Plant Physiol. (1996) 110: 195–202

An Insecticidal *N*-Acetylglucosamine-Specific Lectin Gene from *Griffonia simplicifolia* (Leguminosae)[1]

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***Griffonia simplicifolia* II, an *N*-acetylglucosamine-specific legume lectin, has insecticidal activity when fed to the cowpea weevil, *Callosobruchus maculatus* (F.). A cDNA clone encoding *G. simplicifolia* II was isolated from a leaf cDNA library, sequenced, and expressed in a bacterial expression system. The recombinant protein exhibited *N*-acetylglucosamine-binding and insecticidal activity against cowpea weevil, indicating that glycosylation and multimeric structure are not required for these properties. These results support the hypothesis that genes of the legume lectin gene family encode proteins that function in plant defense against herbivores.**

Lectins are carbohydrate-binding proteins (Barondes, 1988) that may function as cell-recognition molecules (Sharon and Lis, 1989) or as mediators of symbioses between nitrogen-fixing microorganisms and legumes (Diaz et al., 1989). Accumulating evidence suggests that lectins may also be involved in plant defense against fungi and insects (Chrispeels and Raikhel, 1991). Lectins with specificity for GlcNAc residues appear to be insecticidal to many insects, including the cowpea weevil, *Callosobruchus maculatus*, a pest of stored cowpea (*Vigna unguiculata* Walp.) seeds. Most cowpea weevil-active lectins and lectin-like proteins are members of the chitin-binding protein or legume lectin gene families (Chrispeels and Raikhel, 1991).

Proteins of the chitin-binding protein family contain one or more highly conserved 30- to 43-amino acid chitin-binding domains. The chitin-binding domain occurs in several different proteins and is an example of an evolutionarily mobile domain (Raikhel et al., 1993). Certain proteins that appear to function in plant defense against pests, such as wheat germ agglutinin and the basic chitinases (Czapla and Lang, 1990; Murdock et al., 1990; Habibi et al., 1993; Powell et al., 1993), contain the chitin-binding domain.

The legume lectin gene family is a large family of homologous proteins that arose by gene duplication and divergence from an ancestral lectin gene (Chrispeels and Raikhel, 1991). Members of this family, such as PHA, α-amylase inhibitor, and arcelin, appear to function in plant defense against mammals, birds, and insects (Jaffe and Vega Lette, 1968; Jayne-Williams and Burgess, 1974). Legume lectins and lectin-like proteins substantially influence the interactions of seed beetles of the family Bruchidae with seeds of their host plant family Leguminoseae. For example, common bean α-amylase inhibitor poses a major natural barrier to colonization of common bean seeds (*Phaseolus vulgaris*) by the cowpea weevil (Ishimoto and Kitamura, 1989; Huesing et al., 1991). Expression of a gene encoding common bean α-amylase inhibitor resulted in pea (*Pisum sativum*) seed immunity to azuki bean weevil and cowpea weevil (Shade et al., 1994). Arcelin, another protein of the legume lectin gene family from wild *P. vulgaris*, is toxic to the Mexican bean weevil, *Zabrotes subfasciatus* (Osborn et al., 1988), and has been used to develop *Z. subfasciatus*-resistant common bean lines (Kornegay et al., 1993).

Since lectins with specificity for GlcNAc are toxic to cowpea weevil and since lectins of the legume lectin gene family appear to function in plant defense against seed-feeding bruchids, we decided to clone a GlcNAc-specific legume lectin gene. One such lectin is the GlcNAc-specific GS-II. Leaves and seeds of *G. simplicifolia* contain several different homologous lectins that vary in their carbohydrate specificities (Shankar Iyer et al., 1976; Ebisu et al., 1978; Delmotte and Goldstein, 1980; Lamb et al., 1983). Leaf GS-II is composed of two types of subunits, whereas seed GS-II is a tetrameric protein containing a single type of subunit. Both leaf and seed GS-II lectins show similar carbohydrate-binding specificity and antigenicity. Preliminary studies in our laboratory showed that seed GS-II was toxic to the cowpea weevil. In the present paper, we describe the isolation and bioassay of leaf GS-II lectin against cowpea weevil and the cloning of a leaf GS-II lectin gene, which to our knowledge is the first GlcNAc-specific legume lectin gene to be cloned, together with its expression in a bacterial expression system. We demonstrate that glycosylation and multimeric structure are not essential for carbohydrate-binding and insecticidal activities.

MATERIALS AND METHODS

Griffonia simplicifolia Plants

Fresh *Griffonia simplicifolia* seeds were purchased from Forestry Enterprises (Accra, Ghana, West Africa). They were surface-disinfected in 10% laundry bleach for 1 min and rinsed with distilled water. After soaking in distilled

[1] This work was supported in part by USAID under the Bean/Cowpea Collaborative Research Support Program, and by the Office of Agricultural Research, Purdue University, West Lafayette, IN. This is journal paper No. 14,713 of the Purdue Agriculture Experiment Station.

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Abbreviations: Bt, *Bacillus thuringiensis*; CPB, Colorado potato beetles; GS-II, *Griffonia simplicifolia* II; PHA, phytohemagglutinin; WSDT, within seed developmental time.

water for 5 min, the testae were scarified with a razor blade to promote water absorption during germination. They were then held on moist filter paper until they germinated. Seedlings were transferred to soil in pots and grown in the greenhouse under standard conditions.

Isolation of GS-II Lectin from *G. simplicifolia* Leaves

The method of Lamb et al. (1983) was followed with minor modifications. Leaves were excised from the plants and homogenized in extraction buffer (0.1 M phosphate, pH 7.2, 0.15 M isoascorbic acid, 1 mM DTT, and 1 mM PMSF) using a Polytron (Tekmar Co., Cleveland, OH). The homogenate was passed through three layers of cheesecloth, followed by centrifugation at 10,000*g* for 30 min. $(NH_4)_2SO_4$ was added to the supernatant to 40% saturation, and the pH was adjusted to 7.2. After stirring in a cold room for 1 h the solution was centrifuged as above. $(NH_4)_2SO_4$ was added to the supernatant to 80% saturation, and the solution was adjusted to pH 7.2. After overnight stirring in a cold room, the solution was centrifuged (10,000*g*, 30 min). The pellet was resuspended in distilled water, dialyzed, and lyophilized. The lyophilized protein powder was dissolved in 0.1 M NaCl, spun briefly, and loaded on a GlcNAc affinity column (EY Laboratories, San Mateo, CA). After the column was washed with 0.1 M NaCl, the GS-II protein was eluted with buffer (0.2 M GlcNAc, 0.1 M NaCl), followed by dialysis and lyophilization.

Bioassay of Leaf GS-II against Cowpea Weevil

Cowpea weevils (*Callosobruchus maculatus*) were maintained on susceptible cowpeas, California Blackeye No. 5 (CB-5). The cowpea weevil colony originated in Niger, West Africa. The artificial cowpea seed bioassay system developed by Shade et al. (1986) was used to evaluate GS-II against cowpea weevil. Briefly, susceptible CB-5 cowpea seeds were milled into flour, and the flour was wetted with distilled water and injected into a Teflon mold. GS-II was incorporated into the artificial seeds by dissolving it first in distilled water. After freezing on dry ice and liquid nitrogen and lyophilization, the artificial seeds were coated with 8% (w/v) gelatin and infested with bruchids. WSDT was the life history parameter determined; delayed development served as a measure of negative effects of test proteins.

Electrophoresis and Immunoblotting

Purified GS-II was analyzed on 15% SDS-PAGE gels (Laemmli, 1970). Transfer to a nitrocellulose membrane and immunoblot analysis were carried out according to the Bio-Rad technical instruction protocols. Polyclonal rabbit anti-seed GS-II (EY Laboratories) was used as the primary antibody. Goat anti-rabbit immunoglobulins coupled to alkaline phosphatase conjugate were used as the secondary antibody. Molecular masses were determined by comparison to Bio-Rad low-molecular-weight standards.

N-Terminal Amino Acid Sequencing

SDS-PAGE was performed as above, except that the gel was prerun with 0.05 mM glutathione in the upper buffer. The samples were then run with 0.1 mM sodium thioglycolate in the upper buffer to prevent N-terminal blockage. The protein samples were then electroblotted to a polyvinylidene difluoride membrane. The desired band was then excised and sequenced in the Laboratory for Macromolecular Structure, Department of Biochemistry (Purdue University, West Lafayette, IN) using a model 470A ABI protein sequencer (Columbia, MD) based on the Edman degradation.

cDNA Library Construction

Total RNA from young leaves was prepared using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified from total RNA using oligo(dT) cellulose as described in Promega's protocols and applications guide. A λ-ZAP cDNA synthesis kit (Stratagene) was used to prepare the cDNA library. Synthesized cDNA, fractionated through Sephacryl S-400 columns, was ligated to the Uni-ZAP XR vector arm unidirectionally and packaged using Gigapack II Gold (Stratagene). The primary library contained 4×10^5 plaque-forming units.

Cloning and Sequencing of the GS-II Gene

Polyclonal rabbit anti-seed GS-II (EY Laboratories) was used to screen the cDNA library as described in the picoBlue immunoscreening instruction protocol (Stratagene). λ-ZAP clones showing strong reaction with the antibody were isolated, screened to plaque purity, and converted to pBluescript by co-infection with R408 helper phage. The sequence of both strands of cDNA inserts was determined by the Sanger dideoxy nucleotide chain termination method using a sequenase DNA sequencing kit (United States Biochemical). Subcloning and oligonucleotide primers were used to complete sequencing in both strands. Three *gsii* clones were isolated.

5′ Extension of the cDNA Clone

All three *gsii* clones lacked a complete 5′ signal sequence. To obtain this sequence information, we used a variation of anchored or one-sided PCR. Briefly, two overlapping antisense primers, about 200 bp downstream of the 5′ terminus of the *gsii*-1 clone, were synthesized. Primer 2 was nested upstream of primer 1. The sequence of primer 1 was 5′-$G_{(204)}$CTAGCCTCCTTTCCTGTG$_{(186)}$, and the sequence of primer 2 was 5′-$C_{(191)}$CTGTGCTCTTCTCCCAGAG$_{(172)}$. Specific first-strand cDNA was synthesized as follows: 100 ng of antisense primer 1 was added to 20 μg of total RNA template. The mixture was heated at 65°C for 15 min and then cooled on ice. The final transcriptional reaction contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP mix, 4.0 units of RNase inhibitor (United States Biochemical), and 200 units of Moloney murine leukemia virus reverse transcriptase (United States

Biochemical). The reaction volume was adjusted to 50 μL with diethylpyrocarbonate-treated water. The sample was mixed well and incubated at 42°C for 1 h; the reaction was stopped by addition of 150 μL of 10 mM Tris-HCl, 10 mM EDTA (pH 7.5), followed by phenol/chloroform and chloroform extraction. The RNA/cDNA pellet obtained by overnight ethanol precipitation at −80°C was resuspended in 17 μL of H_2O, from which 8.5 μL were used to ligate to 300 ng of the pBluescript vector digested with *Eco*RV and *Kpn*I. From the ligation reaction, 0.2 μL of product was amplified by PCR (Epicentre Technologies, Madison, WI) using the T3 priming site of the vector and primer 1. Following the first PCR reaction, the second PCR amplification was performed using the T3 primer and nested primer 2. Use of a second nested antisense primer greatly reduced the likelihood of reamplification of any clones arising from nonspecific or false priming in the first reaction. Five microliters of the previous PCR product were used as template. The amplification conditions involved denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 1 min. Amplification was run for 21 cycles using a Perkin-Elmer/Cetus thermal cycler. Ten microliters of the second PCR product were subjected to electrophoresis and Southern blotting using an *Eco*RI-*Eco*RI leaf GS-II cDNA fragment as a probe, which was randomly primed with ^{32}P (United States Biochemical). The band showing the appropriate molecular weight and positive result in the Southern blot was excised from the agarose gel, electroeluted, and ligated to a pGEM vector (Promega) digested with *Sma*I for 48 h at 30°C. After transformation of DH5α competent cells, the presence of inserts in the plasmids was determined by small-scale preparation of plasmid DNA. The inserts were sequenced using the T7 primer and primer 2.

Southern Analysis

Genomic DNA was extracted from leaf tissue according to Dellaporta (1983). Thirty micrograms of DNA were digested with *Hin*dIII and *Eco*RI, separated on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled (Pharmacia) DNA fragment encoding the entire mature GS-II protein. The filter was washed at room temperature for 30 min each in 2×, 1×, and 0.1× SSC, each with 0.1% SDS, and finally in 0.1× SSC, 0.1% SDS at 65°C for 1 h.

gsii Gene Expression in pET Bacterial Expression System

The cDNA clone *gsii*-1, encoding the entire mature protein, was amplified by PCR. An ATG translational start signal was added in-frame upstream of the DNA fragment. The 5′ end primer was 5′-ATGGCCAAGCTT-$CATATGG_{(1)}$CTGATACAGTTTGCTTC$_{(18)}$. The 3′ end primer was 5′-CCGGCTCGAGGATCCT$_{(765)}$CACATGT-$CCGTTATGGC_{(748)}$. After PCR and restriction enzyme (*Nde*I and *Bam*HI) digestion, the fragment was ligated to a pET-9c expression vector (Novagen, Madison, WI). The construct was then transferred to *Escherichia coli* strain BL21 (Novagen) and induced with isopropylthio-β-galactoside. The induced recombinant protein was purified on a GlcNAc affinity column and checked by SDS-PAGE. GlcNAc-affinity-purified protein was used to carry out a dose-response analysis with cowpea weevil as described above at dietary levels of 0, 0.2, 0.4, 0.6, 0.8, and 1.0% (w/w).

Expression of GS-II in Different Tissues of *G. simplicifolia*

Leaf, seed, stem, and root tissues were collected and immediately homogenized in 1× protein sample buffer (62 mM Tris, pH 6.8, 3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromphenol blue). After centrifugation, the supernatant of each tissue extract was run on duplicate gels, one being stained with Coomassie blue to test for even gel loading and protein integrity, the other for immunoblotting. Chicken anti-recombinant GS-II was produced by subcutaneous injection and used as the primary polyclonal antibody. Immunoblotting was performed as described above.

RESULTS

Comparison of Leaf and Seed GS-II Lectins

Seed GS-II (EY Laboratories) exhibited only a single band of 30 kD on SDS-PAGE gels, whereas leaf GS-II was composed of subunits of 30 and 22 kD (Fig. 1A). This result confirms that of Lamb et al. (1983), who suggested that seed GS-II contains a single subunit, whereas leaf GS-II is composed of two subunits. Western blot analysis established that only the 30-kD band of leaf GS-II reacts with the polyclonal anti-seed GS-II antibody (Fig. 1B), indicating that the antigenic properties of the large subunit of leaf and seed GS-II are similar. N-terminal amino acid sequences of the 30-kD subunit from leaf and seed GS-II showed 77% identity (Fig. 2).

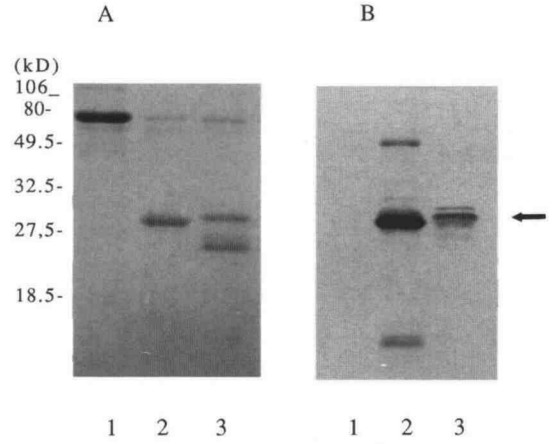

Figure 1. SDS-PAGE and immunoblot of leaf and seed GS-II. A, Coomassie brilliant blue R-250-stained gel. Electrophoresis was carried out in the Laemmli system containing 15% acrylamide. B, Immunoblot of the gel shown in A using polyclonal rabbit anti-seed GS-II antibody. Lane 1, BSA; lane 2, 2 μg of commercial seed GS-II; lane 3, 2 μg of GlcNAc affinity-column-purified leaf GS-II. The 30-kD subunit of leaf GS-II cross-reacting with the polyclonal anti-seed GS-II antibody is marked by an arrow.

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Leaf GS-II: ADTVCFTYPNFGNAVSDLILQ
Seed GS-II: ADTICFTYTNFGLDVSDLTLQGAAK
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Figure 2. N-terminal amino acid sequences of leaf and seed GS-II. Purified leaf GS-II and commercial seed GS-II were subjected to SDS-PAGE and electroblotted to a polyvinylidene difluoride membrane. The band corresponding to the 30-kD subunit of leaf GS-II and the single band of seed GS-II were sequenced based on Edman degradation. Residues that are different between leaf and seed GS-II are highlighted.

Anti-Cowpea Weevil Activity of Leaf and Seed GS-II

Table I shows that incorporation of GS-II from either leaf or seed into artificial seeds significantly delayed the WSDT of cowpea weevil. One percent (w/w) of leaf GS-II in the artificial seeds caused a 32-d delay in WSDT, i.e. a doubling of developmental time.

Cloning the cDNA of Leaf GS-II Lectin

Immunoscreening of the *G. simplicifolia* leaf cDNA library with a commercial polyclonal antibody raised against seed GS-II identified three clones. Nucleic acid sequencing revealed that these clones represented three different homologous transcripts. Differences were mainly in the 3′ untranslated region (Fig. 3). The deduced amino acid sequences perfectly matched the N-terminal amino acid sequence obtained earlier. The several amino acid residues upstream of the N terminus presumably represent part of the signal sequence.

5′ Extension of the *gsii*-1 Clone and Southern Analysis Indicate the Presence of a *gsii* Gene Family

Small-scale plasmid purification and agarose gel electrophoresis of clones derived from PCR amplification of total RNA using *gsii*-1-generated nested antisense primers resulted in 14 transformed DH5α colonies containing inserts of the appropriate size. The 14 clones were sequenced. These clones represented seven different *gsii*-1-like sequences, indicating the presence of a large family of transcribed GS-II lectin genes (data not shown). None of the extended cDNAs were identical to the 5′ coding region of the three cDNA clones isolated earlier. Figure 4 shows the nucleic acid and deduced amino acid sequences derived from 5′ extension of one of the seven clones. The overlapping regions from the cDNA clones and 5′ extension share 88% identity at the nucleic acid level and 74% identity at the amino acid level. Therefore, the original three cDNAs and the seven different cDNAs produced by 5′ extension represent 10 different but highly similar transcripts from *gsii* genes. Because of the high degree of sequence similarity between these cDNAs, we reasoned that the signal sequence obtained from 5′ extension would be very similar to that of the *gsii*-1 cDNA clone and would function in a chimeric protein construction.

Following restriction with *Eco*RI and *Hind*III, several bands differing in intensity on Southern blots were observed (Fig. 5), also indicating a multigene family.

Insecticidal Activity of the Recombinant GS-II Protein

Following ligation of the DNA fragment that codes for the mature GS-II protein to a pET-9c expression vector, transfer of the recombinant DNA into *E. coli* strain BL21, and induction by isopropylthio-β-galactoside, substantial amounts of recombinant GS-II protein were produced (Fig. 6). Nucleic acid and N-terminal amino acid sequencing confirmed that the protein expressed in the bacterial system was identical to the large subunit of the leaf GS-II. GlcNAc-binding specificity was unaffected, since the recombinant protein bound to the GlcNAc affinity column and was eluted with GlcNAc. Bioassay of purified recombinant GS-II using protein concentrations ranging from 0 to 1% (w/w) in artificial seeds against cowpea weevil is presented in Figure 7. For each 1% (w/w) of recombinant GS-II, there was a 7.4-d delay in WSDT with an r^2 of 0.985. Since the recombinant protein is not glycosylated, this result indicates that glycosylation is not essential for the carbohydrate-binding properties or the insecticidal activities of the protein, although it may enhance activity.

GS-II Gene Expression Is Developmentally Regulated

Immunoblots showed the presence of GS-II lectin in all *G. simplicifolia* tissues tested (Fig. 8). However, the expression level was much lower in stem than in other tissues. Two or more isoforms were found in leaves and seeds. These may represent the products of different genes in the multigene family. Alternatively, they may result from some posttranslational modifications, such as differential glycosylation.

DISCUSSION

Several lectins with specificity for GlcNAc have been shown to cause delays in the development of the cowpea weevil (Murdock et al., 1990). As to insecticidal activity, seed GS-II lectin is 10-fold more active than wheat germ agglutinin when compared on a molar basis (J.E. Huesing, R.E. Shade, L.L. Murdock, unpublished data). Leaf GS-II at 1% (w/w) delayed the development of *C. maculatus* by 32 d, which is approximately the same effect of 0.5% seed GS-II. Given the shared immunoreactivity of leaf and seed GS-II and the substantial sequence homology between the 30-kD subunit of leaf and the single subunit of seed GS-II, it may be that the insecticidal activity of leaf GS-II is embodied in the 30-kD subunit only, whereas the 22-kD

Table I. *Effect of seed and leaf GS-II from G. simplicifolia on WSDT of cowpea weevil feeding in artificial cowpea seeds (ACPS)*

Treatments followed by the same letter are not significantly different.

Treatments	WSDT[a]
	d
Intact CB-5 seeds	28.79a
ACPS control	29.85a
ACPS + 0.5% (w/w) seed GS-II	63.29b
ACPS + 1% (w/w) leaf GS-II	61.52b

[a] Student-Newman-Keuls was used for the range test ($\alpha = 0.05$).

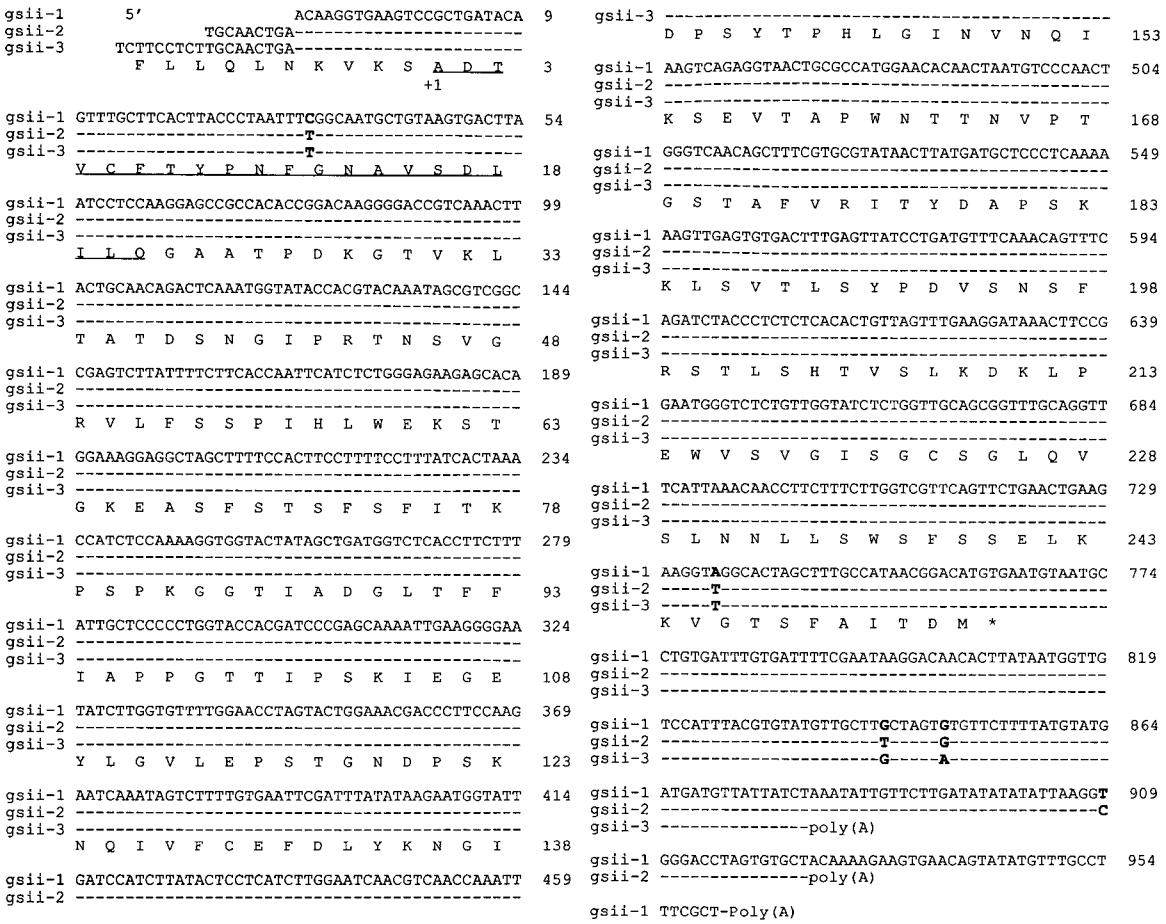

Figure 3. The nucleic acid and deduced amino acid sequences of three cDNA clones, *gsii*-1, *gsii*-2, and *gsii*-3, isolated from a *G. simplicifolia* leaf cDNA library. Identical bases are indicated by dashes. The underlined region matches the N-terminal amino acid sequence from leaf GS-II sequencing; therefore, +1 represents the N terminus of the mature GS-II protein. The sequence in front of the underlined region is believed to be a partial signal sequence. The translation termination site is designated with an asterisk (*).

subunit may either not bind carbohydrate or may differ in carbohydrate specificity. The significance of the 22-kD subunit remains to be elucidated.

The developmental delay that results when cowpea weevil larvae consume a diet containing recombinant GS-II establishes that the recombinant protein is toxic. Compared to native leaf or seed GS-II, however, its potency is lower. The higher relative toxicity of native GS-II might result because the native leaf and seed proteins are multimers linked by disulfide bonds, whereas recombinant GS-II is a monomer. Subunits in multimers may structurally enhance the interactions with their target sites or help protect against proteolytic degradation. A multicystatin from potato tuber reportedly inhibited the development of corn rootworm larvae, whereas cystatin monomers produced by trypsinization of the multicystatin exhibited no growth-retarding effect even though the cystatin fragments inhibited gut activity in vitro (Walsh and Strickland, 1993; Orr et al., 1994). It may be that glycosylation of native GS-II contributes to its biological activity, although it is not essential. However, given that carbohydrate attachment positions in glycosylated legume lectins are not conserved, it has been suggested that the carbohydrate units may not be involved in an important functional role (Pusztai, 1991). Another possible explanation of the lower biological activity of recombinant GS-II is that the 22-kD subunit normally plays a role in enhancing the activity of the 30-kD subunit. Finally, it is possible that the recombinant GS-II protein

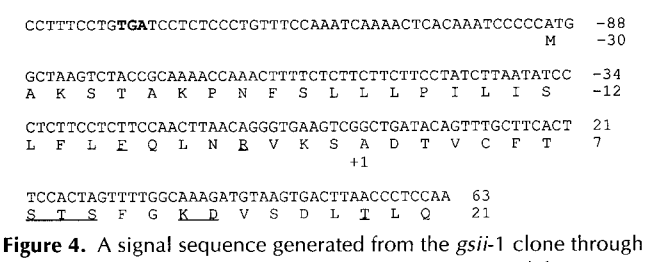

Figure 4. A signal sequence generated from the *gsii*-1 clone through 5′ extension. Two nested antisense primers were used for reverse transcription and PCR to reduce the likelihood of amplification of clones by nonspecific priming. Seven highly homologous clones were obtained. The stop codon preceding the putative translational start codon is highlighted. The N terminus of the mature GS-II is indicated as +1. The amino acid residues that differ from those in the *gsii*-1 cDNA clone are underlined.

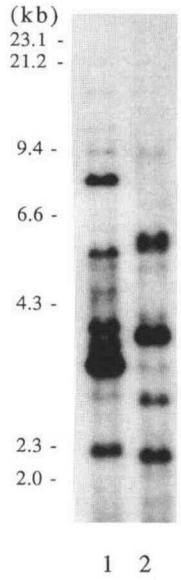

Figure 5. DNA hybridization analysis showing the existence of a GS-II multigene family. Thirty micrograms of genomic DNA from *G. simplicifolia* leaves were treated with *Hin*dIII (lane 1) and *Eco*RI (lane 2), fractionated on a 0.7% (w/v) agarose gel, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled DNA fragment of the *gsii*-1 clone that encodes the entire mature GS-II protein.

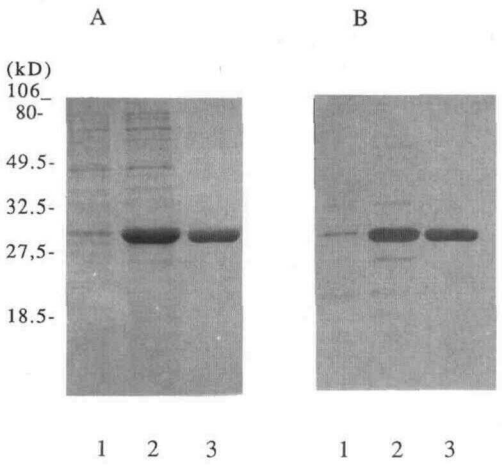

Figure 6. Bacterial expression of the *gsii*-1 gene and purification of the recombinant GS-II. A, SDS-PAGE. B, Immunoblot of the gel shown in A. The DNA fragment encoding the entire mature GS-II protein was ligated to pET-9c expression vector. The construct was transferred to *E. coli* strain BL21 and induced by isopropylthio-β-galactoside. One hundred microliters each of uninduced (lane 1) and induced (lane 2) bacterial cell cultures were spun briefly and dissolved in 20 μL of 1× sample buffer, electrophoresed, and immunoreacted with polyclonal chicken anti-recombinant GS-II antibody. The induced bacterial cells were lysed and passed through a GlcNAc affinity column, and 5 μg of the product are shown in lane 3.

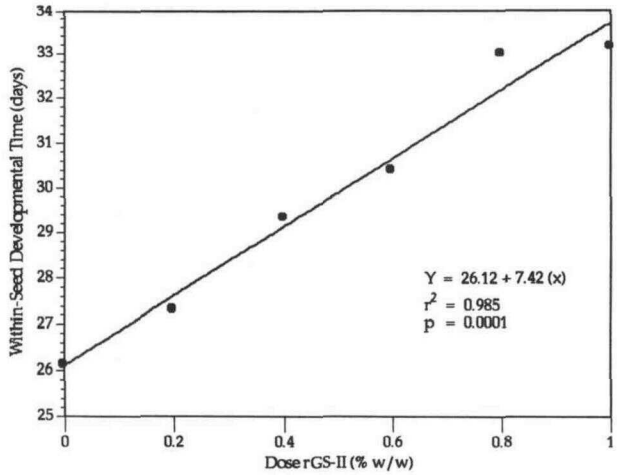

Figure 7. Dose-response analysis of recombinant GS-II (rGS-II) versus cowpea weevil WSDT (days at 26°C and 60% RH). Each data point represents the mean of at least 24 insects.

may not be folded completely normally, so that although it binds GlcNAc, its stability or biological activity is otherwise attenuated.

A putative N-linked glycosylation site is present at Asn^{162} in the mature protein of clone *gsii*-1. Expression of recombinant GS-II in bacteria and subsequent purification on a GlcNAc affinity column indicates that glycosylation is not required for the GlcNAc binding. Bacterially synthesized, unglycosylated PHA-L retains the same leukagglutinating and mitogenic activity as the native lectin (Hoffman and Donaldson, 1987). It thus appears that with the legume lectins, the carbohydrate moieties may not play a crucial role in forming the tertiary structure of the holoprotein and that their biological activity is unaffected by the absence of carbohydrate.

The carbohydrate-binding sites of Con A, PHA, and some Vicieae lectins are formed by six amino acid residues

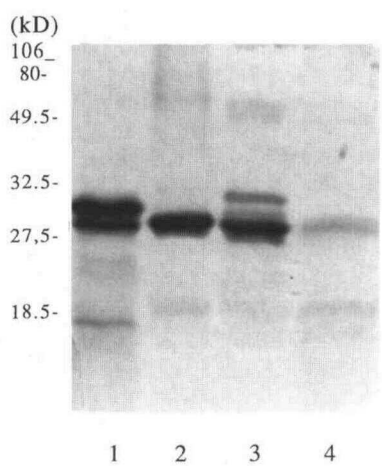

Figure 8. Expression pattern of GS-II protein in *G. simplicifolia* tissues. Shown is an immunoblot of 20 μg of protein from leaf (lane 1), root (lane 2), seed (lane 3), and stem (lane 4) separated by 15% SDS-PAGE, transferred onto nitrocellulose paper, and probed with polyclonal chicken anti-recombinant GS-II antibody.

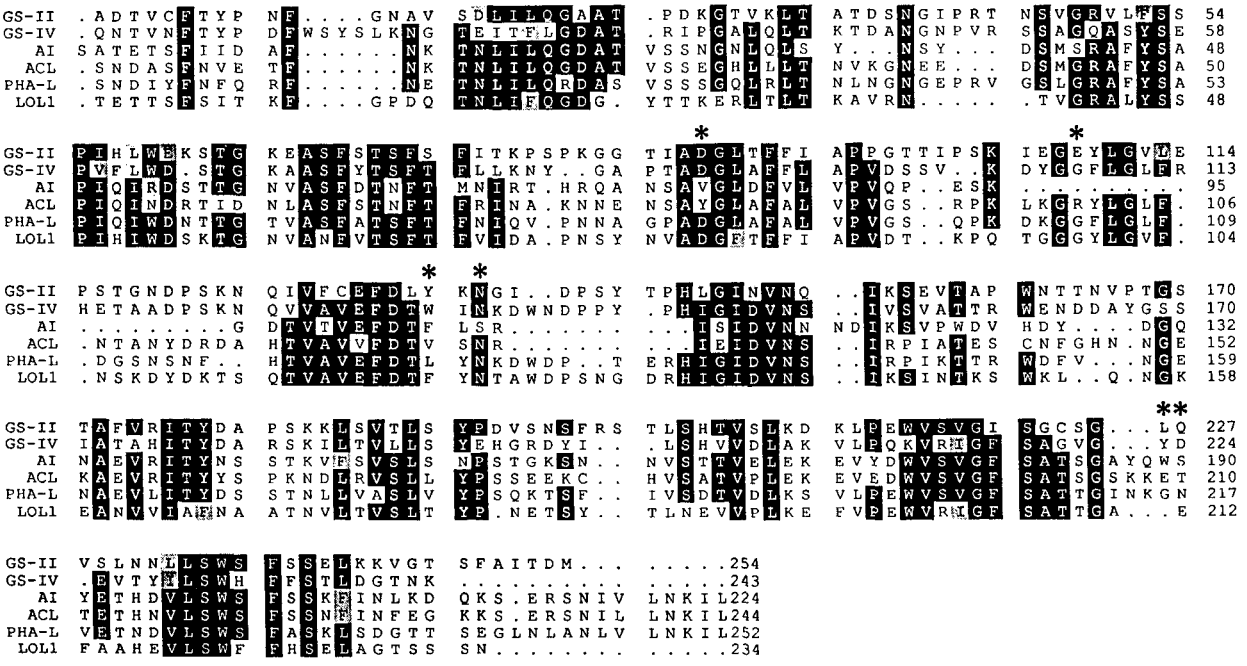

Figure 9. Primary amino acid sequence comparison of some lectins of the legume lectin gene family. GS-II and GS-IV are from *G. simplicifolia*. AI, ACL, and PHA-L represent α-amylase inhibitor, arcelin, and phytohemagglutinin from common bean (*P. vulgaris*). Isolectin 1 (LOL1) is from the *Lathyrus ochrus*. The putative carbohydrate-binding sites are indicated by asterisks (*).

dispersed in linear sequence (Bourne et al., 1990; Rouge et al., 1993). Mirkov and Chrispeels (1993) reported that a mutation in the base sequence of PHA-L, which resulted in an amino acid change from Asn^{128} to Asp^{128} of PHA-L, completely abolished the carbohydrate-binding activity of PHA-L, although the protein structure was not disrupted. Since the three-dimensional structures of legume lectins are probably similar (Sharon and Lis, 1990), GS-II also may bind to GlcNAc through several dispersed amino acids. By primary sequence comparison, we infer that these amino acid residues are probably Asp^{88}, Glu^{108}, Tyr^{134}, Asn^{136}, Leu^{226}, and Gln^{227} or other residues that are near to them (Fig. 9). Differences in amino acids at the carbohydrate-binding sites among different legume lectins might be related to their different carbohydrate-binding specificities.

Differential expression of GS-II protein in various plant tissues could be explained in two ways: (a) a particular GS-II gene could be regulated differently in different tissues; and (b) different genes encoding GS-II proteins could be expressed independently in tissue-specific ways.

Transfer of insecticidal lectin genes into plant crops may have potential for pest management. Development of Bt-engineered potato has demonstrated the feasibility of controlling CPB through transgenic host plant resistance, but resistant biotypes of CPB have already arisen from Bt selection pressure in the laboratory (McGaughey, 1985), emphasizing the need for a continued search for new resistance genes. GS-II causes major delays in CPB development (data not shown). The GS-II gene may therefore represent an alternative or additional candidate gene for potato transformation for CPB resistance. Co-transformation of potato with two genes with different modes of action (e.g. GS-II and Bt) may provide the transgenic plants with not only higher resistance but more durable resistance as well.

ACKNOWLEDGMENTS

We thank Dr. R. Shukle (U.S. Department of Agriculture—Agricultural Research Service) and Dr. M. Narasimhan for their critical review of the manuscript. We also thank J. Clithero for doing DNA sequencing and S. Balfe for helping with the artificial seed bioassay.

Received July 21, 1995; accepted October 3, 1995.

LITERATURE CITED

Barondes SH (1988) Bifunctional properties of lectins: lectins redefined. Trends Biochem Sci **13:** 480–482

Bourne Y, Roussel A, Frey M, Rouge P, Camps J, Cambillau C (1990) Three dimensional structures of complexes of *Lathyrus ochrus* isolectin I with glucose and mannose: fine specificity of the monosaccharide-binding site. Proteins Struct Funct Genet **8:** 365–376

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem **162:** 156–159

Chrispeels MJ, Raikhel NV (1991) Lectins, lectin genes, and their role in plant defense. Plant Cell **3:** 1–9

Czapla TH, Lang BA (1990) Effect of plant lectins on the larval development of European corn borer (Lepidoptera:Pyralidae) and southern corn rootworm (Coleoptera:Chrysomelidae). J Econ Entomol **83:** 2480–2485

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep **1:** 19–21

Delmotte FM, Goldstein IJ (1980) Improved procedure for purification of the *Bandeiraea simplicifolia* I isolectins and *Bandeiraea*

simpicifolia II lectin by affinity chromatography. Eur J Biochem **112:** 219–223

Diaz CL, Melchers LS, Hooykaas PJJ, Lugtenberg BJJ, Kijne JW (1989) Root lectin as a determinant of host-plant specificity in the *Rhizobium*-legume symbiosis. Nature **338:** 579–581

Ebisu S, Shankar Iyer PN, Goldstein IJ (1978) Equilibrium dialysis and carbohydrate-binding studies on the 2-acetamido-2-deoxy-D-glucopyranosyl-binding lectin from *Bandeiraea simplicifolia* seeds. Carbohydr Res **61:** 129–138

Habibi J, Backus EA, Czapla TH (1993) Plant lectins affect survival of the potato leaf hopper (Homoptera:Cicadellidae). J Econ Entomol **86:** 945–951

Hoffman LM, Donaldson DD (1987) Synthesis of mitogenic phytohemagglutinin-L in *Escherichia coli*. Biotechnology **5:** 157–160

Huesing JE, Shade RE, Chrispeels MJ, Murdock LL (1991) α-Amylase inhibitor, not phytohemagglutinin, explains resistance of common bean seeds to cowpea weevil. Plant Physiol **96:** 993–996

Ishimoto M, Kitamura K (1989) Growth inhibitory effects of an α-amylase inhibitor from kidney bean (*Phaseolus vulgaris*) on three species of bruchids (Coleoptera:Bruchidae). Appl Entomol Zool **24:** 281–286

Jaffe WG, Vega Lette CL (1968) Heat-labile growth inhibiting factor in bean (*Phaseolus vulgaris*). J Nutr **94:** 203–210

Jayne-Williams DJ, Burgess CD (1974) Further observation on the toxicity of navy beans (*Phaseolus vulgaris*) for Japanese quail (*Coturnis coturnix japonica*). J Appl Bacteriol **37:** 149–169

Kornegay J, Cardona C, Posso CE (1993) Inheritance of resistance to Mexican bean weevil in common bean, determined by bioassay and biochemical tests. Crop Sci **33:** 589–594

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lamb JE, Shibata S, Goldstein IJ (1983) Purification and characterization of *Griffonia simplicifolia* leaf lectins. Plant Physiol **71:** 879–887

McGaughey WH (1985) Insect resistance to the biological insecticide *Bacillus thuringiensis*. Science **229:** 193–195

Mirkov TE, Chrispeels MJ (1993) Mutation of Asn^{128} to Asp of *Phaseolus vulgaris* leucoagglutinin (PHA-L) eliminates carbohydrate-binding and biological activity. Glycobiology **3:** 581–587

Murdock LL, Huesing JE, Nielsen SS, Pratt RC, Shade RE (1990)Biological effects of plant lectins on the cowpea weevil. Phytochemistry **29:** 85–89

Orr GL, Strickland JA, Walsh TA (1994) Inhibition of *Diabrotica* larval growth by a multicystatin from potato tubers. J Insect Physiol **40:** 893–900

Osborn TC, Alexander DC, SSM, Cardona C, Bliss FA (1988) Insecticidal activity and lectin homology of arcelin seed protein. Science **240:** 207–210

Powell KS, Gatehouse AMR, Hilder VA, Gatehouse JA (1993) Antimetabolic effects of plant lectins and plant and fungal enzymes on the nymphal stages of two important rice pests, *Nilaparvata lugens* and *Nephotettix cinciteps*. Entomol Exp Appl **66:** 119–126

Pusztai A (1991) Structure of lectins. *In* JD Phillipson, DC Ayres, H Baxter, eds, Plant Lectins. Cambridge University Press, Cambridge, UK pp 32–38

Raikhel NV, Lee HI, Broekaert WF (1993) Structure and function of chitin-binding proteins. Annu Rev Plant Physiol Plant Mol Biol **44:** 591–615

Rouge P, Barre A, Causse H, Chatelain C, Porthe G (1993) Arcelin and α-amylase inhibitor from the seeds of common bean (*Phaseolus vulgaris* L.) are truncated lectins. Biochem Syst Ecol **21:** 695–703

Shade RE, Higgins TJV, Pueyo JJ, Murdock LL, Schroeder H, Chrispeels MJ (1994) Transgenic peas expressing the α-amylase inhibitor of the common bean are resistant to the bruchid beetles *C. maculatus* and *C. chinensis*. Biotechnology **12:** 793–796

Shade RE, Murdock LL, Foard DE, Pomeroy MA (1986) Artificial seed system for bioassay of cowpea weevil (Coleoptera: Bruchidae) growth and development. Environ Entomol **15:** 1286–1291

Shankar Iyer PN, Wilkinson KD, Goldstein IJ (1976) An N-acetyl-D-glucosamine binding lectin from *Bandeiraea simplicifolia* seeds. Arch Biochem Biophys **177:** 330–333

Sharon N, Lis H (1989) Lectins as cell recognition molecules. Science **246:** 227–246

Sharon N, Lis H (1990) Legume lectin—a large family of homologous protein. FASEB J **4:** 3198–3208

Walsh TA, Strickland JA (1993) Proteolysis of the 85-kilodalton crystalline cysteine proteinase inhibitor from potato releases functional cystatin domains. Plant Physiol **103:** 1227–1234