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TobpreproHypSys-A Gene Expression and Defense Protein Activity in the Tobacco Wounding Response

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Tobacco hydroxyproline-rich glycopeptide systemin precursor A (TobpreproHypSys-A), from which TobHypSys I and II are released, plays a crucial role in defense responses. Here, we investigated the expression of *TobpreproHypSys-A* and the activity of defense proteins in tobacco organs during wounding. Expression was induced more rapidly in upper, non-wounded leaves than in lower, wounded leaves. At 24 h after mechanical wounding, expression was low in the roots, but increased in the stems and flowers, although to a lesser extent than in the leaves. At 3 or 10 d after insect-wounding, expression did not differ among organs, suggesting that *TobpreproHypSys-A* could be induced globally and continuously throughout such stress. During that period, the activity of two defense proteins -- PPO and PI -- was consistent with the expression of *TobpreproHypSys-A* in various organs. This indicates that those proteins also could be regulated by TobHypSys, both globally and continuously.

Keywords: *Nicotiana tabacum*, polyphenol oxidase, proteinase inhibitor, systemin, wounding response

Sophisticated mechanisms enable higher plants to cope with threats from herbivores and pathogens. One of these means is the production of an array of defense molecules, including antimicrobial toxins and proteins. In solanaceous species, proteinase inhibitors (PIs) can be accumulated systemically after wounding (Green and Ryan, 1972; Heitz et al., 1993; Pearce et al., 1993; Pena-Cortes et al., 1995). This indicates the presence of a mobile factor responsible for the induction of defense proteins. For example, crude extracts of wounded tomato leaves activate PI genes when applied through the cut stems of young excised plants (Pearce et al., 1991). The factor found in that species is systemin (TomSys), an 18-amino acid polypeptide (Pearce et al., 1991). TomSys, the first plant polypeptide hormone to be identified (Ryan, 2000), is proteolytically processed from a 200-amino acid precursor protein, prosystemin (TomproSys) (McGurl et al., 1992). Because of constitutive expression (Dombrowski et al., 1999; Vetsch et al., 2000), the enzymes involved in that processing also may be present at lower levels before wounding occurs. TomSys causes a cascade of intracellular signaling events leading to the expression of defense genes (Farmer and Ryan, 1992; Conconi et al., 1996; Stratmann and Ryan, 1997; Ryan, 2000). After these events are initiated by its release at wounded sites, TomSys interacts with a membrane-bound receptor, SR160, which is a leucine-rich repeat (LRR) receptor kinase with high amino acid identity and domain similarities to the BRI1 receptor kinase from *Arabidopsis* (Li and Chory, 1997; Meindl et al., 1998; Scheer and Ryan, 1999).

The critical role for TomproSys and TomSys in the defense response has been revealed by experiments in which tomato plants are transformed with *TomproSys* cDNA under the control of the constitutive 35S promoter (McGurl et al., 1992, 1994; Orozco-Cardenas et al., 1993). Those that are transformed with *TomproSys* cDNA in the sense orientation constitutively synthesize wound-inducible defensive proteins throughout the plants in the absence of wounding (McGurl et al., 1994). By contrast, transgenic plants expressing antisense *TomproSys* are severely impaired in their systemic PI induction in wounding responses, and are also compromised in their ability to defend against insect larvae (McGurl et al., 1992; Orozco-Cardenas et al., 1993). These experiments, therefore, demonstrate that TomSys is the primary signal of the wounding response.

Systemin and its precursor have been identified in several other solanaceous species, including potato, black nightshade, and bell pepper (Constabel et al., 1998). However, tobacco, another Solanaceae member, does not express a gene homologous to *TomproSys* (McGurl et al., 1992). Moreover, TomSys is inactive in inducing defense-gene expression in tobacco leaves, thereby indicating that the systemin receptor in that species is different (Ryan, 2000). Nevertheless, tobacco plants do exhibit a wound response that systemically activates the synthesis of a family of tobacco trypsin inhibitors homologous to tomato PI II (Pearce et al., 1993). Those transformed with a TomSys receptor gene, *SR160*, generate systemin-signaling, thus demonstrating that the early steps within the systemin signaling pathway in tomato also are present in tobacco (Scheer et al., 2003). Two systemic 18-residue hydroxyproline-rich glycopeptides -- tobacco hydroxyproline-rich systemin I (TobHypSys I) and tobacco hydroxyproline-rich systemin II (TobHypSys II) -- have been biochemically isolated from tobacco leaves (Pearce et al., 2001). Although both share a 165-residue precursor protein, TobpreproHypSys-A, they are not homologous to each other (Pearce et al., 2001), nor do they share any homology with TomSys (Pearce et al., 2001). *TobpreproHypSys-A* is induced systemically when tobacco leaves are wounded (Rocha-Granados et al., 2005), and it plays a crucial role in regulating defense genes for resistance against herbivorous insects (Ren and Lu, 2006). However, no published data have previously revealed the expression of *TobpreproHypSys-A* and the activity of defense proteins in

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different organs during wounding responses. Therefore, our objective here was to analyze this expression and activity in different tobacco organs after responses to various wounding treatments.

MATERIALS AND METHODS

Plant Material and Growing Conditions

Seeds of tobacco (*Nicotiana tabacum* cv. SR1) were surface-sterilized and germinated on plates with Murashige and Skoog (MS) medium containing 0.8% agar. After 10 d in controlled environment chambers at 22°C, the germinated seedlings were transferred to soil in the greenhouse and grown under long days (16-h photoperiod) at 65% humidity. After flowering (about Day 40), the plants were sampled for our experiments.

Mechanical Wounding Treatment

The lower leaves from flowering tobacco plants were crushed with a hemostat three or four times across the main vein (Bergey et al., 1999). Afterward, those plants were further incubated in the greenhouse, and total RNAs were isolated from their roots, stems, leaves, and flowers.

Insect-feeding Trials

Helicoverpa armigera eggs were hatched at 25°C, and their larvae were reared on an artificial diet for 3 d. At the beginning of the feeding trials, 10 larvae were transferred to the leaves of each flowering plant in the greenhouse. After the larvae fed on these plants for 3 or 10 d, total RNAs were isolated from the roots, stems, leaves, and flowers. Activity of two defense proteins, PPO and PI, also was analyzed from those organs.

RNA Extraction and RT-PCR Analysis

Total RNAs from sampled roots, stems, leaves, and flowers were isolated with TRIZOL®, and purified with a Qiagen RNeasy Mini kit. First-strand synthesis of cDNAs was performed with M-MLV reverse transcriptase (Promega). *TobpreproHypSys-A* was amplified with a pair of primers, 5'-ATGAGAGTTCTGTTTCTCATCTACC3' and 5'-TTAATAGGAGTCAAGAGGACGCTG-3'. The tobacco ubiquitin-conjugating enzyme gene, *Ntubc2*, was amplified with another pair of primers, 5'-GAAGAGACTGGTGAGGGATTTTAAG-3' and 5'-GCGCACCTTCCTGTTGTATTCG-3', as a loading control for equal RNA input and also as an internal standard for quantification (Rocha-Granados et al., 2005). The time/temperature profiles employed for *TobpreproHypSys-A* amplification were as follows: initial denaturation at 94°C for 5 min; followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 56°C), and extension (1 min at 72°C). The predicted product size of *TobpreproHypSys-A* was 498 bp. PCR conditions for *Ntubc2* amplification were nearly identical to those used for *TobpreproHypSys-A*, except that 25 cycles were required in the linear phase of the reaction. The predicted product size of *Ntubc2* was 409 bp. PCR products were analyzed by running 10 µL aliquots of the PCR reaction mixtures in 1.0% agarose gels stained with ethidium bromide.

PPO Activity Analysis

PPO activity was assayed spectrophotometrically as described previously (Laukkanena et al., 1999). Tobacco roots, stems, leaves, and flowers (100 mg FW each) were ground in 1 mL of 100 mM cold sodium phosphate (pH 7.0) on ice before the extracts were clarified by centrifugation. The supernatant (100 mL) was added to a reaction mixture (1000 mL) containing 50 mM sodium phosphate (pH 8.7) and 10 mM catechol. The change in absorbance was measured at 410 nm during 5 min of incubation at 25°C.

PI Activity Analysis

Proteinase inhibitor activity was analyzed according to a protocol described previously (Stout et al., 1998). This assay was based on the ability of plant extracts to block chymotrypsin activity, and was reported as a percentage of the inhibition of the control.

RESULTS

Expression of TobpreproHypSys-A in Tobacco Organs after Wounding

After mechanical wounding, expression of *TobpreproHypSys-A* was induced in both wounded and non-wounded tobacco leaves (Fig. 1a). In the lower, wounded leaves, transcript levels increased dramatically at 8 h after treatment, reaching a maximum at 24 h, then decreasing gradually 36 h after mechanical wounding (Fig. 1a). In the upper, (younger) non-wounded leaves, *TobpreproHypSys-A* expres-

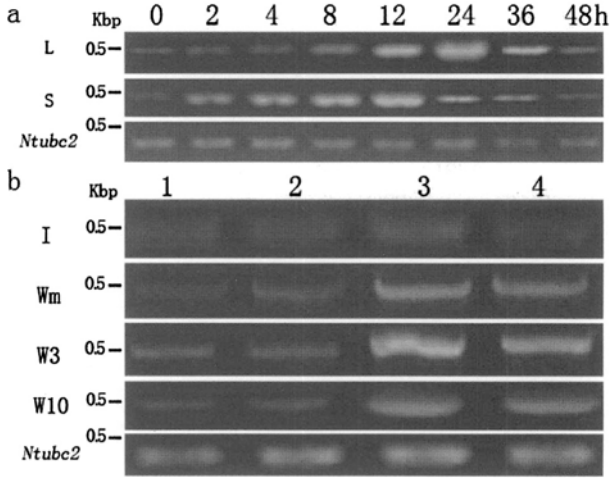

Figure 1. RT-PCR analysis of *TobpreproHypSys-A* expression after wounding. **a,** Total RNAs were isolated from lower wounded (L, indicating local response) and upper non-wounded (S, indicating systemic response) tobacco leaves at 0, 2, 4, 8, 12, 24, 36, or 48 h after mechanical wounding; **b,** total RNAs were isolated from roots (1), stems (2), leaves (3), and flowers (4) of intact plant (I) at 24 h after mechanical wounding (Wm), or at 3 d (W3) or 10 d (W10) after insect-feeding.

sion was rapidly and systemically induced, rising significantly 2 h after mechanical wounding, and increasing to a maximal level at 12 h (Fig. 1a).

In the intact plant, the level of *TobpreproHypSys-A* expression at 24 h after mechanical wounding was low in the roots, somewhat greater in the stems and flowers, and highest in the leaves (Fig. 1b). At 3 or 10 d after the larvae-feeding trials began, expression also was low in the roots, but distinctly increased in the stems and flowers, and to an even greater extent in the leaves (Fig. 1b). Although levels of *TobpreproHypSys-A* transcript were correspondingly decreased in these organs after 10 d, its pattern of expression due to that treatment was not noticeably different from that observed at 24 h after mechanical wounding or 3 d after larvae-feeding (Fig. 1b).

PPO Activity in Tobacco Organs during Insect-feeding

PPO is a crucial defense protein, protecting plants against attacks by insects and pathogens (Ryan, 2000). In our intact plants, its activity was globally low, with no remarkable differences found among organs (Fig. 2). At 3 or 10 d after the feeding trials began, activity increased only slightly in the roots, but more obviously in other organs, especially the leaves. For example, by Day 3, activity was about 3, 10, and 7 times greater in the stems, leaves, and flowers, respectively, compared with the control; at Day 10, those relative increases were 2, 9, and 6 times, respectively (Fig. 2).

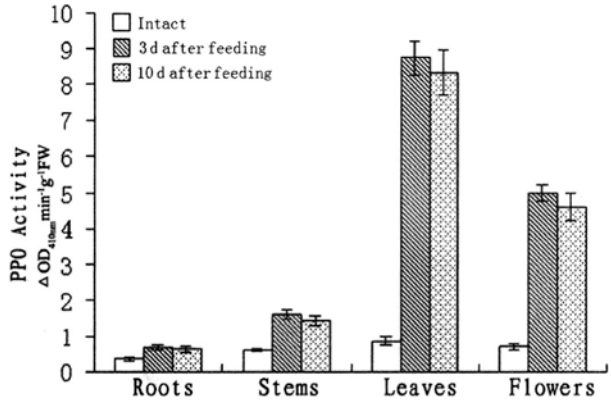

Figure 2. Analysis of PPO activity in intact and larvae-wounded plants. Values represent means ± SD (n=10).

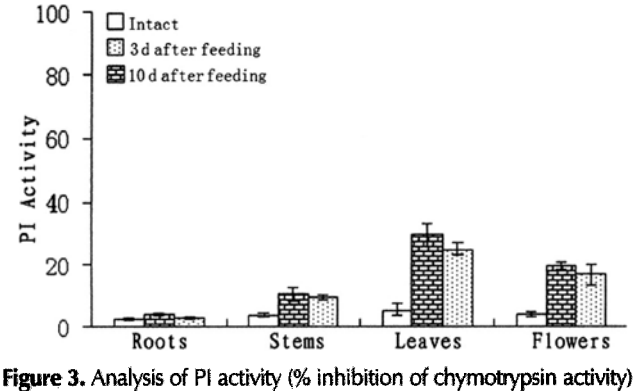

Figure 3. Analysis of PI activity (% inhibition of chymotrypsin activity) in intact and larvae-wounded plants. Values represent means ± SD (n=10).

PI Activity in Tobacco Organs during Insect-feeding

Proteinase inhibitor is an important plant defense protein (Ryan, 2000). In our roots, stems, leaves, and flowers from intact plants, its activity was low, with less than 5% of the chymotrypsin activity being inhibited. At 3 d after the feeding trials, PI activity rose only slightly in the roots, while chymotrypsin activity was distinctly inhibited in the stems (4%), leaves (30%), and flowers (20%; Fig. 3). By Day 10, about 3%, 9%, 24%, and 16% of chymotrypsin activity had been blocked in the roots, stems, leaves, and flowers, respectively (Fig. 3).

DISCUSSION

Characterization of TobpreproHypSys-A differs from that of TomproSys (McGurl et al., 1992; Pearce et al., 2001). TobHypSys I and II, which are released from TobpreproHypSys-A, are not homologous to each other or to TomSys (Pearce et al., 2001). Furthermore, TomSys is inactive when supplied to tobacco plants, indicating that the receptor of TobHypSys I and II varies from that of the TomSys receptor, SR160 (Ryan, 2000). *TobpreproHypSys-A* is systemically induced in mechanically wounded tobacco leaves, and is crucial during herbivore attacks (Rocha-Granados et al., 2005; Ren and Lu, 2006). This indicates that, although distinct differences exist between them, the role of TobpreproHypSys-A (TobHypSys) is similar to that of TomproSys (TomSys) in defense responses (Ryan, 2000). No data have been reported previously that illustrate the expression patterns of *TobpreproHypSys-A* in different tobacco organs during the wounding response. Although the inductivity of *TobpreproHypSys-A* by mechanical wounding differs during various stages of development, its expression generally is induced as soon as wounding occurs in younger leaves, but is delayed in more mature tissues (Rocha-Granados et al., 2005). Our data showed that, in treated tobacco plants, this expression increased more rapidly in the upper, non-wounded leaves than in the lower, treated leaves (Fig. 1a). At 24 h after mechanical wounding, *TobpreproHypSys-A* was induced globally, although transcript levels varied among organs (Fig. 1b). In contrast, herbivore-feeding, which was continuous, elicited a different response, with expression patterns from insect-treated organs at 3 and 10 d being similar to those measured at 24 h after mechanical wounding. This demonstrates that the expression of *TobpreproHypSys-A* was maintained at a higher level during larvae-feeding, and it provides a critical clue for our understanding of the plant wounding response to herbivory.

In tobacco leaves, *TobpreproHypSys-A* has a function similar to that of TomproSys, i.e., modulating two defense proteins, PPO and PI (Ren and Lu, 2006), that play an important role in plant resistance against herbivorous insects (Ryan, 2000). During the larvae-feeding trials, their activities were consistent with the expression of *TobpreproHypSys-A* in all organs, suggesting that the expression of defense genes is regulated by TobHypSys throughout the plant. Nevertheless, the degree of *TobpreproHypSys-A* expression, and activity by those defense proteins, was correlated with the extent of their functioning in different tissues and organs;

i.e., whereas the roots were not prone to feeding by insects, the leaves were the most vulnerable to such attacks and other wounding agents.

ABBREVIATIONS

PI, proteinase inhibitor; PPO, polyphenol oxidase; TobpreproHypSys, tobacco hydroxyproline-rich glycopeptide systemin precursor; TobHypSys, tobacco hydroxyproline-rich glycopeptide systemin; TomproSys, tomato prosystemin; TomSys, tomato systemin

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LITERATURE CITED

Bergey DR, Orozco-Cardenas M, Moura DS, Ryan CA (1999) A wound- and systemin-inducible polygalacturonase in tomato leaves. Proc Natl Acad Sci USA 96: 1756-1760

Conconi A, Miquel M, Ryan CA (1996) Changes in the intracellular lipid composition and free fatty acids of tomato leaves in response to wounding. Plant Physiol 111: 797-803

Constabel CP, Yip L, Ryan CA (1998) Prosystemin from potato, black nightshade, and bell pepper: Primary structure and biological activity of predicted systemin polypeptides. Plant Mol Biol 36: 55-62

Dombrowski JE, Pearce G, Ryan CA (1999) Proteinase inhibitor-inducing activity of the prohormone prosystemin resides exclusively in the C-terminal systemin domain. Proc Natl Acad Sci USA 96: 12947-12952

Farmer EE, Ryan CA (1992) Octadecanoid jasmonate precursors activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell 4: 129-134

Green TR, Ryan CA (1972) Wound-induced proteinase inhibitor in plant leaves: A possible defense mechanism against insects. Science 175: 776-777

Heitz T, Geoffroy P, Stintzi A, Fritig B, Legrand M (1993) cDNA cloning and gene expression analysis of the microbial proteinase inhibitor of tobacco. J Biol Chem 268: 16987-16992

Laukkanena H, Haggman H, Kontunen-Soppela S, Hohtola A (1999) Tissue browning of in vitro cultures of Scots pine: Role of peroxidase and polyphenol oxidase. Physiol Plant 106: 337-343

Li J, Chory J (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 90: 929-938

McGurl B, Pearce G, Orozco-Cardenas M, Ryan CA (1992) Structure, expression, and antisense inhibition of the systemin precursor gene. Science 255: 1570-1573

McGurl B, Orozco-Cardenas M, Pearce G, Ryan CA (1994) Overexpression of the prosystemin gene in transgenic tomato plants generates a systemic signal that constitutively induces proteinase inhibitor synthesis. Proc Natl Acad Sci USA 91: 9799-9802

Meindl T, Boller T, Felix G (1998) The plant wound hormone systemin binds with the N-terminal part to its receptor but needs the C-terminal part to activate it. Plant Cell 10: 1561-1570

Orozco-Cardenas M, McGurl B, Ryan CA (1993) Expression of an antisense prosystemin gene in tomato plants reduces resistance toward *Manduca sexta* larvae. Proc Natl Acad Sci USA 90: 8273-8276

Pearce G, Strydom D, Johnson S (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. Science 253: 895-898

Pearce G, Johnson S, Ryan CA (1993) Purification and characterization from tobacco (*Nicotiana tabacum*) leaves of six small, wound-inducible, proteinase isoinhibitors of the potato inhibitor II family. Plant Physiol 102: 639-644

Pearce G, Moura DS, Stratmann J, Ryan CA (2001) Production of multiple plant hormones from a single polyprotein precursor. Nature 411: 817-820

Pena-Cortes H, Fisahn J, Willmitzer L (1995) Signals involved in wound-induced proteinase inhibitor gene expression in tomato and potato plants. Proc Natl Acad Sci USA 92: 4106-4113

Ren F, Lu YT (2006) Overexpression of tobacco hydroxyproline-rich glycopeptide systemin precursor A gene in transgenic tobacco enhances resistance against *Helicoverpa armigera* larvae. Plant Sci 171: 286-292

Rocha-Granados Cdel M, Sanchez-Hernandez C, Martýnez-Gallardo NA, Ochoa-Alejo N, Delano-Frier JP (2005) The expression of the hydroxyproline-rich glycopeptide systemin precursor A in response to (a)biotic stress and elicitors is indicative of its role in the regulation of the wound response in tobacco (*Nicotiana tabacum* L.). Planta 222: 794-810

Ryan CA (2000) The systemin signaling pathway: Differential activation of plant defensive genes. Biochim Biophys Acta 1477: 112-121

Scheer JM, Ryan CA (1999) A 160-kD systemin receptor on the surface of *Lycopersicon peruvianum* suspension-cultured cells. Plant Cell 11: 1525-1535

Scheer JM, Pearce G, Ryan CA (2003) Generation of systemin signaling in tobacco by transformation with the tomato systemin receptor kinase gene. Proc Natl Acad Sci USA 100: 10114-10117

Stout MJ, Brovont RA, Duffey SS (1998) Effect of nitrogen availability on expression of constitutive and inducible chemical defenses in tomato, *Lycopersicon esculentum*. J Chem Ecol 24: 945-963

Stratmann JW, Ryan CA (1997) Myelin basic protein kinase activity in tomato leaves is induced systemically by wounding and increases in response to systemin and oligosaccharide elicitors. Proc Natl Acad Sci USA 94: 11085-11089

Vetsch M, Janzik I, Schaller A (2000) Characterization of prosystemin expressed in the baculovirus/insect cell system reveals biological activity of the systemin precursor. Planta 211: 91-97