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Hydroxyproline-rich glycopeptide signals in potato elicit signalling associated with defense against insects and pathogens

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ABSTRACT

HypSys peptides are 18–20 amino acids glycopeptide defense signal first discovered in tobacco and tomato that activate expression of defensive genes against insect-herbivores. Discovery of their orthologs in other Solanaceaous and nonsolanaceous plants demonstrated their possible ubiquitous nature and species specific functional diversity. In our continued search to establish the paradigm of defense signalling by HypSys peptides, we isolated a cDNA from potato leaves encoding putative analogs of tomato HypSys peptides flanked by conserved proteolytic cleavage sites. The gene encoding the cDNA was a member of a gene family in the tetraploid genome of potato and its expression was transcriptionally activated by wounding and methyl jasmonate. The deduced precursor protein contained a leader peptidase splice site and three putative HypSys peptides with conserved N- and C-termini along with central proline-rich motifs. In defense signalling, the three HypSys peptides elicit H_2O_2 generation in vivo and activate several antioxidant defensive enzymes in young potato leaves. Similar to potato systemin, the HypSys peptides activate the expression of octadecanoid pathway genes and protease inhibitors for insect defense. In addition, the HypSys peptides also activate the essential genes of the innate pathogen defense response in young potato leaves, acting as common elicitors of signalling associated with anti-herbivore and anti-pathogen defense in potato.

1. Introduction

Peptide elicitors, which activate innate immunity of plants, are either derived from plants or by molecules from invading organisms [1,2]. The first endogenous plant derived peptide signal, systemin, was discovered in tomato and later found to be functionally conserved in potato, pepper and nightshade; the members of the Solanoideae subfamily of the Solanaceae family [3]. When systemin was added to tomato cell suspension culture, it rapidly alkalinized the cellular medium via the blockage of a proton pump in the cell membranes [4,5]. In peptide application assays as well as in constitutively expressing transgenic plants, systemin was demonstrated to act as a primary defense signal against herbivory and a powerful inducer of host protease inhibitors [1,6,7].

Although tobacco plants lack any gene sequence orthologous to prosystemin, systemic activation of protease inhibitor genes in leaves in response to wounding was demonstrated [8]. A search for a functionally similar peptide to systemin in tobacco was undertaken via a suspension cell assay. Small aliquots (1–10 μL) of HPLC-purified fractions of tobacco leaf extracts, when added to 1 mL aliquots of tobacco suspension cells, produced a rapid medium alkalinizing response (the alkalinization assay). Purification of the alkalinizing peaks in tobacco led to the discovery of two hydroxyproline-rich (HypSys) glycopeptides, both 18 amino acids in length, as alternative peptide elicitors of defense responses [9]. Subsequently, in tomato, three HypSys glycopeptides [10,11] were identified and these HypSys peptides were considered to function cooperatively with systemin to provide the strong defense response found in tomato [12]. Interestingly, in members of the Solanaceae, the structural motifs of the HypSys peptides are conserved and processed from a common polyprotein precursor. In tobacco, both HypSys peptides are processed from a common 165 amino acid precursor encoded by a single gene (*Ntpre*proHypSys) [9]. Similarly, in tomato, all three HypSys peptides are generated from a 146 amino acid precursor (SlproHypSys) [10]. However, it is not known with certainty whether different HypSys peptides, co-regulated and encoded on the same precursor protein, have different functions or specificities in inducing defense responses.

Based on their abilities to alkalinize the external medium of cell suspension cultures, HypSys glycopeptides were identified in petunia (*Petunia hybrida)* [13], and in a member of the Convolvulaceae

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family, sweet potato (*Ipomoea batatas)* [14]. A biochemical purification of HypSys glycopeptides from blacknightshade (*S. nigrum*) revealed three HypSys peptides and an orthologous cDNA was isolated [15]. The conserved structural features of the proHypSys sequences include an N-terminal secretory signal for localization to the cell wall matrix. During passage through the secretory system, HypSys peptides are post-translationally modified with hydroxylation of multiple proline residues followed by glycosylation with pentose sugars [16].

At the transcriptional level, *preproHypSys* expression increases in response to methyl jasmonate (MeJ), mechanical wounding, and also to infestations of *Bemisia tabaci* and *Manduca sexta* larvae in tobacco [17]. In tobacco, tomato and black nightshade, HypSys peptides, when supplied through cut petioles, strongly induced the synthesis of protease inhibitors in a similar fashion to systemin in tomato. Therefore, systemin and HypSys peptides, although dissimilar in sequence, have been classified together as functional homologs, both co-regulating the wound response by inducing early defense signals in the octadecanoid pathway to activate defense genes against herbivory [12,18]. In petunia, supplying young plants with HypSys did not induce protease inhibitor activity but instead increased the expression of a *defensin 1* gene known to be involved in protection from pathogen attack, indicating a species specificity of HypSys function and the possibility of functional diversity within a family of signalling peptides [13]. Thus, petunia HypSys is more functionally related to the AtPep family of defense peptides, which have been shown to be involved in defense against pathogen attack [19].

In potato, the only known defense peptide is systemin, which is analogous to tomato systemin and induces protease inhibitors against herbivores. Here, we report the identification of *StpreproHypSys* cDNA from potato which is orthologous to its counterparts in tomato and other members of the Solanaceae and contains three putative HypSys glycopeptides. The putative processed StHypSys glycopeptides are similar to tomato HypSys peptides with 78–85% identity in amino acid sequence. Interestingly, synthetic potato HypSys peptides activate several essential genes of horizontal pathogen resistance in addition to activating jasmonate-mediated insect defense. Also, StHypSys peptides elicit activation of defense enzymes to combat free radical generation and oxidative stress, a common denominator of insect and pathogen attack. To the best of our knowledge this is the first demonstration of plant derived defense peptides acting as common elicitors to both insect defense as well as pathogen defense signalling.

2. Material and methods

2.1. Isolation of StpreproHypSys cDNA

The cDNA sequence coding for the proHypSys precursors in tobacco, tomato, petunia and black nightshade contain a conserved stretch of 30 nucleotides towards the 5/end of the nucleotide sequence coding for the HypSys peptides. From these sequences, a degenerative oligonucleotide primer, 5/-GGAGCTNAAGCAAGAACTTTRCTAGNAAAT-3/(where N represents G/C/T/A, and R is G/A), was synthesized for 3/RACE-PCR (Ambion, Austin TX) to seek orthologs of HypSys coding gene in potato. A 700 bp amplified product was cloned by TOPO (Invitrogen, Carlsbad CA), sequenced, and found to contain *orf* encoding stretches of amino acid sequence homologous to *SlpreproHypSys*. To obtain the complete cDNA sequence, 5/RACE-PCR was performed using a reverse primer specific to an internal sequence that overlapped the 3/RACE-PCR product by 186 bp: 5/-TCCTTCTCCCAAGCATGAA-3/(potato inner).

2.2. Southern-blot analysis

Genomic DNA was extracted from young leaves of a single potato plant by the CTAB (cetyltrimethyl ammonium bromide) method described by Doyle and Doyle [20]. DNA samples were purified by phenol: chloroform extraction. Purified DNA (10 μg) was restriction digested with *Bam*HI, *Eco*RI, *Hind*III, and *Sst*I, run on a 0.8% agarose gel, and blotted onto a Hybond N^+ membrane (Amersham Biosciences, Piscataway NJ). The membranes were hybridized to [^{32}P]-dCTP labelled *StpreproHypSys* specific probes.

2.3. Mechanical wounding and MeJ treatment of plants

Potato plants (*Solanum tubersum* L.) were grown from True Potato Seeds (TPS) in glasshouses. Four-week-old plants with six to eight expanded leaves were transferred to a growth chamber at least 12 h before using in the wounding experiments or MeJ treatment. For wounding, the fifth and sixth leaves from the top were wounded repeatedly across the mid-vein using a hemostat. From the wounded plants the unwounded systemic upper leaves were collected at 0, 2, 4, 6, 8, and 10 h following the mechanical injury for performing time course experiment. The corresponding leaves from the unwounded plants served as controls for each time point. The leaf samples were frozen in liquid nitrogen and kept at −80 °C until used. For MeJ treatment the plants were sprayed with solutions of 125 μL of MeJ in 500 mL of double distilled water containing 0.1% Triton X-100. The leaf samples were collected for time course experiments 0, 1, 2, 4, 6, and 8 h after spraying, immediately frozen in liquid nitrogen, and kept at −80 °C until used.

2.4. RNA isolation and Northern blot analysis

For RNA isolation of Northern blot analysis, approximately 500 mg of leaf material consisting of pooled leaf sample from three independent plants was ground to fine powder in liquid N_2, and total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad CA) according to the manufacturer's protocol. Fifteen microgram total RNA of each sample was run on 1.2% formaldehyde agarose gels, blotted on Hybond N membranes (Amersham Biosciences, Piscataway NJ), and hybridized overnight with [^{32}P]-dCTP labelled probes at 65 °C. Ethidium bromide-stained rRNA-bands were used to ensure equal loading. Following hybridization, membranes were washed twice with 2X SSC/0.1% SDS for 10 min each at 55 °C, followed by two washes each with 0.5X SSC/0.1% SDS for 10 min and two washes with 0.1X 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.

2.5. In vivo detection of H_2O_2

Fully expanded third or fourth leaves from the apex from four week old plants were excised and supplied with peptides dissolved in water or water as control. H_2O_2 accumulating in the leaves was visually detected using 3, 3-diaminobenzidine (DAB) as substrate [21]. After 2 h of peptide application, the leaves were incubated in 1 mg/ml DAB solution, pH 3.8, for 2 h in the dark at room temperature. The leaves were decolorized by boiling in ethanol for 10 min. After cooling, the leaves were transferred to fresh ethanol at room temperature and analyzed.

H_2O_2 was estimated according to Alexieva et al. [22]. Leaf tissue (0.5 g) was crushed in 10 ml of 0.1% trichloroacetic acid (TCA) and centrifuged at 1000 g for 30 min at 4 °C. The clear supernatant was used for the assay. The reaction mixture consisted of 0·5 mL supernatant, 0·5 mL of 0.1 mM potassium phosphate buffer and 2 mL of freshly prepared 1 M KI. The reaction mixture was incubated for 1 h in the dark and absorbance measured at 390 nm. A standard curve