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Two Wound-Inducible Soybean Cysteine Proteinase Inhibitors Have Greater Insect Digestive Proteinase Inhibitory Activities than a Constitutive Homolog[1]

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Diverse functions for three soybean (*Glycine max* L. Merr.) cysteine proteinase inhibitors (CysPIs) are inferred from unique characteristics of differential regulation of gene expression and inhibitory activities against specific Cys proteinases. Based on northern blot analyses, we found that the expression in leaves of one soybean CysPI gene (*L1*) was constitutive and the other two (*N2* and *R1*) were induced by wounding or methyl jasmonate treatment. Induction of *N2* and *R1* transcript levels in leaves occurred coincidentally with increased papain inhibitory activity. Analyses of kinetic data from bacterial recombinant CysPI proteins indicated that soybean CysPIs are noncompetitive inhibitors of papain. The inhibition constants against papain of the CysPIs encoded by the wound and methyl jasmonate-inducible genes (57 and 21 nM for N2 and R1, respectively) were 500 to 1000 times lower than the inhibition constant of L1 (19,000 nM). N2 and R1 had substantially greater inhibitory activities than L1 against gut cysteine proteinases of the third-instar larvae of western corn rootworm and Colorado potato beetle. Cysteine proteinases were the predominant digestive proteolytic enzymes in the guts of these insects at this developmental stage. N2 and R1 were more inhibitory than the epoxide *trans*-epoxysuccinyl-L-leucylamide-(4-guanidino)butane (E-64) against western corn rootworm gut proteinases (50% inhibition concentration = 50, 200, and 7000 nM for N2, R1, and E-64, respectively). However, N2 and R1 were less effective than E-64 against the gut proteinases of Colorado potato beetle. These results indicate that the wound-inducible soybean CysPIs, N2 and R1, function in host plant defense against insect predation, and that substantial variation in CysPI activity against insect digestive proteinases exists among plant CysPI proteins.

Proteinaceous CysPIs, which specifically inhibit sulfhydryl proteinase activities, are distributed ubiquitously among animal, plant, and microorganism species. The animal homologs have been categorized into three major families based on sequence similarities (Barrett, 1987). Stefins are low-molecular-mass CysPIs (about 11 kD) that do not contain disulfide bonds or carbohydrate groups; cystatins are 13-kD proteins that contain two disulfide bonds and are glycosylated; and kininogens contain three CysPI domains and presumably are derived from tandem repeats of cystatin genes. It is interesting that crystallographic data do not resolve a functional involvement for disulfide bonds in the interaction between cystatin and papain, i.e. stefins and cystatins interact with papain via the same topological associations (Bode et al., 1988; Stubbs et al., 1990; Turk and Bode, 1991). Cathelin may represent a new family of CysPIs (Ritonja et al., 1989), since sequence homologies of these CysPIs to members of the other three families are very low. Crystallographic data from the interaction between chicken cystatin or human stefin and papain resolve three functional domains within CysPIs. Central to the interaction with Cys proteinases is the first hairpin loop, which includes the conserved QxVxG motif and is flanked by an N-terminal "trunk" and a second hairpin loop at the C terminus. The cystatin N-terminal trunk contains a G-9 and an A-10 bond that facilitate in making the Cys proteinase substrate site inaccessible. The second hairpin loop has P-W residues that mediate binding of this domain to the Cys proteinase.

Plant CysPIs have sequence similarities to stefins and cystatins, but do not contain Cys residues (Abe et al., 1987, 1992; Kondo et al., 1990; Fernandes et al., 1993). The majority are single CysPI domain peptides, but those identified in species of the Solanaceae possess multiple CysPI domains (Hildmann et al., 1992; Waldron et al., 1993). In plant CysPIs, the QxVxG pentapeptide is conserved in the appropriate spatial context to be included in the first hair-

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Abbreviations: BANA, *N*-benzoyl-DL-arginine-β-napthylamide; CPB, Colorado potato beetle; CysPI, Cys proteinase inhibitor; E-64, *trans*-epoxysuccinyl-L-leucylamide-(4-guanidino)butane; GST, glutathione *S*-transferase; IPTG, isopropylthio-β-galactoside; K_i, inhibition constant; M-JA, methyl jasmonate; WCR, western corn rootworm.

pin loop, and the conserved P-W residues in what could be the second hairpin loop. These have been determined to be essential for CysPI activity (Arai et al., 1991; Urwin et al., 1995). The N-terminal trunk region, however, is apparently dispensable to the function of plant CysPIs, since deletion of the N-terminal 21 or 24 amino acids of oryzacystatin I did not result in substantial loss of papain inhibitory activity (Abe et al., 1988; Chen et al., 1992).

In contrast to the substantial progress achieved in characterizing the plant CysPI structure, physiological function of these proteins is not well understood. Two physiological functions have been proposed: regulation of protein turnover and host plant defense against insect predation and, perhaps, pathogens. Function has been inferred in seed maturation, since CysPI activity varies during seed development in a pattern that is opposite to that of Cys proteinase activity (Fernandes et al., 1991). CysPIs may protect cytosolic metabolism from the effects of incidental rupturing of protein bodies and release of Cys proteinases (Baumgartner and Chrispeels, 1976).

The implication that plant CysPIs function in defense against insect predation is based on analyses of data from in vitro assays that indicate that these proteins inhibit digestive Cys proteinases in insect guts, and bioassays that provide evidence that these proteins possess insecticidal activities (Hines et al., 1991; Liang et al., 1991; Orr et al., 1994) against coleopteran insects. CysPI gene expression is induced by wounding or by M-JA (a presumed intermediate in the wounding signal transduction cascade) treatment in potato leaves (Hildmann et al., 1992). Recently, we determined that only specific genes of the soybean (*Glycine max* L. Merr.) CysPI family are induced by wounding, which implies dichotomous functions for CysPIs, i.e. for plant growth and development and defense (Botella et al., 1996).

We report that bacterial recombinant proteins encoded by soybean CysPI genes function as noncompetitive inhibitors of papain. The proteins encoded by the wound- and M-JA-inducible *N2* and *R1* genes have comparable K_is, and these are more than 2.5 orders of magnitude lower than the K_i of L1, which is encoded by a gene that exhibits relatively constitutive expression (19,000, 57, and 21 nM for L1, N2, and R1, respectively). Similarly, N2 and R1 have substantially greater inhibitory activities against the gut Cys proteinases of WCR and CPB than L1, indicating that wound-inducible proteins are involved in plant defense against insect predation.

MATERIALS AND METHODS

Plant Material and Treatments

Greenhouse-grown soybean (*Glycine max* L. Merr. cv Del Soy) plants with three trifoliate leaves were used in experiments. M-JA (Bedoukian Research, Danbury, CT) was dissolved in ethanol to a concentration of 45.5 mM and was then diluted with water to obtain a solution of 45.5 μM. Leaves of M-JA-treated plants were sprayed with this solution and those of control plants were sprayed with 0.1% ethanol at 6-h intervals (Hildmann et al., 1992). Wounding treatment was administered to leaves using a circular file as previously described (Botella et al., 1994). Wounding treatment was repeated after 20 h, and leaves were collected 4 h later.

Northern Blot Analyses

Total RNA was isolated from soybean leaves as previously described (Niu et al., 1993). The RNA was separated on 1.5% formaldehyde-agarose gels and transferred to a nitrocellulose membrane. Hybridization was performed in a solution of 6× SSC (1× SSC contains 150 mM NaCl and 15 mM sodium citrate, pH 7.0), 2× Denhardt's reagent, and 0.1% SDS containing 2×10^6 cpm/mL ^{32}P-labeled DNA probe at 68°C for 20 h (Sambrook et al., 1989). After hybridization, filters were washed in 1× SSC and 0.1% SDS at room temperature for 20 min and then washed twice in 0.2× SSC and 0.1% SDS at 68°C for 20 min. The filters were subjected to autoradiography at −80°C for 20 h using an intensifying screen.

Recombinant CysPI Proteins

PCR products of the open reading frame of *L1*, *N2*, and *R1* (GenBank accession nos. U51853, U51854, and U51855, respectively) were inserted into pGEX-KG in frame with the *GST* gene (Guan and Dixon, 1991). *Escherichia coli* strain DH5α was used to express the recombinant proteins. The GST-CysPI fusion proteins were purified by affinity chromatography using glutathione-agarose beads (Sigma), and GST was separated from the CysPI by thrombin hydrolysis. Expression of recombinant proteins was induced by the addition of IPTG to a final concentration of 0.2 mM, and the culture was 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 (Sigma), 2 mM EDTA, 0.1% β-mercaptoethanol, and 0.2 mM PMSF, pH 7.3). The cells were disrupted at 4°C 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 and incubated with slow agitation overnight at 4°C. The 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 column was then equilibrated with 5 mL of buffer C (50 mM Tris-HCl, 150 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% β-mercaptoethanol, pH 8.0). Then, 4 μg of thrombin (Sigma) was added onto the column and mixed with the beads. The beads were incubated with gentle agitation at room temperature for 3 h. The recombinant protein was eluted with buffer D (150 mM NaCl, 16 mM Na_2HPO_4, and 4 mM NaH_2PO_4, pH 7.3) in a total volume of 5 mL. The protein solution was dialyzed against 4 L of deionized water at 4°C for 24 h. The water was changed twice during the dialysis period. The protein concentrations were determined using a Bio-Rad protein assay kit, and proteins were analyzed for purity on 15% SDS gels (Sambrook et al., 1989).

Determination of Soybean CysPI Constants against Papain

K_i values were determined essentially as described by Abe et al. (1994). Recombinant soybean CysPI in 0.2 mL of reaction solution (0.25 M sodium phosphate buffer, 2.5 mM EDTA, and 25 mM β-mercaptoethanol, pH 6.0) was incubated with 7.5 μg of papain in 0.1 mL of solution (25 mM sodium phosphate buffer, pH 6.0) at 37°C for 5 min. Then, 0.2 mL of 1 mM BANA (Sigma) substrate was added to the reaction mixture, and incubation was continued for another 10 min. The reaction was terminated by the addition of 1 mL of 2% HCl in ethanol. The color product was developed by the addition of 1 mL of 0.06% *p*-dimethylaminocinnamaldehyde (Sigma) in ethanol. A_{540} was measured, and the velocity of the reaction was expressed as $\Delta A_{540}\ h^{-1}\ mL^{-1}$. K_i values were determined from a double reciprocal plot of the data (Dixon et al., 1979).

Assay for Papain Inhibitory Activity in Soybean Leaves

Leaves were frozen in liquid nitrogen and ground to a fine powder. The leaf powder was extracted with 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl and 2.0 mM EDTA (4 mL extraction buffer g^{-1} fresh weight of tissue). The samples were extracted by vortexing for 10 s and centrifuged at 12,000*g* for 15 min. The papain inhibitory activity of 100 μL of supernatant was assayed using BANA as the substrate (Abe et al., 1994).

Measurement of Soybean CysPI Activities against Insect Digestive Cys Proteinases

The alimentary tracts were dissected from the third-instar larvae of WCR (*Diabrotica virgifera* Le Carte) and CPB (*Leptinotarsa decemlineata* Say), which were obtained from the Department of Entomology (Purdue University, West Lafayette, IN). Whole guts (30 of WCR or 8 of CPB) were homogenized in 100 μL of 200 mM sodium acetate buffer (pH 5.0) using a glass pestle in a microfuge tube at 4°C, and the homogenate was centrifuged at 12,800*g* for 5 min. The supernatant was removed and diluted to 1 gut equivalent in 10 μL of buffer. Proteolytic activity was assayed using [^{3}H]methemoglobin as a substrate (Wieman and Nielsen, 1988). The reaction mixture included 50 μL of [^{3}H]methemoglobin (0.4 μCi/mL), 10 μL of 50 mM Cys, 10 μL of crude gut extract, 10 μL of recombinant soybean CysPI at different concentrations, and 20 μL of 20 mM sodium acetate buffer in a final volume of 100 μL. After incubation at 37°C for 10 min, the reaction was terminated by the addition of 100 μL of 10% (w/v) TCA. The mixture was held on ice for 20 min and then centrifuged at 12,000*g* for 5 min. The radioactivity in a 150-μL aliquot of the supernatant was determined by liquid scintillation spectrometry.

RESULTS

Wounding and M-JA Induce Differential Soybean CysPI Gene Expression and Activity

Three unique soybean cDNA CysPI clones, pL1, pN2, and pR1, were obtained from an immature embryo λZap II cDNA library (GenBank accession nos. U51853, U51854, and U51855, respectively). Since none of the deduced soybean CysPI sequences contains an N-terminal M, apparently the cDNAs do not encode full-length open reading frames (Fig. 1). At the N terminus, N2 is one residue longer than oryzacystatin I (Abe et al., 1987), and R1 and L1 are 7 and 9 residues shorter, respectively (Fig. 1). As with other plant CysPIs, the soybean proteins are conspicuous by the absence of Cys residues, indicating that disulfide linkages do not contribute to the functional structure of the proteins. The essential residues in the first and second hairpin loops are conserved in all of the soybean CysPIs, spatially located in appropriate positions downstream of the N termini

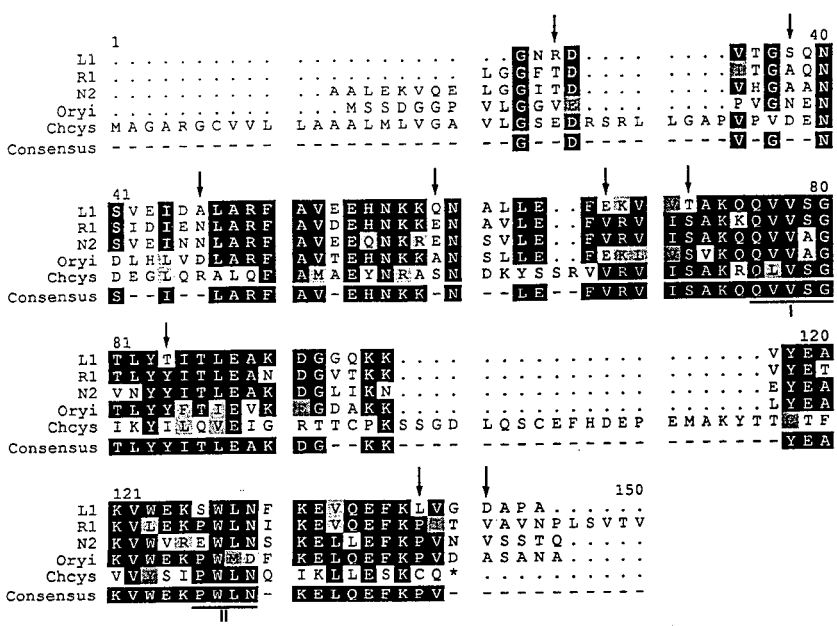

Figure 1. L1, N2, and R1 have sequence homology with oryzacystatin I and chicken cystatin. The predicted amino acid sequences of L1, N2, and R1 proteins were aligned with oryzacystatin I (Oryi; Abe et al., 1987) and chicken cystatin (Chcys; Colella et al., 1989) using the PileUp program (Genetics Computer Group, Madison, WI). Gaps introduced to optimize alignment comparisons are designated by dots. Conserved amino acid residues are shaded. The conserved domains that form the first and second hairpin loops (Machleidt et al., 1989) in Chcys, which interact with papain, are underlined and designated as I and II, respectively. Divergent amino acid residues among L1 and N2 and R1, which could be candidate residues contributing to the differences in CysPI activities of these proteins, are indicated by arrows. Dashes indicate gaps in consensus sequence.

(Machleidt et al., 1989). Recombinant N2 and R1 inhibited papain activity to a degree comparable to other plant CysPIs (Table I; Abe et al., 1994). These data confirm the likelihood that the N terminus is not essential for CysPI activity (Abe et al., 1988). Furthermore, crystallographic data indicate that the tertiary structure of CysPIs is not dependent on the N-terminal sequence (Bode et al., 1988; Stubbs et al., 1990). The substantial papain inhibitory activity exhibited by N2, despite the absence of the P residue in the second hairpin loop domain (position 126 in Fig. 1; Bode et al., 1988), may indicate that a P-W motif is not essential for the function of this loop domain in the interaction of the plant CysPIs with papain, as has been inferred for the interaction between cystatin and papain (Bode et al., 1988).

Wounding or M-JA treatment induced *N2* and *R1* expression in soybean leaves (Fig. 2A). Coincident with the induced expression of these CysPI genes was increased CysPI activity in leaves (Fig. 2B). Residual CysPI activity in soybean leaves was correlated with constitutive expression of *L1* and presumably attributable to L1. Since *L1* expression is not up-regulated by wounding or M-JA, the greater CysPI activity in soybean leaves, mediated by these inducers, is presumably due to N2 and R1 production.

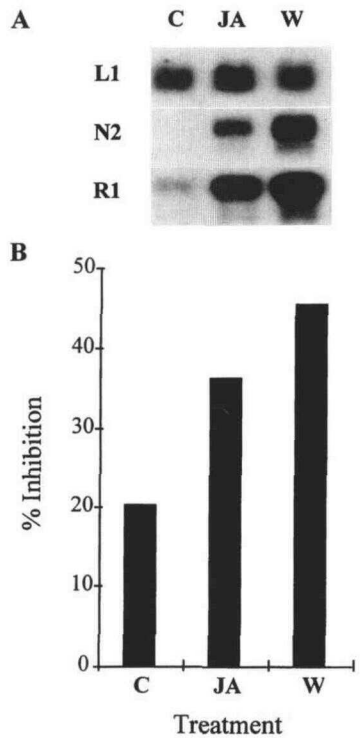

Figure 2. *N2* and *R1* mRNA accumulation and papain inhibitory activity are induced in leaves by wounding and M-JA treatments. A, Northern blot analysis of soybean CysPI transcripts; B, papain inhibitory activities of extracts from untreated (C), M-JA-treated (JA), or wounded (W) leaves. For the northern blot analysis, 10 μg of total leaf RNA were loaded onto a 1.5% formaldehyde-agarose gel. The RNA was probed with ^{32}P-labeled cDNA encoding L1, N2, or R1. The blot was sequentially probed after removal of previous radiolabeled probe according to the method of Sambrook et al. (1989). For analysis of papain inhibitory activity, leaf extracts were obtained as detailed in "Materials and Methods." Relative inhibitory activities are expressed as percentages of inhibition of papain proteolytic activity, average of 12 data points per treatment, where 100% is the activity in the assay mixture containing extraction buffer without cell extract.

Kinetics of Soybean CysPI Activity

Bacterial recombinant soybean CysPI proteins were produced using the GST expression system (Guan and Dixon, 1991). The open reading frames of *L1*, *N2*, or *R1* cDNAs were amplified by PCR and inserted into the pGEX-KG vector. Purification on a glutathione affinity column and thrombin cleavage resulted in the isolation of recombinant proteins with the appropriate apparent molecular mass of 11 kD (Fig. 3). The proteins of the wounding- and M-JA-inducible genes *N2* and *R1* (Fig. 2A) had substantially greater papain inhibitory activities than did L1 (Table I). R1 recombinant protein had inhibitory activity comparable to the isolated soybean seed CysPI that is encoded by *R1* (Hines et al., 1991; data not shown). The K_is for N2 and R1 were 2.5 to 3 orders of magnitude lower than that of L1. The substantial difference in papain inhibitory activity between N2 or R1 and L1 indicates that residues other than those in the QxVxG motif and the W, in the first and second hairpin loops, respectively, mediate high CysPI activity. Presumably, the important residues are common in N2 and R1 but differ in L1 (Fig. 1). The K_i values of N2 and R1 against papain are comparable to those of corn CysPI (CC-1) and oryzacystatin I, 37 and 32 nM, respectively (Abe et al., 1994).

L1 and R1 are noncompetitive inhibitors of papain, as indicated by the Lineweaver-Burk plots (Fig. 4). N2 also exhibited noncompetitive kinetics of inhibition against papain (data not shown). Cystatins are apparently reversible, competitive inhibitors of papain (Barrett, 1987). However, our results indicating noncompetitive inhibition of papain by soybean CysPIs are supported by the "docking" model of CysPI interaction with Cys proteinases proposed from crystallographic data (Bode et al., 1988; Stubbs et al., 1990) and the inhibition kinetic data of CC-1 (Abe et al., 1994).

Table I. *N2 and R1 proteins exhibit greater papain inhibitory activity than L1*

K_is of recombinant soybean CysPI proteins for papain were determined from Lineweaver-Burk plots (Dixon et al., 1979).

Soybean CysPI	K_i
	nM
L1	19,000
N2	57
R1	21

Soybean CysPIs Differentially Inhibit Insect Gut Proteinases

The digestive proteinases in alimentary tracts isolated from the third-instar larvae of WCR or CPB were substantially inhibited by recombinant L1, N2, and R1 (Fig. 5; Table II). Virtually all proteinase activity in the guts of these insects was attributable to Cys proteinases (Fig. 5; Orr

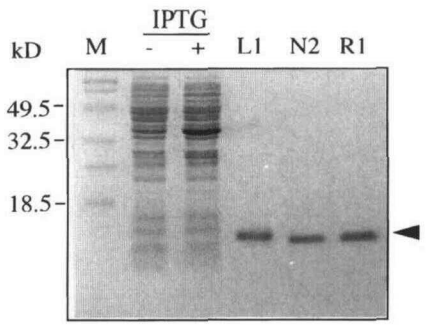

Figure 3. Soybean CysPI expression in the GST fusion protein system and purification of recombinant L1, N2, and R1 proteins. Bacterial lysates and purified recombinant proteins were analyzed by 15% SDS-PAGE. Expression of GST-CysPI fusion proteins was induced by IPTG. The fusion proteins were affinity-purified by glutathione affinity chromatography. Recombinant CysPIs were separated from GST by thrombin hydrolysis. Lane M, Molecular mass protein standards; lane −, lysate from uninduced bacteria; lane +, lysate from IPTG-induced bacteria overexpressing GST-L1 fusion protein; and lanes L1, N2, and R1, thrombin-cleaved and -eluted L1, N2, and R1, respectively.

et al., 1994), although numerous Cys proteinase isoforms may contribute to this activity (Gillikin et al., 1992). Kunitz inhibitor was virtually inactive against the insect gut proteases, indicating that trypsin activity was not prevalent. As with papain, N2 and R1 inhibited gut proteinase activities of both insects to a greater degree than L1. However, the soybean CysPIs were substantially less active against CPB than WCR gut proteinases. The substantially greater inhibitory activities of N2 and R1 against WCR digestive proteinases indicate the potential of these as WCR insecticides. E-64 is a potent irreversible inhibitor of Cys proteinases that was isolated originally from *Aspergillus japonicus*. The C-2 atom of the inhibitor forms a covalent ether link with the C-25 active site residue in papain (Yamamoto et al., 1991). Although N2 and R1 exhibited comparable inhibitory activities against WCR gut proteinases (Fig. 5A), N2 had greater activity against CPB gut proteinases than did R1 (Fig. 5B), indicating that the inducible isoforms of soybean CysPIs have differential activities against diverse Cys proteinases. Multiple defense CysPIs may represent a mechanism by which host plants negate the effects of multiple insect gut proteinase isoforms, and perhaps a way to cope with the capacity of insects to adapt gut proteinase activity (qualitatively or quantitatively) to a proteinase inhibitor challenge (Jongsma et al., 1995).

DISCUSSION

It is assumed that plants perceive insect predation similarly to how they perceive wounding, and that they utilize a common signal transduction cascade or common components of different pathways to initiate plant defense responses (Farmer and Ryan, 1992; Hildmann et al., 1992). Therefore, it is presumed that some wound-inducible genes, such as those that encode Ser proteinase inhibitors and CysPIs, likely function in host plant defense against insect predation. We provide additional confirmation that some soybean CysPIs have this function. CysPIs encoded by wound-inducible *N2* and *R1* have greater inhibitory activities against both papain and digestive Cys proteinases of two coleopteran insects, WCR and CPB, than the CysPI encoded by the constitutively expressed *L1*. Cys proteinases are the principal protein digestive enzymes in the guts of many coleopteran insects, including WCR and CPB (Murdock et al., 1987; Orr et al., 1994; Fig. 5). Our results provide evidence that specific CysPIs function in host plant insect resistance and, perhaps, that unique CysPIs function more effectively against specific insect pests.

Recombinant soybean CysPIs were substantially more active against gut proteinases of WCR than those of CPB (Fig. 5; Table II); N2 and R1 had numerous times greater inhibitory activities than E-64 against WCR gut proteinases. The Cys proteinases in guts of CPB are apparently less sensitive to soybean CysPIs than those of WCR, at least

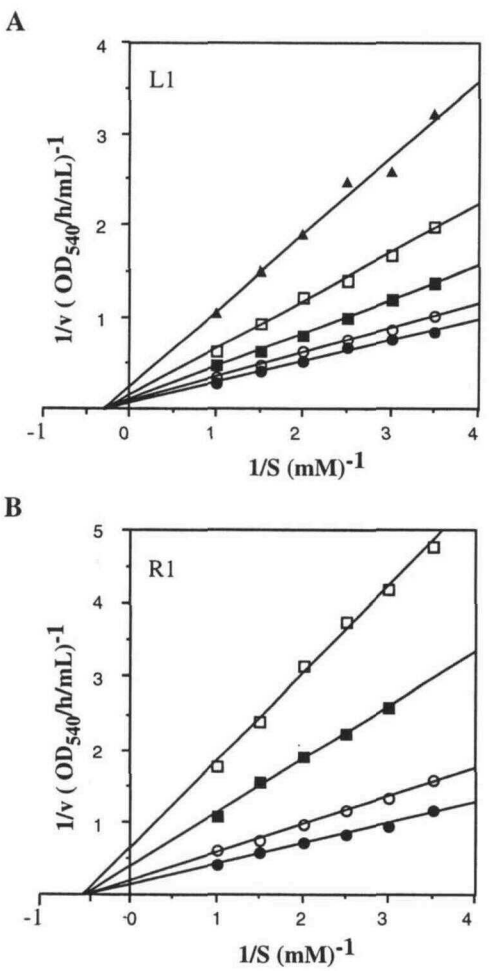

Figure 4. Inhibition of papain activity by soybean CysPIs is noncompetitive. Inhibition kinetic data are illustrated in Lineweaver-Burk double-reciprocal plots. CysPI activity was determined using various concentrations of BANA as substrate. A, Plot of L1 kinetic data. ●, No inhibitor in the assay mixture; ○, 25 μg of L1; ■, 50 μg of L1; □, 75 μg of L1; ▲, 100 μg of of L1. B, Double-reciprocal plot of R1 kinetic data. ●, no inhibitor; ○, 0.3 μg of R1; ■, 0.6 μg of R1; □, 0.75 μg of R1.

those proteinases isolated from third-instar larvae. Another possibility is that the recombinant soybean CysPIs are not stable in CPB gut extracts. Orr et al. (1994) determined that tryptic fragments of potato multicystatin were effective inhibitors of gut Cys proteinase activities of WCR and southern corn rootworm, but did not inhibit the growth of WCR or southern corn rootworm in feeding bioassays, despite the fact that the multicystatin from which these were derived were active in these assays. Co-feeding of the tryptic fragments with potato carboxypeptidase resulted in growth inhibition of southern corn rootworm but not WCR. The authors proposed that monodomain cystatins are vulnerable to biochemical degradation in insect guts in vivo (Orr et al., 1994). However, this scenario may not be applicable to every monodomain CysPI, because we have determined that 0.2% of recombinant N2 protein painted onto the surface of potato leaves substantially reduced CPB larval feeding, leading to weight loss and delayed larval development (Y. Zhao, M.A. Botella, L. Subramanian, X. Niu, S.S. Nielsen, R.A. Bressan, and P.M. Hasegawa, unpublished data).

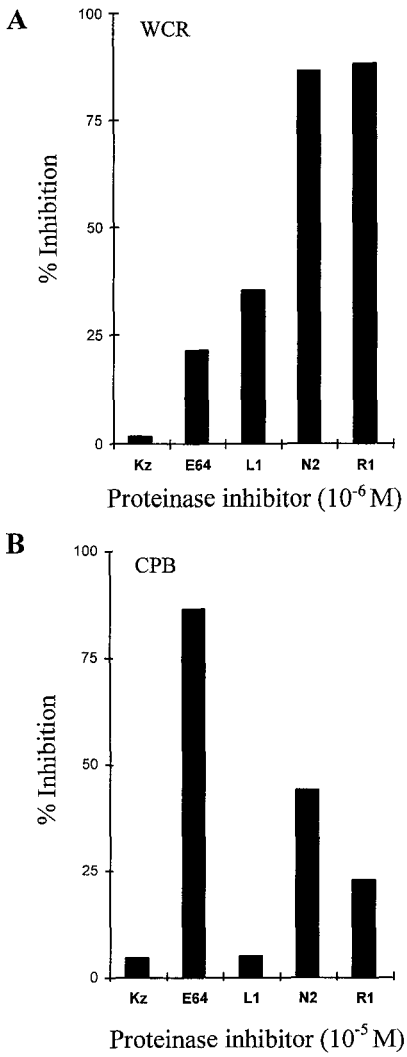

Figure 5. Wound-inducible N2 and R1 proteins have greater insect digestive proteinase inhibitory activities than does constitutive L1 protein. Inhibition of midgut proteolytic activity from third-instar larvae of WCR (A) or CPB (B) by Kunitz trypsin inhibitor (Kz), E-64 (E64), or recombinant soybean CysPIs proteins L1, N2, and R1 was assayed using [^{3}H]methemoglobin as substrate. The relative inhibitory activities are expressed as percentages of inhibition of 100% papain activity (without inhibitor), average of 6 (A) or 12 (B) data points per treatment.

Table II. *Inhibition by soybean CysPIs of proteinase activities in guts of WCR larvae*

The inhibitory activities of the CysPIs were measured as described in Figure 5. Fifty percent inhibition values were derived from a plot of data illustrating inhibition of WCR gut proteinase activity as a function of CysPI concentration.

Inhibitor	Concentration for 50% Inhibition
	nM
E-64	7000
L1	4000
N2	50
R1	200

Although L1, N2, and R1 have 60 to 70% sequence identity (Fig. 1), L1 has more than 2.5 orders of magnitude less papain inhibitory activity than N2 or R1 (Table I). Also, N2 and R1 have substantially different inhibitory effects on WCR and CPB gut proteinases. From this information can be inferred some insights pertaining to critical motifs or perhaps residues that mediate differences in papain and specific insect gut proteinase inhibitory activities that would direct future structure and function research using site-specific mutagenesis. Candidate motifs or residues that are responsible for the differences in papain and WCR gut proteinase inhibitory activities are those that are common to N2 and R1 but differ in L1 (Fig. 1), particularly those that are contained in other CysPIs with high inhibitory activities. Some of these are probably also involved in the lower efficacy of N2 and R1 against CPB gut proteinases. Residues that diverge between N2 and R1 may be the basis for the difference between the inhibitory activities of these CysPIs against CPB gut proteinases.

Potential critical residues can be substituted or deleted to improve the inhibitory activity or target enzyme specificity. Known motifs or residues essential for CysPI activity, such as the QxVxG and W, must exist in all of these proteins; therefore, it is presumed that other motif and residue differences impact target enzyme specificity. Residues that increase inhibitory activity or enhance target specificity are candidates for modification that could improve CysPI efficacy and host plant defense against insects and nematodes (Urwin et al., 1995). Deletion of D86 of oryzacystatin-I resulted in 14-fold lower K_i values against papain and a cyst nematode (*Globodera pallida*) gut proteinase, and substantially reduced growth and development of *G. pallida* in tomato roots (Urwin et al., 1995). Conversely, substitutions for P83, W84, and D86 reduced CysPI activity of oryzacystatin I. Strategies to mediate host plant insect resistance with CysPIs must be focused against the myriad of Cys proteinase isoforms that are used for protein digestion by insects and are induced in insect guts by exposure to an inhibitor (Orr et al., 1994; Jongsma et al., 1995).

Presently, molecular diversity is attained by surveying plants for alternative forms of proteins, but in the future methods of directed protein evolution will likely result in more efficacious CysPI isoforms.

In addition to the involvement in plant defense, CysPIs presumably have roles in growth and development. This is indicated by the diverse functions of Cys proteinases in processes such as protein turnover (Baumgartner and Chrispeels, 1976; Holwerda and Rogers, 1992; Kalinski et al., 1992; Michaud et al., 1994; Yu and Greenwood, 1994), stress responses (Schaffer and Fischer, 1988; Koizumi et al., 1993), and protein processing (Shapira and Nuss, 1991). If we assume an analogy with organisms as diverse as nematodes and humans, CysPIs may function to regulate the programmed cell death required for plant development and senescence (Martin and Green, 1995; Steller, 1995). A nodule-specific plant Cys proteinase that is assumed to function in the symbiosis between *Frankia* and *Alnus glutinosa*, perhaps in the alteration of root morphology that results in nodulation (Goetting-Minesky and Mullin, 1994) has been identified. A Cys proteinase has been implicated in the senescence-specific proteolysis that occurs during pea ovary senescence (Cercós and Carbonell, 1993). *SAG12*, which encodes a Cys proteinase, has been identified because of its specific up-regulation during leaf senescence of Arabidopsis (Gan and Amasino, 1995). *SAG12* may be a plant homolog of the interleukin-1β-converting enzyme family that is currently believed to be an essential component of programmed cell death (Hengartner and Horvitz, 1994; Martin and Green, 1995; Steller, 1995; Thompson, 1995). Consequently, regulation of Cys proteinases may be fundamental in the control of cell death, including those processes that mediate plant growth and development. It is possible that future research will determine that CysPIs have a substantial function in the regulation of these processes.

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