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Differential Expression of Soybean Cysteine Proteinase Inhibitor Genes during Development and in Response to Wounding and Methyl Jasmonate[1]

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Three cysteine proteinase inhibitor cDNA clones (pL1, pR1, and pN2) have been isolated from a soybean (*Glycine max* L. Merr.) embryo library. The proteins encoded by the clones are between 60 and 70% identical and contain the consensus QxVxG motif and W residue in the appropriate spatial context for interaction with the cysteine proteinase papain. *L1*, *R1*, and *N2* mRNAs were differentially expressed in different organs of plants (juvenile and mature) and seedlings, although *N2* mRNA was constitutive only in flowers. *R1* and *N2* transcripts were induced by wounding or methyl jasmonate (M-JA) treatment in local and systemic leaves coincident with increased papain inhibitory activity, indicating a role for R1 and N2 in plant defense. The *L1* transcript was constitutively expressed in leaves and was induced slightly by M-JA treatment in roots. Unlike the chymotrypsin/trypsin proteinase inhibitor II gene (H. Peña-Cortés, J. Fisahn, L. Willmitzer [1995] Proc Natl Acad Sci USA 92: 4106–4113), expression of the soybean genes was only marginally induced by abscisic acid and only in certain tissues. Norbornadiene, a competitive inhibitor of ethylene binding, abolished the wounding or M-JA induction of *R1* and *N2* mRNAs but not the accumulation of the wound-inducible *vspA* transcript. Presumably, ethylene binding to its receptor is involved in the wound inducibility of *R1* and *N2* but not *vspA* mRNAs. Bacterial recombinant L1 and R1 proteins, expressed as glutathione *S*-transferase fusion proteins, exhibited substantial inhibitory activities against vicilin peptidohydrolase, the major thiol endopeptidase in mung bean seedlings. Recombinant R1 protein had much greater cysteine proteinase inhibitor activity than recombinant L1 protein, consistent with the wound inducibility of the *R1* gene and its presumed role in plant defense.

Injury to plants due to phytophagous insects or mechanical damage induces accumulation of proteinase inhibitor proteins (Ryan, 1981, 1990). Inhibitor proteins with specificity against Ser proteinases have been well characterized and there is direct evidence of their involvement in host plant defense against herbivorous insects (Johnson et al., 1989; Ryan, 1990). Accumulation of proteinase inhibitors occurs both locally, at the site of injury, and systemically, in other organs of the plant distal to the primary wound site (Brown and Ryan, 1984; Peña-Cortés et al., 1988; Ryan, 1990). It now seems that, at least in solanaceous species, local and systemic accumulation of Ser proteinase inhibitors is a component of the plant defense response that is controlled by signal transduction cascades, which are initiated by wounding or pest or pathogen attack and ultimately regulate the transcription of genes encoding these proteins (Farmer and Ryan, 1992).

Proteinaceous CysPIs are widely distributed in plants and have been isolated from a number of sources (Ryan, 1981, 1990), including rice (Abe et al., 1987a), cowpea (Fernandes et al., 1993), maize (Abe et al., 1992), and soybean (*Glycine max* L. Merr.; Hines et al., 1992) seeds. However, little has been determined about their function in plants. It seems that these proteins are involved in plant defense against insects, particularly those in the coleopteran and hemipteran orders (Liang et al., 1991; Walsh and Strickland, 1993). Several families of these insects are unique in their utilization of Cys proteinases (rather than Ser proteinases) for protein digestion (Houseman, 1978; Houseman and Downe, 1983; Murdock et al., 1987). The use of Cys proteinases may be an evolutionary adaptation that enables these insects to consume legume seeds and other plant materials that contain high levels of Ser proteinase inhibitors (Ryan, 1990). Protein fractions from soybean seeds contain CysPI activity (Murdock et al., 1987; Brzin et al., 1990; Hines et al., 1991, 1992), and the CysPI isolated from hypocotyl-rich fractions is a member of the cystatin superfamily based on N-terminal sequence data (Brzin et al., 1990; Hines et al., 1991). The purified soybean seed protein effectively inhibited papain and human cathepsin B Cys proteinase activities but did not inhibit trypsin or chymotrypsin (Brzin et al., 1990; Hines et al., 1991).

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Abbreviations: CysPI, Cys proteinase inhibitor; GST, glutathione *S*-transferase; [^{3}H]MetHem, [^{3}H]methemoglobin; M-JA, methyl jasmonate; NBD, norbornadiene; Pin1 and Pin2, proteinase inhibitor I and II, respectively; SA, salicylic acid; Vpase, vicilin peptidohydrolase.

Although it is known that plant CysPIs are encoded by gene families (Abe et al., 1987a; Kondo et al., 1990; Fernandes et al., 1993; Waldron et al., 1993; Walsh and Strickland, 1993), little is known about the differential regulation of these genes. Wounding and exogenous application of M-JA or ABA induced accumulation of an mRNA in potato leaves that has sequence homology to plant CysPI genes (Hildmann et al., 1992). These data provide rudimentary information for a hypothesis that a common (or perhaps analogous) wound-inducible intracellular signal transduction pathway activates Ser proteinase inhibitor and CysPI genes in response to insect or pathogen attack (Farmer and Ryan, 1992).

In this paper we present data that show that soybean CysPIs are encoded by a multigene family and that these CysPI genes are differentially regulated in organs of seedlings and plants. Expression of two CysPI genes (*R1* and *N2*) was induced through wound or M-JA treatment in leaves both locally and systemically. Another gene (*L1*) is constitutively expressed in this organ. Similar to the potato CysPI gene (Hildmann et al., 1992) but dissimilar to the Ser proteinase inhibitor *Pin2* gene (Peña-Cortés et al., 1995), expression of the soybean CysPI genes was only marginally up-regulated by ABA in specific tissues. The ethylene action inhibitor NBD eliminated the induction of the CysPI transcripts in leaves mediated by wounding or M-JA treatment. Recombinant fusion L1 and R1 proteins inhibited Vpase activity, with the CysPI encoded by the wound-inducible *R1* gene having substantially greater inhibitory activity. R1 encodes the CysPI protein isolated from soybean seeds that substantially inhibited the proteolytic activity of crude extracts of larval midguts of several coleopteran insects, to an even greater extent than L-*trans*-epoxylsuccinyl-leucylamido-(4-guanidino)-butane, a specific and irreversible CysPI (Hines et al., 1991). Wound- and M-JA-inducible CysPI transcript accumulation in leaves was accompanied by increased papain inhibitory activity.

MATERIALS AND METHODS

Soybean (*Glycine max* L. Merr. cv Del Soy) plants were used as the sources of experimental material. Plants were grown in containers containing vermiculite in a greenhouse and supplemented with one-half-strength Hoagland solution (Jones, 1982). Plant materials were harvested, frozen in liquid nitrogen, and stored at −80°C until processing.

Wounding, ABA, M-JA, Salicylic Acid, Ethylene, and NBD Treatments

Plants were wounded using a circular file as previously described (Botella et al., 1994b) or sprayed with 100 μM ABA, 45.5 μM M-JA, or 1 mM SA dissolved in 0.1% ethanol or 0.1% ethanol as a control (Hildmann et al., 1992; Xu et al., 1993). Experiments were conducted on plants with three trifoliate leaves and treatments were applied to the first and third leaves from the apex (local application). These leaves, along with second leaves from the apex (systemic), stems, and roots, were collected and total RNA was isolated from each for northern analyses. The systemic leaves were covered with plastic wrap during the spray treatments (Xu et al., 1993). NBD treatment of soybean plants was conducted in 25-L plastic chambers at a final concentration of 2000 parts per million. Plants were pretreated for 1 h with NBD, followed by wounding or M-JA treatment, and then they were again returned to the chamber for an additional 24 h of NBD treatment.

RNA Extraction and Gel-Blot Hybridization

Tissues were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Total RNA was extracted using the guanidinium thiocyanate-phenol-chloroform method as previously described (Botella et al., 1994a). RNA was quantified spectrophotometrically, separated on 1.2% formaldehyde agarose gels, visualized with ethidium bromide, and transferred to a membrane (Sambrook et al., 1989). Probe labeling and hybridization procedures were essentially as described by Botella et al. (1994a). *Eco*RI-*Xho*I CysPI inserts, including 3′ untranslated regions from pL1, pN2, and pR1 (GenBank accession nos. U51853, U51854, and U51855, respectively), were used as probes. The *VspA* probe for gel blots was the entire cDNA insert described by Mason and Mullet (1990). RNA blots were stripped in 2 mM Tris (pH 8.0) and 2 mM EDTA at 70°C for 15 min prior to reprobing. With the exception of those shown in Figure 2, all northern blots were stripped and reprobed with the different CysPI isoform probes. A 25S rRNA probe was used as an internal standard for gel loading and RNA transfer (Delcasso-Tremousaygue et al., 1988).

Measurement of Papain Inhibitory Activity in Leaf Extracts

CysPI activities in leaf extracts were expressed as inhibition of papain endopeptidase activity using the [^{3}H]MetHem assay (Hines et al., 1991). Papain activity was determined as the time-dependent release of TCA-soluble peptides from [^{3}H]MetHem. Leaf liquid nitrogen powders were extracted in 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl and 2 mM EDTA (2 mL g^{-1} fresh weight of tissue) and the slurry was centrifuged at 350,000*g* for 30 min. Papain (0.25 μM) was incubated with 5 μL of plant extract (or buffer) in the presence of 5 mM Cys in a total volume of 50 μL for 10 min at 37°C. All reagents were prepared in the buffer solution described above. After incubation, the assay medium was cooled on ice for 2 min before the addition of 50 μL of 1 mg/mL ice-cold [^{3}H]MetHem (10,000 cpm). The reaction mixture was then incubated for 10 min at 37°C, after which it was rapidly cooled on ice while 100 μL of 20% TCA was added to terminate the proteolytic action of papain. The tubes that contained the reaction mixture were then centrifuged at 15,000 rpm for 5 min and then 50 μL of the supernatant was added to 4 mL of scintillation liquid (Fisher Scientific) and the radioactivity was determined. This proteolytic reaction mixture contained excess [^{3}H]MetHem so that it was not substrate limited. CysPI activity was expressed as a percentage of inhibition of papain activity relative to the buffer control.

Bacterial Recombinant Protein Production

PCR products of the open reading frame of *L1* and *R1* cDNAs (Fig. 1) were inserted into pGEX-KG in-frame with the GST gene (Guan and Dixon, 1991). The *Escherichia coli* strain DH5α was used to express the recombinant proteins. Expression of recombinant proteins was induced by the addition of isopropylthio-β-galactoside to a final concentration of 0.2 mM and then the cultures were allowed to grow for an additional 4 h. The cells were then collected by centrifugation at 10,000*g* for 10 min. The cell pellet from 1 L of culture was suspended in 6 mL of buffer A (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1% Triton X-100, 2 mM EDTA, 0.1% β-mercaptoethanol, 0.2 mM PMSF, pH 7.3). The cells were disrupted at 4°C with three bursts of 2 min each using a sonicator. After the sample was centrifuged at 12,000*g* for 20 min, the supernatant was collected and mixed with 2 mL of glutathione-agarose beads (Sigma) and incubated overnight at 4°C with slow agitation. The glutathione-agarose beads were packed into a spun column and washed with 50 mL of buffer B (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, and 1% Triton X-100, pH 7.3). The GST-CysPI fusion protein was eluted with 4 mL of buffer D (10 mM GSH in 50 mM Tris-HCl, pH 8.0). The protein solution was dialyzed against 4 L of deionized water for 24 h at 4°C. The H_2O volume was changed twice during the dialysis period. The protein concentrations were determined using the Bio-Rad protein assay kit and the proteins were analyzed on 12% SDS gel (Sambrook et al., 1989).

Vpase Activity

Vpase was purified from the cotyledons of 5-d-old mung bean seedlings using a combination of ammonium sulfate precipitation and chromatography on Sephacryl S-200 HR (Pharmacia) and DEAE-Trisacryl M columns (BioSepra, Marlborough, MA). Inhibition of Vpase by recombinant soybean GST-CysPI fusion proteins was measured using a modification of the procedure described previously (Wilson et al., 1988). The inhibitor sample was mixed with 2.75 units of Vpase and assay buffer (50 mM sodium citrate and 5 mM β-mercaptoethanol, pH 5.7) in a total volume of 100 μL. After the sample incubated for 10 min at 25°C, 1 mL of 1% (w/v) azocasein (Sigma) in assay buffer was added and incubation continued for an additional 2 h at 37°C. The reaction was terminated by the addition of 100 μL of 50% (w/v) TCA and the protein precipitate was removed by centrifugation. The supernatant was mixed with an equal volume of 2 N NaOH and the A_{430} was determined. One unit of Vpase produced a change in A of 2.0 under these conditions (1.0/h).

RESULTS

Isolation of cDNA Clones Encoding Soybean CysPIs

Poly(A)$^+$ RNA from immature soybean embryos (combined from different stages of embryogeny) was used as a template both for reverse transcription-PCR and for the construction of a cDNA library in λZAPII (Stratagene). The primers used for reverse transcription-PCR were degenerate oligonucleotides encoding the DEHNKKENA motif of the N-terminal sequence obtained from the purified soybean seed CysPI (Brzin et al., 1990; Hines et al., 1991) and the antisense of the KELQEF motif in the C-terminal region of oryzacystatin I (Abe et al., 1987a, 1987b; Kondo et al., 1990). A 180-bp PCR fragment was obtained that encoded a portion of the soybean seed CysPI based on comparison with available amino acid sequence information (Brzin et al., 1990; Hines et al., 1991). This cDNA fragment was used as a probe to screen the immature embryo cDNA library, which resulted in the isolation of three unique cDNA clones (pL1, pR1, and pN2).

The amino acid sequence deduced from pR1 is identical, with one exception, to the available N-terminal sequence of

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                                                                   --->
     1                                                                    70
Gm   .......... .......... .......... .......... ..GFTDITGA QNSIDIENLA RFAVDEHNKK
R1   .......... .......... .......... .......... LGGFTDITGA QNSIDIENLA RFAVDEHNKK
L1   .......... .......... .......... .......... ..GNRDVTGS QNSVEIDALA RFAVEEHNKK
N2   .......... .......... .......... ..AALEKVQE LGGITDVHGA ANSVEINNLA RFAVEEQNKR
Vu   .......... .......... .......... .......MAA LGGNRDVAGN QNSLEIDSLA RFAVEEHNKK
Zm   MRKHRIVSLV AALLVLLALA AVSSTRSTQK ESVADNAGML AGGIKDVPAN ENDLQLQELA RFAVNEHNQK
Os1  .......... .......... .......... ..MSSDGGPV LGGVEPV.GN ENDLHLVDLA RFAVTEHNKK
Os2  .......... .......... ........MA EEAQSHAREG GRHPRQPAGR ENDLTTVELA RFAVAEHNSK

     --->       *   A                              B  <---
     71                                                                          140
Gm   ENAVLEFVRV KSAKKQVVSG .......... .......... .......... .......... .......... .
R1   ENAVLEFVRV ISAKKQVVSG TLYYITLEAN D.GVTKKVYE TKVLEKPWLN IKEVQEFKPI TVAVNPLSVT V
L1   QNALLEFEKV VTAKQQVVSG TLYTITLEAK D.GGQKKVYE AKVWEKSWLN FKEVQEFKLV GDAPA..... .
N2   ENSVLEFVRV ISAKQQVVAG VNYYITLEAK D.GLIKNEYE AKVWVREWLN SKELLEFKPV NVSSTQ.... .
Vu   QNALLEFGRV VSAQQQVVSG TLYTITLEAK D.GGQKKVYE AKVWEKPWLN FKELQEFKHV GDAPA..... .
Zm   ANALLGFEKL VKAKTQVVAG TMYYLTIEVK D.GEVKKLYE AKVWEKPWEN FKQLQEFKPV EEGASA.... .
Os1  ANSLLEFEKL VSVKQQVVAG TLYYFTIEVK E.GDAKKLYE AKVWEKPWMD FKELQEFKPV DASANA.... .
Os2  ANAMLELERV VKVRQQVVGG FMHYLTVEVK EPGGANKLYE AKVWERAWEN FKQLQDFKPL DDATA..... .
```

Figure 1. Alignment of amino acid sequences deduced from pR1 (R1), pL1 (L1), and pN2 (N2), with the N-terminal sequence of the soybean seed CysPI, Gm (Brzin et al., 1990) and amino acid sequences deduced from cowpea, Vu (Fernandes et al., 1993); rice, Os1 and Os2 (Abe et al., 1987a; Kondo et al., 1990); and maize, Zm (Abe et al., 1992) cDNAs. The conserved QxVxG motif and W that function in the interaction between the CysPI and the proteinase (Bode et al., 1988; Stubbs et al., 1990; Turk and Bode, 1991) are designated by A and B, respectively. The asterisk identifies the only residue that differs between R1 and the N-terminal sequence of the soybean seed protein (Brzin et al., 1990). The amino acid locations of the respective primers used for reverse transcription-PCR and their orientations are indicated with arrows. Deduced amino acid data from pL1, pN2, and pR1 are based on nucleotide sequence information from both strands of each cDNA listed in GenBank as accession nos. U51853, U51854, and U51855, respectively.

the soybean seed CysPI (Brzin et al., 1990; Hines et al., 1991; Fig. 1). This seed CysPI has been shown to inhibit substantially western corn rootworm digestive Cys proteinases (Hines et al., 1991). In overall sequence, R1 is 67% identical with the peptide encoded by pL1 (L1), and L1 and R1 are 61 and 64%, respectively, identical with the peptide encoded by pN2 (N2). The soybean CysPIs have high sequence similarity with other plant CysPIs, including L1, which is 88% identical to the cowpea CysPI protein (Fernandes et al., 1993). All three proteins include the QxVxG motif and the W in the appropriate sequence topology to be contained in the first- and second-hairpin loop domains, respectively, that interact with Cys proteinases (Fig. 1; Bode et al., 1988; Stubbs et al., 1990; Turk and Bode, 1991).

Plant cystatins can be categorized generally into two groups, based on whether they contain single or multiple CysPI domains. The domains within potato multicystatin can be proteolytically separated with trypsin and each of these fragments possesses CysPI activity (Walsh and Strickland, 1993; Waldron et al., 1993). The only other plant multicystatin protein identified to date is also from a solanaceous species, tomato (Bolter, 1993). The mRNAs encoding the multicystatins are about 2 kb, whereas those encoding single cystatin domain proteins, such as the soybean CysPIs, are about 0.6 kb (Fig. 2). The transcripts detected by *L1* and *R1* inserts were of comparable size, but the *N2* mRNA was larger (Fig. 2). Data illustrating the differential regulation of *L1* and *R1* provide evidence that the *R1* probe does not detect the *L1* transcript (Figs. 3 and 4). That the *L1* probe does not detect the *R1* transcript is less obvious but the evidence leads to this likelihood. In Figure 2, the blot data illustrated in the last two lanes on the right (probed with *L1* and *N2* or *R1* and *N2*) allow a direct comparison of relative *L1* and *R1* transcript abundances, since *N2* mRNA was constant in both instances and thus was an internal standard verifying that comparable RNA amounts were represented in each lane. If the *L1* probe had hybridized to both transcripts, then presumably the mRNA abundance detected would have been a combination of both *L1* and *R1* transcripts and substantially greater than that detected by the *R1* probe. From the blot of

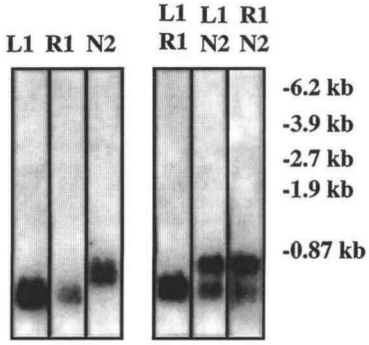

Figure 2. Transcripts detected by soybean cDNA inserts that encode L1, R1, and N2 CysPIs. Northern blots of total RNA (10 μg) from leaves, after wounding, hybridized with ^{32}P-labeled pL1, pR1, or pN2 insert (lanes labeled L1, R1, and N2, respectively) or a mixture of probes as indicated. Transcript sizes indicated on the right are based on RNA markers.

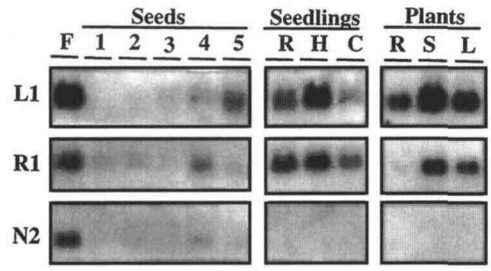

Figure 3. Expression of soybean CysPI genes is differentially regulated in plant tissues. Northern blot analyses were conducted using 10 μg of total RNA extracted from flowers immediately after anthesis (F) and seeds 1, 2, 3, 4, or 5 weeks postpollination; roots (R), hypocotyls (H), or cotyledons (C) of 1-week-old seedlings, or roots (R), stems (S), or leaves (L) of plants with three trifoliate leaves. A ^{32}P-labeled pL1 (L1), pR1 (R1), or pN2 (N2) insert was used as probe.

seedling root RNA (Fig. 3), it seems that the *L1* probe did not detect the *R1* transcript, at least to the same extent as did the *R1* probe. No multicystatin transcript could be detected, even at lower hybridization stringencies.

Developmental Expression of Soybean CysPI Genes

RNA gel-blot analyses revealed abundant levels of *L1*, *R1*, and *N2* transcripts in flowers (Fig. 3). Comparatively, mRNA levels in seeds throughout embryogeny were quite low. The presence of all three CysPI mRNAs in seeds was

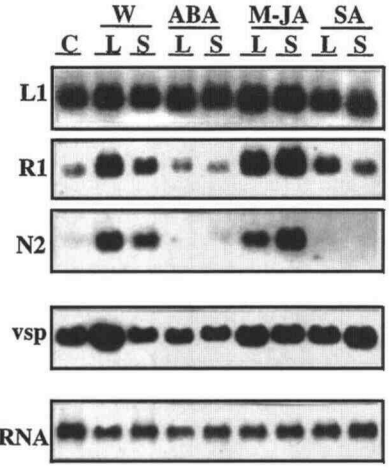

Figure 4. Mechanical wounding or M-JA treatment induces *R1* and *N2* mRNA accumulation in leaves of soybean plants. Northern analyses were conducted using total RNA (10 μg) isolated from leaves 24 h after mechanical wounding (W) or spray treatment with 100 μM ABA, 45.5 μM M-JA, or 1 mM SA. Control leaves were sprayed with 0.1% ethanol (C), the same solution in which ABA, M-JA, and SA were dissolved. Experiments were conducted on plants with three trifoliate leaves and treatments were administered to the first and third leaves (local application, L). These leaves and the second leaf (systemic, S) were collected for RNA isolation. A radiolabeled pL1 (L1), pR1 (R1), or pN2 (N2) insert was used as a probe. A soybean *vspA* cDNA (vsp) insert was used as a positive control for wounding and M-JA treatments (Mason and Mullet, 1990). The 25S rRNA (rRNA) probe was used as an internal standard for RNA loading and nitrocellulose transfer.

expected, since the cDNA library was prepared from RNA isolated from a compilation of embryos at various stages of maturation. The abundance of the individual transcripts was consistent with the low frequency of CysPI cDNAs in the immature embryo library. Also, the level of *R1* mRNA correlated well with the low level of R1 protein that was present in mature soybean seeds (Hines et al., 1991). *L1* and *R1* transcripts were present in all seedling and plant organs, with the exception that *R1* mRNA was not detected in the roots of plants at the three-trifoliate leaf stage. The *N2* transcript was not detected in vegetative tissues of seedlings or plants unless the filters were substantially overexposed, and even then the *N2* mRNA signal was barely detectable (not shown).

R1 and *N2* Transcript Accumulation Induced by Wounding or M-JA Treatment

Wounding elicited both local and systemic accumulation of *R1* and *N2* transcripts in soybean leaves (Fig. 4). M-JA treatment induced local accumulation of the transcripts and, within the limits of our experimental conditions, caused increased systemic levels of *R1* and *N2* mRNAs. The local and systemic induction of *R1* and *N2* mRNAs is analogous to the wound-inducible transcriptional regulation of *Pin1* and *Pin2*, which encode Ser proteinase inhibitors involved in defense of solanaceous species against insects (Peña-Cortés et al., 1988, 1995; Farmer and Ryan, 1990). Wound induction of these genes is mediated through signal transduction cascades that involve jasmonate and other intermediates (Farmer and Ryan, 1992). A similar local and systemic induction of a transcript by wounding or M-JA treatment in potato leaves was detected using a probe that encoded the partial sequence of a protein with similarity to oryzacystatin (Hildmann et al., 1992). Wounding or M-JA treatment also stimulated the accumulation of *vspA*-detectable (Mason and Mullet, 1990) mRNA (Fig. 4). However, in this experiment leaves of control plants had higher than typical levels of this transcript, which led to an attenuation in the degree of induction that was detected in leaves after wounding or M-JA treatment. Levels of *vspA*-detectable mRNA were somewhat variable in soybean leaves used in different experiments, apparently modulated by variation in the plant-growing conditions of the greenhouse (Mason and Mullet, 1990). A small wounding induction of *L1* mRNA accumulation was observed in leaves, but this increase was very small relative to the constitutively high levels present prior to treatment (Fig. 4). The signaling molecule that was translocated from the first and third leaves and induces systemic *R1* and *N2* mRNA accumulation after wounding or M-JA treatment did not affect *N2* and only marginally increased *R1* transcript levels in stems (Fig. 5). Perhaps stem cells are either unresponsive to the systemic signaling molecule or the potentially responsive cells do not have access to the molecule. M-JA induced only *L1* mRNA levels in roots and no effect from wounding on abundances of any transcript could be detected in this organ (Fig. 5).

ABA did not induce accumulation of soybean CysPI transcripts in leaves or roots (Figs. 4 and 5) and only slightly increased *R1* mRNA in stems. As was the case with potato, ABA had little effect on wound-inducible CysPI genes, indicating that in soybean plants, as in species of the Solanaceae, this hormone is not involved in the regulation of all defense genes. In contrast, *Pin2* expression is substantially up-regulated by ABA (Peña-Cortés et al., 1995). SA induced *R1* transcript abundance in leaves and stems (Figs. 4 and 5). SA induces systemic acquired resistance (Gaffney et al., 1993) but also has been determined to prevent wound-induced gene expression of *Pin2* genes, apparently by blocking jasmonic acid synthesis (Peña-Cortés et al., 1993).

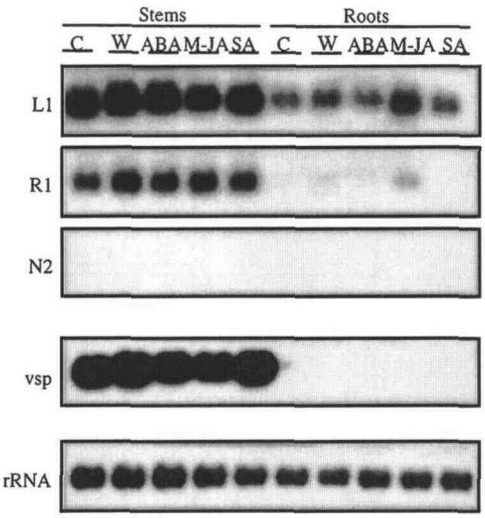

Figure 5. *L1*, *R1*, and *N2* message levels in soybean stems and roots 24 h after leaves were wounded or treated with ABA, M-JA, or SA. The first- and third-trifoliate leaves were mechanically wounded (W) or sprayed with 100 μM ABA, 45.5 μM M-JA, or 1 mM SA. Control plants were sprayed with 0.1% ethanol (C). Probes were ^{32}P-labeled inserts from pL1 (L1), pR1 (R1), pN2 (N2), *vspA* (vsp), or rRNA (rRNA) cDNAs.

Wounding and M-JA Induction of CysPI Gene Expression Requires Ethylene

Pretreatment of plants with NBD prevented *R1* and *N2* transcript induction in leaves by wounding or M-JA treatment (Fig. 6). From these results, it is apparent that ethylene is required to regulate the expression of *R1* and *N2* by these inducers and seems to function downstream of wounding and M-JA in the signaling cascade. However, apparently, ethylene alone, at least at physiological levels, does not induce *R1* or *N2* transcript abundance. Although wounding and pathogen infection generally cause stress ethylene production (Paradies et al., 1980; Felix and Boller, 1995), the relationship between M-JA and ethylene production is less evident. Perhaps ethylene potentiates M-JA function in the wounding signal transduction pathway that mediates specific induction of soybean CysPI genes, as has been shown for induction of pathogenesis-related protein gene expression (Xu et al., 1994). It is interesting that NBD did not inhibit wounding or M-JA induction of *vspA* gene expression.

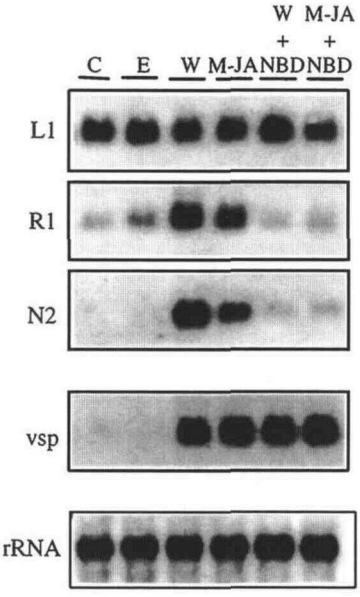

Figure 6. NBD inhibits wounding and M-JA induction of *R1* and *N2* gene expression in soybean leaves. RNA was isolated from leaves of untreated plants (C) or leaves of plants that were wounded (W) or M-JA treated. Where applicable, plants were pretreated for 1 h with NBD prior to wounding (W + NBD) or M-JA treatment (M-JA + NBD). Probes were ^{32}P-labeled inserts from pL1 (L1), pR1 (R1), pN2 (N2), *vspA* (vsp), or rRNA (rRNA) cDNAs.

Recombinant L1 and R1 Inhibit a Plant Cys Proteinase Present in Mung Bean Cotyledons

To test whether the proteins encoded by the CysPI genes were functional, L1 and R1 were expressed as recombinant GST-fusion proteins in *E. coli* and tested against Vpase, the major sulfhydryl endopeptidase present in the cotyledons of mung bean seedlings. Vpase is a member of the Cys proteinase family of proteolytic enzymes (Baumgartner and Chrispeels, 1977). Since N-terminal deletion of 21 or 23 amino acids from oryzacystatin I did not affect CysPI activity of recombinant oryzacystatin, it was anticipated that recombinant L1 and R1 would possess functional inhibitory activity (Abe et al., 1988; Chen et al., 1992). R1 had substantially greater Vpase activity than L1, despite the near 70% identity in primary sequence (Fig. 7). Presumably, the basis for the difference in inhibitory activity is due to motifs or residues that differ between the proteins, and these are candidates for mutation in structure and function analyses.

Wound- and M-JA-Induced Papain Inhibitory Activity in Soybean Leaves

To further corroborate that induction of *R1* and *N2* mRNAs upon wounding or M-JA treatment are related to the defense function of the R1 and N2, the CysPI activities of leaves were measured after these treatments. Illustrated in Figure 8 are the papain inhibitory activities from leaves that were the source of RNA for the blots shown in Figure 4. Inhibition of papain Cys proteinase activity is a measure of CysPI activity (Turk and Bode, 1991; Bolter, 1993). Substantial induction of CysPI activity was detected in leaves (Fig. 8) after wounding (local) or M-JA treatment (local and systemic). These levels of CysPI activity correlated well with the induction of *R1* and *N2* transcripts (Fig. 4), with the exception that, although the transcripts were systemically induced after wounding, only a small increase in CysPI activity was detected. Perhaps a basal level of transcript accumulation is necessary before a discernible increase in CysPI activity can be measured, or perhaps translation does not occur effectively in these tissues. A similar discrepancy is observed with SA-treated material, in which the *R1* mRNA induction was not correlated with an induction of inhibitor activity. Low CysPI activity in control

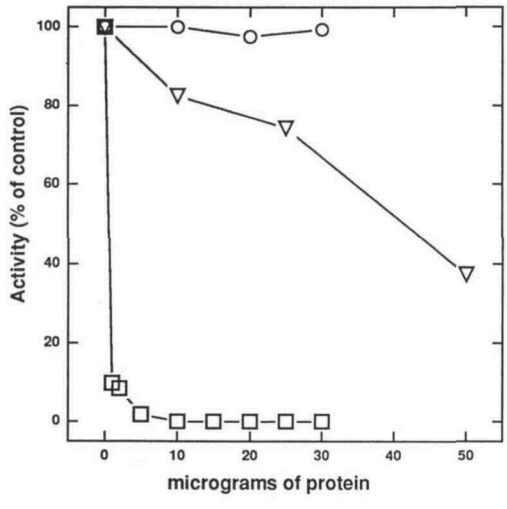

Figure 7. Recombinant soybean CysPIs inhibit Vpase activity. Vpase activity in the presence of recombinant GST (○), GST-L1 (△), and GST-R1 (□) was measured and expressed as a percentage of Vpase activity in the absence of added protein.

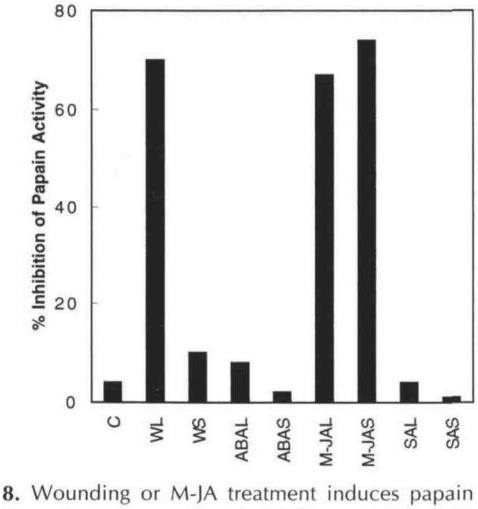

Figure 8. Wounding or M-JA treatment induces papain inhibitory activity in soybean leaves. Papain inhibitory activity of leaf extracts from untreated plants (C) or plants 24 h after wounding, local (WL) or systemic (WS), or treated with ABA (local, ABAL, or systemic, ABAS), M-JA (M-JAL or M-JAS), or SA (SAL or SAS). Samples correspond to those illustrated in Figure 4. Data are the averages of three replicate samples.

leaves, despite constitutively high levels of *L1* mRNA, is indicative that L1 is not an effective inhibitor of papain, at least relative to R1 and N2. It is also possible that the abundant *L1* mRNA present in leaves is not efficiently translated to inhibitor protein.

DISCUSSION

Extracellular proteinases in the lumen of insect guts hydrolyze dietary protein and the amino acids are assimilated as essential nutrients (Murdock et al., 1987; Ryan, 1990). It has been determined in many species of the Hemiptera and Coleoptera that Cys proteinases account for the majority of proteolytic activity responsible for protein digestion in the gut (Murdock et al., 1987, 1988; Ryan, 1990; Gillikin et al., 1992; Michaud et al., 1993). Some of these have been identified as capthepsin-like Cys proteinases of the papain superfamily (Thie and Houseman, 1990; Lemos and Terra, 1991; Houseman and Thie, 1993; Michaud et al., 1993; Bertl and Storer, 1995). Presumably, inhibition of these proteinases would substantially attenuate protein digestion and essential amino acid assimilation. In fact, CysPIs have been shown to inhibit insect growth and development (Murdock et al., 1988; Hines et al., 1990; Liang et al., 1991; Chen et al., 1992; Orr et al., 1994) and evidence has linked this inhibition to CysPIs' alteration of digestive proteolytic activities in the gut (Hines et al., 1991; Liang et al., 1991; Orr et al., 1994).

It is generally inferred that wounding and pathogen attack elicit analogous plant defense responses, including the induction of proteinase inhibitors (Ryan, 1990). Also, the current hypothesis assumes that a common signal transduction pathway may mediate the induction of wounding and defensive genes, although the elicitors at the site of wounding or insect attack are apparently different (Ryan, 1990; Doares et al., 1995). Local and systemic wound induction of *R1* and *N2* gene expression in soybean leaves suggests a role for these CysPIs in plant defense. M-JA induction of *R1* and *N2* mRNA accumulation also supports this proposed function for these proteins, since this hormone is a likely intermediate in the wounding signal transduction pathway leading to plant defense against insects (Farmer and Ryan, 1990; Farmer et al., 1992; Doares et al., 1995). Wounding and JA treatment also induced the accumulation of papain inhibitory activities in soybean leaves, coincident with increased *R1* and *N2* mRNA levels. Previously, it was determined that M-JA-induced papain inhibitor activity in tomato leaves was associated with the induction of an 87-kD protein (Bolter, 1993). This protein is consistent in size with potato multicystatin, indicating that a multidomain CysPI exists in tomato as it does in potato (Hildmann et al., 1992; Orr et al., 1994). However, wounding did not induce papain inhibitory activity in tomato leaves (Bolter, 1993) despite the fact that in potato leaves this inducer caused an increase in CysPI mRNA (Hildmann et al., 1992). Perhaps in Solanaceae species there is a difference in the signaling cascade whereby wounding induces the transcriptional activation of CysPI genes but does not result in the production of active inhibitor protein, as it does in soybean.

It is noteworthy that the soybean seed CysPI encoded by the *R1* cDNA substantially inhibited proteolytic activities of crude larval gut extracts of five different insects (Brzin et al., 1990; Hines et al., 1991), further implicating the defense function of R1 protein. Furthermore, R1 had substantially greater CysPI activity in the Vpase assay than the protein encoded by the *L1* gene, which is constitutively expressed in leaves. Recombinant N2 also has considerably greater CysPI activity than L1 (Zhao et al., 1996). There are several reasons why a plant would express multiple CysPI homologs in the same organ for insect defense. The digestive tracts of western corn rootworm larvae contain numerous Cys proteinases (Gillikin et al., 1992), which means that it is unlikely that a single CysPI would be effective against each isoform and thereby inhibit proteolytic digestion of these insects sufficiently to retard growth and development in a significant manner. Challenging of *Spodoptera exigua* larvae with *Pin2* protein (P12) in transgenic plants resulted in an adaptation by the insect so that growth was unaffected (Jongsma et al., 1995). The larvae responded to chymotrypsin/trypsin inhibitor exposure by producing proteinases that were no longer inhibited by P12. Induction of multiple CysPIs by the plant in response to insect attack would require a substantially more complicated response by the pest to alleviate the protein digestion impediment. Furthermore, numerous CysPIs would result in multifaceted selection pressure on the insect, thereby reducing the efficacy at which the organism could evolve resistant biotypes (Brattsten, 1991).

Physiological plant function for soybean CysPIs in growth and development can be alluded to from data illustrating the temporal and tissue-specific expression of the transcripts. From these data it can be suggested that L1 and, to a lesser degree, R1, have prominent roles in plant metabolism. Cys proteinases are responsible for the mobilization of amino acids from storage proteins in seeds and tubers (Wilson et al., 1988; Watanabe et al., 1991; Holwerda and Rogers, 1992; Michaud et al., 1994; Yu and Greenwood, 1994). It has been proposed that CysPIs regulate Cys proteinases during seed maturation and germination, periods when proteins must be accumulated for storage and then hydrolyzed for amino acid assimilation, respectively (Valevski et al., 1991). The isolation of the soybean CysPI cDNAs from an embryo library is consistent with this possible function of these proteins. A senescence-specific Cys proteinase has been detected in pea ovaries (Ceros and Carbonell, 1993) and Cys proteinases have been suggested to function in the latter stages of outer integument development (Nadeau et al., 1996). Low temperature-induced accumulation of an mRNA encoding a Cys proteinase was detected in tomato fruit (Schaffer and Fischer, 1988) and wounding and osmotic stresses induced Cys proteinase genes (Koizumi et al., 1993; Linthorst et al., 1993). Presumably, CysPIs are also required to regulate Cys proteinases that are important to other biochemical processes in other plant tissues. The cytosolic localization of an endopeptidase inhibitor (now assumed to be a CysPI) has led to a proposed function in the protection of metabolic machin-

ery from the detrimental effects of proteolysis that results from incidental rupturing of Cys-proteinase-containing vesicles (Baumgartner and Chrispeels, 1976), a process that might increase in stress situations.

Possible alternative functions for the CysPIs can be suggested from results indicating that plant interactions with a nematode, virus, and the nitrogen-fixing actinomycete *Frankia* are facilitated by Cys proteinases of the papain superfamily (Shapira and Nuss, 1991; Goetting-Minesky and Mullin, 1994; Urwin et al., 1995). Oryzacystatin I expressed in tomato roots substantially inhibited the growth, development, and fecundity of the cyst nematode *Globodera pallida* (Urwin et al., 1995), implicating CysPIs as potential deterrents of these organisms. Two virus-encoded, papain-like proteinases undergo autocatalysis in chestnut blight fungal cells, a process that causes the virus to mediate hypovirulence of the fungal pathogen (Shapira and Nuss, 1991; Nuss, 1992). *Frankia*-induced nodule formation in symbiosis with *Alnus glutinosa* is accompanied by the expression of a Cys proteinase (Goetting-Minesky and Mullin, 1994). It is suggested that the production of the Cys proteinase is a plant response to prevent invasion by *Frankia*. If this is the case, then CysPIs would serve a valuable function in reducing the inhibitory activity of the proteolytic enzyme and facilitate the symbiosis. We suggest also the possibility that R1 and N2 function in plant defense by attenuating programmed cell death that is likely induced by insect predation in a manner similar to pathogen infection (Greenberg et al., 1994; Staskawicz et al., 1995). *SAG12* from *Arabidopsis thaliana* encodes a Cys proteinase that may function in leaf senescence (Gan and Amasino, 1995a, 1995b). Leaf senescence is a type of programmed cell death (Thompson, 1995). One hypothesis might be that insect predation elicits local and systemic programmed cell death responses in plants that are mediated, at least in part, by Cys proteinases, which in turn must be controlled by CysPIs to restrict the extent of cell and tissue degradation (Greenberg et al., 1994; Staskawicz et al., 1995). Pathogens or pests that can elicit uncontrolled programmed cell death in plants would have a selective advantage in nutrient acquisition, particularly if they have evolved resistance to induced defensive proteins. Thus, although programmed cell death may represent a defensive response, it is imperative that the plant has the capacity to regulate this process.

Finally, our results indicate that increased *R1* and *N2* gene expression induced by wounding or M-JA involves ethylene, since NBD suppresses the induction elicited by these inducers. Furthermore, ethylene either functions downstream of M-JA in the signaling cascade or perhaps potentiates its effects. It is possible that systemic *R1* and *N2* transcript accumulation is mediated through systemin, which also induces ethylene production (Felix and Boller, 1995). In the same experiment NBD did not affect message levels detected by *vspA*, a wound- and M-JA-inducible soybean gene (Mason and Mullet, 1990). Wounding-mediated local induction of *win2* also does not require ethylene (Weiss and Bevan, 1991). These results delineate a dichotomy in the defense signal transduction pathway that occurs subsequent to wounding and accounts for differential expression of unique genes. Ethylene may be a specific signal that induces the expression of pest defensive genes in soybean upon insect predation that, perhaps, delineates this response from the induction that is caused by wounding.

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