentration of less than 2 pmol/plant (5). Both peptides are derived from a single hydroxyproline-rich glycoprotein of 165 amino acids, called Nicotiana tabacum proHypSys (NproHypSys). The expression of the precursor gene NtpreproHypSys is inducible by NtHypSys peptides, methyl jasmonate, wounding, and elicitors (5, 14). The gene is strongly induced during attacks by Manduca sexta larvae and adult white flies (Bemisia tabaci) (14). Transgenic tobacco plants overexpressing NtpreproHypSys under control of the 35S promoter have enhanced resistance toward Helicoverpa armigera larvae (15), suggesting that constitutive processing of the NtpreproHypSys precursor protein occurs in the transgenic plants in the absence of wounding, similar to tomato plants overexpressing prosystemin (16).

An ortholog of NtpreproHypSys in tomato leaves, called LepreproHypSys (6), encodes a protein precursor of 146 amino acids in which three hydroxyproline-rich glycopeptides are harbored. The three peptides have been isolated from tomato

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leaves and shown to be powerful inducers of the synthesis of protease inhibitors I and II in leaves when supplied through cut petioles (6). LepreproHypSys is expressed in leaves in response to methyl jasmonate vapors, to wounding, and by supplying young tomato plants with the three LeHypSys peptides through their cut stems at low nanomolar concentrations.

In seeking HypSys peptides in leaves of other Solanaceae species, we have isolated two genes from petunia (Petunia hybrida) and called them PhpreproHypSys I and II. The genes are orthologs of NtpreproHypSys and LepreproHypSys. Three PhHypSys peptides were isolated from petunia leaf extracts that were encoded by PhpreproHypSys I, but no peptides encoded by PhpreproHypSys II were found during the isolations. The three PhHypSys peptides were named I, II, and III. In contrast to the tobacco and tomato HypSys peptides, the PhHypSys peptides induce neither anti-herbivore protease inhibitor activity in leaves nor polyphenol oxidase activity, but the peptides did activate the expression of defensin 1, a gene associated with inducible defense of plants against pathogens (17–19). The data presented here support the roles of endogenous plant defense-signaling peptides from different plant species as amplifiers of the defense signaling pathways for inducible defense genes that have evolved in their unique ecological niches.

**EXPERIMENTAL PROCEDURES**

**Peptide Assays**—Petunia suspension cells utilized for the alkalination assay were maintained as previously described (8), and the medium was adjusted to pH 5.6 with KOH. Cultures were maintained by transferring 3 ml of cells to 45 ml of media every 7 days and were assayed 3–5 days after transfer. Cultured cells were continually shaken at 160 rpm at all times. One hour before assaying for alkalinating activity, 1 ml of cells was aliquoted into each well of 24-well cell culture cluster plates and allowed to equilibrate on a flat bed shaker. Aliquots from HPLC2 fractions or from solutions of purified peptides (1–10 μl) were added to the cells, and the pH was monitored for 20 min. Petunia plants used for protease inhibitor and polyphenol oxidase assays were grown in chambers under 17 h of light (300 μEinsteins m⁻² s⁻¹) at 28 °C and 7 h of darkness at 17 °C. Petides were supplied to excised plants through their cut stems for 1 h, and the plants were placed in 20-ml vials containing distilled water and incubated in closed plexiglass boxes for 24 h at 200 microeinstein m⁻² s⁻¹ before crushing the leaves with a mortar and pestle to express juice for protease inhibitor protein (13) and polyphenolase activity assays (20).

**Peptide Isolation**—Four-week-old petunia plants, before onset of flowering, were sprayed with methyl jasmonate as previously described (5). After 15 h, the leaves were collected (10 kg), ground in liquid nitrogen, and stored at –20 °C. Frozen leaves (~1 kg) were homogenized in a 4-liter blender for 2 min using 2.4 liters of 1% trifluoroacetic acid, and the leaf juice was filtered through a layer of Miracloth (Calbiochem). The liquid was centrifuged at 10,000 × g for 20 min. The acidic supernatant was adjusted to pH 4.5 with 10N NaOH and recentrifuged at 10,000 × g for 20 min. After readjusting the supernatant to a pH of 2.5 with trifluoroacetic acid, the clear solution was applied to a 3 × 25-cm, 40 μm, C18 reversed phase flash column (Bondesil, Varian Analytical Instruments, Walnut Creek, CA) equilibrated with 0.1% trifluoroacetic acid, H₂O. Elution was performed at 8 p.s.i. with compressed nitrogen gas. After loading, the column was washed with 0.1% trifluoroacetic acid, H₂O, followed by 250 ml of 40% methanol, 0.1% trifluoroacetic acid to elute peptides. Methanol was removed using a rotary evaporator, and the remaining liquid was lyophilized to dryness. The dry weight yield from 10 consecutive extractions was 7.8 g. The dry powder was dissolved in 30 ml of 0.1% trifluoroacetic acid, H₂O, centrifuged at 10,000 × g for 10 min and applied to a Sephadex G-25 column (4 × 40 cm) equilibrated with 0.1% trifluoroacetic acid, H₂O in three sequential runs (10 ml each). Eight-ml fractions were collected, and the alkalination activity was assayed as described above using 10 μl of each fraction/ml of cells. The activity was found primarily in fractions eluting at 1–1.5 void volumes, which were pooled and lyophilized. The dry powder (150 mg) was dissolved in 5 ml of 0.1% trifluoroacetic acid, and after centrifugation and filtration, two 2.5-ml aliquots were separately chromatographed on a C18-HPLC preparative column (218TP1022, 10 μm, 22 × 250 mm; Vydc, Hesperia, CA) with a flow rate of 4 ml/min. After 5 min, a gradient was applied from 0 to 40% acetonitrile, 0.1% trifluoroacetic acid over 90 min. The absorbance was monitored at 225 nm. Fractions were collected at 1-min intervals, and 10-μl aliquots were analyzed for alkalinating activity. Three major peaks of activity were detected, and fractions 42–46 (peak 1), fractions 49–52 (peak 2), and fractions 54–55 (peak 3) were each pooled separately and lyophilized. The yields were 56, 40, and 10 mg, respectively. The alkalinating activity peaks were subjected to strong cation exchange chromatography on a poly-SULPHOETHYL Aspartamide column (5 μm, 4.6 × 200 mm; The Nest Group, Southborough, MA). The column was equilibrated in 5 mM potassium phosphate, pH 3, in 25% acetonitrile, and, after dissolving and centrifuging, each peak was loaded onto the column in 1-ml aliquots, and after 2 min, a 90-min gradient was applied to 100% elution buffer, consisting of 5 mM potassium phosphate, 500 mM potassium chloride, pH 3, in 25% acetonitrile. Absorbance was monitored at 225 nm. A flow rate of 1 ml/min was employed, and fractions were collected at 1-min intervals. Aliquots of 5 μl from each fraction were used for alkalination assays. Peak 1 eluted in fractions 53–55, peak 2 in fractions 42–44, and peak 3 in fractions 56–59. Fractions of each peak were pooled, lyophilized, and further purified by reversed phase C18 chromatography at pH 6 (column 218TP54, 5 μm, 4.6 × 250 mm, Vydc, Hesperia, CA). The dried material was dissolved in 1 ml of column equilibration buffer, 10 mM potassium phosphate, pH 6, and, after centrifugation, the clear supernatant was applied to the column with a flow rate of 1 ml/min. After 2 min, a 90-min gradient was applied from 0 to 40%, consisting of 10 mM potassium phosphate, pH 6, in 50% acetonitrile. Absorbance was monitored at 210 nm, and aliquots of 5 μl were used to determine alkalinating activity. Peak 1 was identified in fractions 37–39, peak 2 in fractions 49–51, and peak 3 in fractions 54–58. Each peak was pooled and lyophilized for further purification. The lyophilized peaks were dis-
solved in 1 ml of 0.1% trifluoroacetic acid, H₂O and centrifuged, and the supernatants were loaded onto a narrow bore C18 column (218TP52; 5 μm, 2.1 × 250 mm; Vydac) and eluted with methanol/trifluoroacetic acid at a flow rate of 0.25 ml/min. After 2 min, a 90-min gradient was applied from 0 to 60% methanol, 0.05% trifluoroacetic acid. Fractions were collected at 1-min intervals, and the absorbance was monitored at 214 nm. Alkalinating activity was determined with 2 μl of each eluted fraction. Peak 1 eluted in fractions 61–63, peak 2 in fractions 62–64, and peak 3 in fractions 63–66.

As a final purification step and to quantify the material in each of the three peaks, the same narrow bore reversed phase C18-HPLC column employed above was utilized with an acetonitrile gradient. The fractions containing Peaks 1–3 were pooled, methanol was removed with a SpeedVac concentrator, and the remaining solution was loaded onto the column. After 2 min, a 90-min gradient was applied from 0 to 40% acetonitrile, 0.1% trifluoroacetic acid. The flow rate was 0.25 ml/min, and absorbance was monitored at 210 nm. Alkalinating activity was assayed after adding 1 μl of each fraction to 1 ml of petunia cells. The activity eluted in fractions 48–49 for peak 1, 53–54 for peak 2, and 61–63 for peak 3. The peaks were quantified by comparing peak areas relative to known quantities of synthetic tobacco HypSys I and II. The yield for peak 1 was 100 pmol, peak 2 was 96 pmol, and peak 3 was 470 pmol.

**Peptide Analysis and Synthesis**—Amino-terminal sequencing was performed using Edman chemistry on a Procise model 492 protein sequencer (Applied Biosystems, Foster City, CA). Matrix-assisted laser desorption-ionization spectra were obtained using a PerSeptive Biosystems (Framington, MA) Voyager time of flight mass spectrometer equipped with a nitrogen laser (337 nm). α-Cyano-4-hydroxycinnamic acid (Aldrich) was used as the matrix. Peptide synthesis was performed as described (5). Carbohydrate contents were estimated from the changes in mass of each peptide after mild acid hydrolysis.

**cDNA Isolation**—The cDNAs that were previously isolated from tobacco and tomato each contain a 30-nucleotide region overlapping the processing site of the leader peptide. From these sequences, a consensus, degenerative oligonucleotide primer, 5′-GGAGCTNAAGCAAGAACTTTRCTAG-NAAAAT-3′ (where N represents G/C/T/A, and R is G/A), was synthesized for 3′ RT-PCR (Ambion, Austin, TX) to seek orthologs of the two genes in petunia. A product was produced from tobacco HypSys I and II. The peak for 1 was 100 pmol, 2 was 96 pmol, and 3 was 470 pmol.

**DNA Blot Analysis**—Genomic DNA was isolated from young leaves from a single plant of petunia according to the CTAB method described by Doyle and Doyle (21). DNA samples were restriction-digested with BamHI, EcoRI, HindIII, and XbaI, size-fractionated on a 0.8% agarose gel, and Southern blotted onto a Hybond N+ membrane (Amersham Biosciences). The blots were hybridized to [32P]dCTP-labeled specific probes.

**Expression Analyses**—Three-week-old plants having 6–8 expanded leaves were analyzed for expression levels of the two genes. The lower leaves of petunia plants were wounded with a hemostat across the midvein. Expression of the genes in both wounded and upper unwounded leaves was assayed by blot analyses with RNA collected at 0, 1, 2, 4, 6, 8, 12, and 24 h following the injury. At the time of collection, the leaf samples were immediately frozen in liquid nitrogen, ground to a fine powder in a mortar and pestle, and stored at −80 °C. Total RNA was isolated with TRIZOL reagent (Invitrogen) according to the manufacturer’s protocol, and 15 μg of each sample was separated by electrophoresis on 1.2% formaldehyde-agarose gels, blotted on Hybond N membranes (Amersham Biosciences), and hybridized with [32P]dCTP-labeled specific probes at 65 °C. 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 5 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 from 4 to 24 h at −80 °C.

**MelA Treatment**—Three-week-old plants were sprayed with a solution of 125 μl of MelA in 500 ml of double-distilled water containing 0.1% Triton X-100. The leaf samples were collected for assays at 0, 1, 2, 4, 6, 8, 12, and 24 h after spraying and immediately frozen in liquid nitrogen and stored at −80 °C.
RESULTS

Isolation of PhpreproHypSys I and II cDNAs—A consensus sequence derived from a highly conserved region of 30 nucleotides within the coding regions of NtpreproHypSys (5) and LepreproHypSys (6) (Fig. 1) was used to synthesize a primer for RT-PCR to search for an ortholog in petunia (Fig. 1B). Two cDNAs were isolated that encoded proteins of 214 and 207 amino acids, respectively (Fig. 2, A and B). The gene coding for the 214-amino acid protein, called PhpreproHypSys I, exhibited 34.2% identity with the tomato HypSys precursor and 24.7% identity with the tobacco precursor, whereas the gene coding for the 207-amino acid protein, called PhpreproHypSys II, exhibited 42.7% identity with the tobacco HypSys precursor and 20.8% identity with the tomato precursors. The deduced proteins from both genes contained leader sequences as well as internal regions of 18–21 amino acids that were rich in proline and serine residues resembling the regions in the tobacco and tomato precursor proteins from which the HypSys peptides of tobacco and tomato were derived.

Isolation and Characterization of Bioactive Petunia Leaf Hydroxyproline-rich Glycopeptides—Extracts were prepared from petunia leaves using protocols that led to the isolations and purifications of HypSys peptides from tobacco and tomato leaves (5, 6). Column fractions from purification steps were assayed using the alkalization response of suspension-cultured petunia cells (cf. “Experimental Procedures”). Three bioactive peaks were identified (Fig. 3A) and purified to apparent homogeneity, and their amino acid sequences and molecular masses (Fig. 3B) indicated that all three peptides were encoded in the PhpreproHypSys I gene (Figs. 2A and 3C). Peak 2 contained two isoforms that were identical except at residue 7, which was a serine in one isoform and a proline in the other. Commercial Petunia hybrida varieties are tetraploid, and the isoforms are a common result of polyploidy. The isoform containing the serine at residue 7 is the HypSys peptide encoded in PhpreproHypSys I (Fig. 2). The purified peptides had identical biological activities when assayed in the alkalization assay at low nanomolar concentrations. Peptides 1 and 2 contained 10 pentose units and one hexose unit, determined using mass spectrometric analyses, whereas peptide 3 contained 3–6 pentose units and no hexose, which may account for the broad peak 3 (Fig. 3B). The three peptides, because of their similarities in sizes, their hydroxyproline-rich regions, and being derived from an ortholog of tobacco and tomato HypSys precursors, were named PhHypSys I, II, and III. In Fig. 4, the amino acid sequences of HypSys peptides from petunia are compared with HypSys peptides from tobacco and tomato. The fourth putative peptide identified in the petunia precursor protein (cf. Fig. 2A, residues 41–59) is enriched in serine and proline residues and was tentatively named PhHypSys IV; it is not shown, since no peptide matching this sequence was detected during the purification of HypSys peptides. Additionally, no HypSys peptides were detected that matched any sequences in PhpreproHypSys II.

Expression Analyses of PhpreproHypSys I and PhpreproHypSys II—To analyze wound inducibility of the two genes, 3-week-old petunia plants were wounded with a hemostat across the midveins of the sixth to eighth leaves from the apex, total RNA was extracted from both the wounded leaves and upper unwounded leaves, and transcript levels were assayed over time by gel blot analyses using oligonucleotide probes specific for each gene. PhpreproHypSys I mRNA was wound-inducible and peaked in lower wounded leaves within 4 h and in upper unwounded leaves at about 6 h (Fig. 5A). The time course expression of PhpreproHypSys II was different, being constitutively expressed in leaves of unwounded plants and not increased in response to wounding, either in wounded leaves or in upper unwounded leaves (Fig. 5A). PhpreproHypSys I was rapidly and strongly induced by MeJA within 1 h, but PhpreproHypSys II expression gradually increased over the 6-h duration of the experiments (Fig. 5B).

In young tomato plants, protease inhibitor and polyphenol oxidase activities increase in response to wounding and exposure to MeJA vapors. Therefore, protease inhibitor and polyphenol oxidase activities were assayed in petunia leaves in response to wounding and MeJA. These assays were also performed in leaves in response to PhHypSys peptides, supplied through their cut petioles. We found no evidence for an increase in protease inhibitor activities or polyphenol oxidase activity in any of these experiments (data not shown). We therefore considered the possibility that the HypSys peptides may be activating defense genes against pathogens, such as defensin 1, rather than against herbivores, as we had recently found with AtPep1 and homologs in Arabidopsis (7). To determine if wounding petunia leaves would induce the expression of defensin 1, leaves of young plants were wounded, and the expression of defensin 1 was assayed in leaf RNA using RT-PCR at 0, 4, 12, and 24 h. In Fig. 6, the expression of the gene is shown to increase through 4–12 h and then decrease by 24 h.

Petunia leaves were supplied through the cut petioles with 8 pmol of PhHypSys I, II, and III, and increases in the expression of defensin 1, a component of the innate immune response in plants (17, 19), was assayed in leaf extracts 4 h later. defensin 1 was expressed in response to all three of the peptides (Fig. 7A), indicating that the peptides, as with wounding, were activating the defensin 1 gene. PhHypSys I, II, and III were chemically synthesized but lacking the carbohydrate residues found in the native peptides, and they were assayed for their abilities to cause an alkalization response. The synthetic peptides were active at micromolar concentrations rather than at nanomolar concentrations as found with the native peptides, and they were assayed for their ability to cause an alkalization response. The synthetic peptides were active at micromolar concentrations rather than at nanomolar concentrations as found with the native peptides, indicating the importance of the carbohydrates to bioactivity. Supplying the synthetic peptides to leaves through their cut

Hydroxyproline-rich Glycopeptide Defense Signals from Petunia

FIGURE 2. Nucleotide and predicted amino acid sequences of two petunia cDNAs selected with the primer from Fig. 1. A, petunia proline-rich cDNA I. The putative signal sequence is italicized. A dashed underline indicates a 30-nucleotide region found in tomato and tobacco peptide precursors (5, 6) from which a consensus oligonucleotide primer was synthesized for 3′ RT-PCR amplification of petunia orthologs. Proline-rich regions within the sequences that are similar to defense peptides previously isolated from tobacco and tomato leaves are underlined with a solid line. B, petunia proline-rich cDNA II. The underlined regions are as described in A.
Hydroxypoline-rich Glycopeptide Defense Signals from Petunia

DISCUSSION

HypSys peptides in tobacco (5) and tomato (6) leaves are powerful inducers of anti-herbivore defense genes. The isolation of these peptides and their precursor genes prompted a search for similar peptides and genes in leaves of other Solanaceae species. A petunia (Petunia hybrida) HypSys precursor gene was sought using RT-PCR with mRNA from leaves of plants treated with MeJA 15 h before harvesting (MeJA induces the expression of HypSys precursor genes in tobacco and tomato leaves). An oligonucleotide primer with a consensus sequence based on a highly conserved 30-bp region found in tomato and tobacco preproHypSys genes (Fig. 1) successfully identified two petunia leaf cDNAs encoding proteins homologous to the tobacco and tomato HypSys precursors (Fig. 2, A and B). The precursor genes were named PhpreproHypSys I and PhpreproHypSys II, consistent with the tobacco and tomato precursor genes (5, 6). The proteins encoded by the two proteins contained 214 and 207 amino acids, respectively, that included four proline-rich regions flanked by charged residues (Fig. 2, A and B). Three of the four regions were identical to peptides isolated from a petunia leaf extract from PhpreproHypSys I (Fig. 3, A and B). Proline residues of the purified peptides were post-translationally modified with hydroxyl and glycosyl groups (Fig. 3C), consistent with the HypSys peptides characterized from tobacco and tomato leaves. Three peptides were named PhHypSys I, II, and III, although the fourth encoded peptide (Fig. 2A, residues 41–59), which was not isolated, was named PhHypSys IV. If this peptide is found, then the precursor will be the first example of a plant polypeptide that produces more than three bioactive peptides. No peptides were purified that were encoded by PhpreproHypSys II.

The amino acid sequences of the three HypSys peptides from petunia are compared in Fig. 4 with those of tobacco and tomato leaves. Hydroxypoline-rich regions are in red.

**FIGURE 3. Identification and isolation of bioactive peptides in extracts from petunia leaves.** A, alkalination assays of fractions of a petunia leaf extract fractionated by C18 reversed-phase HPLC with an acetonitrile gradient (see “Experimental Procedures”). Ten-microliter aliquots from 1-ml fractions eluting from the column were added to 1 ml of suspension cultured petunia cells and, after 20 min, the pH of the medium was recorded. B, final HPLC purifications of the three peptides from A. The broad peak 3 peptide reflects a heterogeneity of attached carbohydrate adducts. C, amino acid sequences and carbohydrate residues of the three peptides from B. O, hydroxypoline. The locations of the carbohydrate residues have not been established.

**FIGURE 4. A comparison of HypSys peptides isolated from petunia, tobacco, and tomato leaves.** Hydroxypoline-rich regions are in red.

**TABLE 1.**

| Peptide    | Peptide Backbone Sequence | Mass    | Carbohydrate Residues |
|------------|---------------------------|---------|-----------------------|
| Peptide 1  | RSLHKSOOTOKPSDEQGQ        | 2147.3  | 10                    |
| Peptide 2  | RDIHLSQQOAKPDAHTGQ        | 2285.4  | 10                    |
| Peptide 3  | RGKRLPOOAEOYDPOYHQ        | 2182.3  | 3                      |

1 Mass includes the one hexose of 162 MU that is not released upon acid hydrolysis.

petioles at 25 nmol/leaf resulted in the expression of defensin within 2 h (Fig. 7B).

**FIGURE 4. A comparison of HypSys peptides isolated from petunia, tobacco, and tomato leaves.** Hydroxypoline-rich regions are in red.
the hydroxyproline-rich motifs, each peptide contains several carbohydrate residues, mainly pentoses, that may contribute to the conformations required for receptor recognition. While cell surface leucine-rich repeat receptor kinases have been isolated for the systemin and Pep1 peptides, a receptor for a HypSys peptide has not yet been identified.

The PhpreproHypSys peptide precursor protein, deduced from the PhpreproHypSys I sequence, is initially synthesized with a leader sequence and is presumed to be synthesized through the secretory pathway, where post-translational hydroxylations and glycosylations occur. A pSORT program (26) analysis suggests that the nascent PhpreproHypSys I is sequestered in the cell wall, similar to the LepreproHypSys protein (12). The three PhHypSys peptides derived from the precursor contain N-terminal Arg residues that are part of a GR motif that may be a component of the processing site (Fig. 2A). GR residues are also found in the LepreproHypSys precursor at the N termini processing sites of the three tomato HypSys peptides and at the N terminus of one of the two tobacco HypSys peptides (5, 6). The GR motif is a strong candidate for being part of the recognition sequence for a processing enzyme, and the loss of the Gly at the N termini in several HypSys peptides suggests that an exopeptidase may trim the N termini. Each of the petunia peptides terminates in a Gln residue, as do two of the three tomato HypSys peptides (Fig. 4), but in the two tobacco precursor proteins (5), no Gln residues are found near the C termini.

PhpreproHypSys I is expressed in petunia leaves in response to wounding (Fig. 5A) in both the wounded leaves and in the upper unwounded leaves (Fig. 5A). This systemic wound response is similar to that found with prosystemin (16), NtpreproHypSys (5), and LepreproHypSys (6). In contrast, PhpreproHypSys II is constitutively expressed in leaves and does not increase in response to wounding. PhpreproHypSys I is strongly expressed in response to MeJA, whereas PhpreproHypSys II expression increases slowly with time (Fig. 5A). Further investigations of the tissue- and cell-specific localization of PhpreproHypSys II expression and its encoded protein and peptides may reveal the role of this gene in petunia plants.

Our finding that the PhHypSys peptides activated expression of defensin 1, an inducible defense protein, supports the proposal that the roles of plant peptide defense signals are to amplify signaling pathways that regulate diverse defense responses against predators and/or pathogens (27–29) and strengthens an emerging paradigm in which the induction of endogenous peptides in response to pest and pathogen attacks strengthens the inducible defense responses that have evolved in each plant species.

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