Journal of Nematology 40(2):138–146. 2008.

Protease Inhibitor Expression in Soybean Roots Exhibiting Susceptible and Resistant Interactions with Soybean Cyst Nematode

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Abstract: Protease inhibitors play a role in regulating proteases during cellular development and in plant defense. We cloned and sequenced cDNA encoding six protease inhibitors expressed in soybean roots infected with soybean cyst nematode (SCN) and determined their expression patterns. Four of these protease inhibitors are novel and have not been reported previously. Using RT-PCR, we measured the relative transcript levels of each protease inhibitor in roots of the soybean cv. Peking inoculated with either SCN TN8 to examine the expression of protease inhibitors during the susceptible interaction or with SCN NL1-RHg representing the resistant interaction. Within 12 to 24 hours, mRNA transcripts encoding five of the six protease inhibitors were more highly elevated in soybean roots exhibiting the susceptible interaction than the resistant interaction. Transcripts encoding two protease inhibitors possessing Kunitz trypsin inhibitor domains were induced 37- and 27-fold in the susceptible interaction within 1 dpi, but were induced only 5- to 7-fold in roots displaying the resistant interaction. Our results indicate that soybean roots recognize differences between these two SCN populations before the nematodes initiate a feeding site, and accordingly the roots express transcripts encoding soybean protease inhibitors differentially. These transcripts were generally less abundant in roots exhibiting the resistant interaction.

Key words: host-parasitic relationship, molecular biology, protease inhibitors, resistance, RT-PCR, soybean, soybean cyst nematode

Soybean cyst nematode (SCN; *Heterodera glycines)* is the major pest of soybean (*Glycine max*) in the US and is responsible for an estimated 7% loss in soybean annually. This represents approximately $700 million in losses to the US farmer each year and is more than the losses from all other soybean pests combined (Wrather and Koenning, 2006). Crop rotation and planting of soybean varieties resistant to specific races of SCN alleviate but do not eliminate the problem.

SCN is an obligate, sedentary root endoparasite that invades soybean, then migrates toward the vascular tissue. Approximately 18 to 48 hours post-inoculation (hpi), the nematode selects a pericycle or endodermal cell, inserts its stylet and begins to feed. The selected cell and surrounding cells undergo a transformation, wherein the neighboring cell walls deteriorate, cytoplasmic changes occur and a syncytium is formed (Endo and Veech, 1970; Riggs et al., 1973). Interestingly, in soybean cv. Peking, syncytia form in both resistant and susceptible interactions. In the susceptible interaction, the syncytium continues to grow; however, in the resistant interaction, the syncytium degenerates within 4 days post- inoculation (dpi) (Riggs et al., 1973; Kim et al., 1987).

Concurrent with the different cytoplasmic changes that occur within the root during nematode invasion, there are changes in gene expression. Several investigators have studied the response of soybean to SCN at the molecular level to identify genes important to susceptibility and resistance. Alkharouf et al. (2004) and Alkharouf and Matthews (2004) compared expressed sequence tags (EST) from cDNA libraries of roots of soybean at 0.5, 2 to 4, and 6 to 8 dpi of cv. Peking with SCN isolate NL1-RHg, which is avirulent on cv. Peking, to identify genes expressed during the resistance response. Furthermore, genes expressed in the syncytium formed by SCN NL1-RHg in susceptible cv. Kent roots were identified by EST analysis of a cDNA library made from mRNA isolated from syncytial cells collected by laser capture microdissection (Klink et al., 2005). Numerous genes were identified that are expressed at the nematode feeding site during the susceptible or compatible interaction. Khan et al. (2004) used cDNA microarrays to determine the transcript levels of soybean genes in the susceptible response two days after inoculation of soybean cv. Kent roots with SCN NL1-RHg. This investigation was extended using a larger cDNA microarray and additional time points from 6 hpi to 8 dpi (Alkharouf et al., 2006). When Affymetrix soybean microarrays containing over 35,600 oligonucleotide probe sets became available, several laboratories studied gene expression in soybean roots infected with SCN over time (Ithal et al., 2007a; Klink et al., 2007b; Putoff et al., 2007) and in syncytia from roots exhibiting resistant or susceptible phenotypes (Ithal et al., 2007b; Klink et al., 2007a). Through these studies, several hundred genes were identified that have increased or decreased transcript levels within roots of susceptible and resistant soybean, including several genes that encoded protease inhibitors.

Protease inhibitors play a role in regulating endogenous proteases during development and in defense of plants against insect and nematode attack (Laskowski and Kato, 1980; Sijmons et al., 1994; Urwin et al., 1995, 1998; Gatehouse, 2002; Lawrence and Koundal, 2002; Samac and Smigocki, 2003). Protease inhibitors can be separated into several diverse multigene families. They

Received for publication March 24, 2008.
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The authors thank Veronica Martins for expert technical assistance and acknowledge the continuing support provided by the United Soybean Board under grant 5214 to BFM. The authors are indebted to Drs. Andrea Skantar, Leslie Wanner and Ann Smigocki, United States Department of Agriculture, and Dr. Mark Halsey, United Soybean Board, for careful reading of the manuscript. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture.
This paper was edited by Isgouhi Kaloshian.

have been studied in different tissues, including leaves, flowers, seeds and roots. Protease inhibitors are major constituents of seeds of some plants, including soybean. In soybean seeds, there are two major classes of serine protease inhibitors, the Bowman-Birk and the Kunitz trypsin inhibitors. These inhibitors may comprise up to 6% of the total protein in the seed. Wounding and mechanical damage are known to induce protease inhibitor proteins in leaves. The development of many insects is affected by plant protease inhibitors targeted to midgut serine proteases (Lopes et al., 2004). However, some insects may overcome inhibition by protease inhibitors present in transgenic plants or in artificial diets by expressing alternate forms of protease not sensitive to inhibition by the protease inhibitor (Ferry et al., 2005; Vila et al., 2005).

SCN invades the soybean root and initiates a syncytium or feeding site within 18 to 48 hpi (Riggs et al., 1973; Endo, 1991; Mahalingam and Skorupska, 1996). In the susceptible interaction of soybean cv. Peking interacting with SCN population TN8, the syncytium continues to grow through 8 dpi. In contrast, the syncytium begins to degrade in the resistant interaction of cv. Peking with SCN NL1-RHg within 4 dpi (Endo, 1965). Therefore, gene expression in the soybean root during this time interval may be of great consequence to susceptibility and resistance. Our earlier gene expression analysis using cDNA microarrays indicated that several protease inhibitors were differentially expressed in soybean roots at early time points after inoculation with SCN (Khan et al., 2004; Alkharouf et al., 2006). In this paper, we describe the cloning and expression pattern of six genes expressed in soybean roots infected with SCN with high similarity to protease inhibitors, specifically at microarray addresses E01D05, D03G05, D10B03, B10D02, B08G06 and D06D04 (Khan et al., 2004). Two genes encode proteins closely related to two Kuntiz trypsin inhibitors (E01D05 (*GmKTI-1*) and D03G05 (*GmKTI-2*)); one gene codes a more distantly related Kunitz trypsin inhibitor (B08G06 (*GmKTI-3*)); two encode protease inhibitors with Bowman-Birk domains (B10D02 (*GmBBI-1*) and D06D04 (*GmBBI-2*)); and one has high similarity to a potato inhibitor I (D10B03 (*GmPPI*)). In addition, we describe their expression in soybean cv. Peking roots from 0.5, 3 and 8 dpi with SCN population NL1-RHg (race 3; Hg 7) and with TN8 (race 14; Hg 1.3.6.7) to determine their transcript levels in soybean roots during the resistant and susceptible responses, respectively.

MATERIALS AND METHODS

Plant and nematode material: The SCN population NL1-RHg and its growth have been described previously (Klink et al., 2007a). This population reacts with soybean differentials in the manner of race 3 and yields a resistant response from soybean cv. Peking. The SCN population TN8 yields a susceptible response from soybean cv. Peking. Both SCN populations were maintained similarly on soybean cv. Kent. Both populations can be obtained from the SCN Stock Center (Dr. Terry Niblack, University of Illinois, Champaign-Urbana, IL).

Nematodes were grown, harvested, collected and used to inoculate soybean roots as described by Klink et al. (2007b). Roots were inoculated with 2,000 J2/root. On average, roots were infected with approximately 290 nematodes by 12 hpi. Three or more roots were harvested at each time point, 0.5, 1, 2, 4 and 8 dpi, as described in Klink et al. (2007b). RNA was extracted using the method of Mujer et al. (1996). Three independent biological replicates were performed.

cDNA cloning and analysis: Microarray clones representing *GmKTI-1*, *GmKTI-2*, *GmKTI-3 GmBBI-1, GmBBI-2* and *GmPPI* were reported previously as being one-pass sequenced from the 5′ end before isolating the insert for printing on microarray slides (Khan et al., 2004; Alkharouf et al., 2004, 2006). Here, we sequenced the complete insert from these clones in both directions using the ABI Big Dye Terminator Cycle Sequencing Kit and the ABI Prism 3100 Genetic Analyzer (Perkin-Elmer, Applied Biosystems, Foster City, CA). All inserts contained 5′ putative ATG start sites except clones *GmBBI-1* and *GmBBI-2*, which were lacking their 5′ ends. To obtain the 5′ ends, PCR primers were designed to amplify the sequences from a cDNA library of soybean cv. Peking infected with SCN NL1-RHg for 2 d. Nested gene-specific primers for *GmBBI-1* were designed starting at the 115 bp position 5′GGAATTGAGCGAGTGCAAATACAAG 3′ and at the 165 bp position 5′ GTTGCAAGGTTCATAACAGAAGTTGG 3′. The vector primer was 5′CAGCTATGACCATGATTACGCCAAG 3′ C. Nested gene-specific primers were also designed for *GmBBI-2* at the 70 bp position 5′CTGCACATTTACACATTGGAGAG 3′ and at the 160 bp position 5′CGTCAATGCATTGACACAGTCCAG 3′. The vector primer was 5′ GAAATTAACCCTCACTAAAGGG 3′.

For the other genes, reverse primers 90 bp and 140 bp from the 5′ end were designed. The first round of PCR used the gene-specific 3′ primer at the 140 bp position for a linear reaction using 1µl of the cDNA library in a 25 µl reaction. The second round of PCR used 1µl of the first reaction as a template in a 50 µl reaction mix containing the vector primer and the gene-specific primer at the 90 bp position. Both rounds of PCR used High Fidelity Platinum Taq (Invitrogen, Carlsbad, CA) for 35 cycles. PCR products were gelpurified and cloned into the PCR4 TOPO vector (Invitrogen, Carlsbad, CA). The clones were sequenced, and the new DNA sequence was aligned with the previously known sequence using Lasergene software (DNASTAR Inc., Madison, WI). The 5′ ATG start codon and 3′ stop codon were located, and a translated sequence was obtained for searching and alignment

with known plant sequences. Nucleotide and predicted amino acid sequences for the six clones were compared to those in GenBank, EMBL and Swiss Prot databases using BLAST tools (Altschul et al., 1997). Amino acid sequences were predicted using ExPASy Translate (http://us.expasy.org/tools/dna.html). Multiple sequence alignment and cladograms were conducted using ExPASy ClustalW2 (http://us.expasy.org/tools/#align; Larkin et al., 2007) using the neighbor-joining method.

Real-Time, Reverse-Transcriptase PCR: RNA was extracted from roots at 0, 0.5, 1, 2, 4, and 8 dpi and treated with DNase I to remove genomic DNA. The concentration of each RNA was adjusted to 1 to 2 µg/µl, diluted 1:1000, and used as template in PCR controls to demonstrate the absence of contaminating genomic DNA as described in Matthews et al. (2004). DNase I-treated RNA samples served as template for reverse transcription to cDNA using SuperScript First Strand-Synthesis System for real time RT-PCR (Invitrogen, Grand Island, NY) with oligo d(T) as the primer, according to manufacturer's instructions. Real time RT-PCR assays were conducted in triplicate as described by Alkharouf et al. (2006) using templates from three independent biological replicates. DNA accumulation was measured using SYBR green fluorescence and ROX as the reference dye. Serial dilutions were used over a four-log range. PCR efficiencies were equal. Ct values and relative abundance of transcripts were calculated from standard curves using software supplied with the Stratagene MX3000P Real-Time PCR. Data analysis was performed according to the $2^{-\Delta\Delta C}$t method (Livak and Schmittgen, 2001). The relative abundance of gene-specific transcripts (Table 1) was compared to the abundance of two reference genes, ribosomal 21S protein (CF921751) and elongation initiation factor 3 (SSH1C11), with similar results. Data is presented using ribosomal 21S protein as reference. Both genes were chosen based on microarray data, which indicated that these two genes did not fluctuate over the SCN time course infections. SYBR green dissociation curves and gel electrophoresis indicated that each gene-specific primer pair produced single PCR products. Real time RT-PCR incubation times for clones *GmBBI-1*, *GmBBI-2* and *GmKTI-1* were 10 min for DNA dissociation at 96°C, then 42 cycles of 30 sec at 96°C, 60 sec at 45°C, and 30 sec at 72°C. Incubation times for clones *GmKTI-2*, *GmKTI-3*, *GmPPI* and E13G06 were 10 min for DNA dissociation at 96°C, then 42 cycles of 30 s at 96°C, 60 sec at 52°C, and 30 sec at 72°C.

RESULTS

Cloning and identification of protease inhibitors: Although cDNA microarrays provide information on general trends of gene expression, cross-hybridization between closely related gene family members or to domains of other genes can confound results. Furthermore, the cDNA were only one-pass sequenced at the 5′end before amplification and printing. Although one-pass sequencing is useful, it does not provide complete information about the clone. Therefore, we confirmed the identity of each of the selected genes encoding protease inhibitors (Table 1) by cloning full-length cDNA copies of each, deducing the amino acid translations of the coding regions and comparing the amino acid sequences with public databases to identify similar genes.

The two highly similar serine protease inhibitors, *GmBBI-1* and *GmBBI-2*, have not been characterized in-depth previously. Characteristic cysteine residues are conserved between these two proteases and among other serine proteases (Fig. 1). *GmBBI-1* encodes a protein of 117 amino acids, while *GmBBI-2* encodes a protein of 118 amino acids. Both are most highly related to ground nut (*Apios americana*) trypsin inhibitor (BAF50740.1; blastp $2e^{-32}$ and $3e^{-25}$, respectively). Also, both contain a highly conserved Bowman-Birk domain, and both are similar to the serine proteinase inhibitor CAA56254 (blastp $3e^{-29}$ and $3e^{-21}$, respectively; McGurl

TABLE 1. Primers used for RT-PCR to measure transcript levels of six proteinase inhibitors relative to 40S ribosomal protein S21. The GenBank accession number of the full cDNA sequence and amplicon size are provided.

Gene function	Gene name	GenBank accession	Primer sequence (5′–3′)	Amplicon size (bp)
Bowman-Birk	*GmBBI-1*	EU444602	AATAATGAGCATGTTCCAGTG GGCCTAGTAGCTTTTATTCATT	122
Bowman-Birk	*GmBBI-2*	EU444599	ATGTGCAGATATTGGAGAA CTTGGCCTAGTAGCTTTTATTA	219
Kunitz trypsin	*GmKTI-1*	EU444598	AAAGGGCGGCGGAATAGA CTGGCAGACCCTTGAGAATAAC	224
Kunitz trypsin	*GmKTI-2*	EU444603	CCCCATTTTCCCAGGTG GGCTTATCCCAGGTATGCT	173
Kunitz trypsin	*GmKTI-3*	EU444601	AAACCCCTCCTAGCAGCAGAAC AAGGGTCACGGATAACATAAAGAG	198
Potato inhibitor	*GmPPI*	EU444603	CATGGCCTGAGCTAGTTGGAGT ACCCTATCACAGCGGAAATCAG	130
40S_Ribosomal S21		CF921751	CTAAGATGCAGAACGAGGAAGG GAGAGCAAAAGTGGAGAAATGG	168

FIG. 1. Amino acid sequence line-up of the two serine protease inhibitors, *GmBBI-1* and *GmBBI-2*, with similarity to *G. max* Bowman-Birk protease inhibitors. The asterisk (*) represents amino acid conserved between the two sequences. Cysteine residues characteristic of Bowman-Birk serine protease inhibitors are shaded in grey.

et al. 1995) found in flowers, leaves and roots of alfalfa (*Medicago sativa*). There are 24 amino acid differences between the two protease inhibitors *GmBBI-1* and *GmBBI-2*, all of which are in the C-terminal 60% of the protein (Fig. 1). Although they are not highly related to the Bowman-Birk family of inhibitors reported from *Glycine* spp., blastp comparisons indicate that *GmBBI-1* and *GmBBI-2* contain the conserved motif of Bowman-Birk protease inhibitors ($6e^{-10}$ and $3e^{-5}$, respectively) and that they are most similar to *Glycine soja* Bowman-Birk isoinhibitor A2 (BAB86784; $5e^{-07}$). ClustalW2 analysis reveals *GmBBI-1* and *GmBBI-2* are a novel group of soybean protease inhibitors representing novel soybean Bowman-Birk protease inhibitors (Fig. 2).

Clone *GmKTI-1* contains a highly conserved Kunitz trypsin inhibitor domain ($4e^{-47}$) and is a variant of *G. max* Kunitz trypsin inhibitor p20–1 (AB028441.1 tblastx $1e^{-110}$; BAA82254 blastp $3e^{-109}$) with only four amino acid mismatches. The cDNA encodes a 207 amino acid protein with a deduced molecular weight (MW) of 22,674, while the Kunitz trypsin inhibitor p20 encodes 206 amino acids (Ashida et al., 2000). *GmKTI-1* is also closely related to *G. max* Kunitz trypsin inhibitor-S (X80039; e value of $6e^{-84}$) reported by Gotor et al. (1995).

Another clone, *GmKTI-2*, also contains a conserved domain from the Kunitz family of protease inhibitors (cd00178; smart00452; pfam00197). It has perfect identity with soybean trypsin inhibitor AF128268.1 (tblastx $4e^{-171}$; AAF87095.1 blastp $1e^{-84}$; 100% match). However, *GmKTI-2* contains 43 more amino acids at its 5′ end, including another met start site (Fig. 3). Interestingly, DNA sequence alignment of AG128268 with *GmKTI-2* reveals that *GmKTI-2* contains an adenine 'A' 21 nt 3′ to the ATG start site that is absent in the 5′ untranslated region of AG128268. When 'A' is inserted into the sequence of AF128269 at this position, the frame shift places 43 amino acids of AF128268, including the met start site, in-frame, and the encoded amino acids exactly match those encoded by *GmKTI-2*. Furthermore, *GmKTI-2* has high similarity to and the same met start site as chickpea (*Cicer arietinum*) Kunitz proteinase inhibitor-1 (AJ276263; $1e^{-73}$), pea (*Pisum sativum*) (AJ011398; $4e^{-70}$) and barrel medic (*Medicago truncatula*) AF526372. Furthermore, the chickpea and Medicago sequences do not have a met in the region matching the proposed met start site of AG128268. Calculated from the 5′-most met site, *GmKTI-2* encodes a protein of 211 amino acids with a MW of 22,676.

GmKTI-3 encodes a novel soybean Kunitz trypsin protease inhibitor comprised of 214 amino acids with a predicted MW of 23,655. This sequence is closely related to prostrate sesbania (*Sesbania rostrata*) (AJ441323; $8e^{-88}$) protease inhibitor pi1 and chickpea trypsin inhibitor (AJ850055; $1e^{-77}$) found in epicotyls.

Clone *GmPPI* encodes a novel 70 amino acid proteinase inhibitor with a predicted MW of 7,766. This sequence has no close matches to any reported *G. max* sequences and contains a conserved potato inhibitor I family domain (pfam00289; $4e^{-17}$); it has strong amino acid sequence identity with *M. truncatula* proteinase inhibitor I13 (ABE83038; blastp $9e^{-23}$).

Expression of PI: Relative amounts of transcripts were determined using real time RT-PCR (Fig. 4) for each gene at 0.5, 1, 2, 4 and 8 dpi in susceptible and resistant

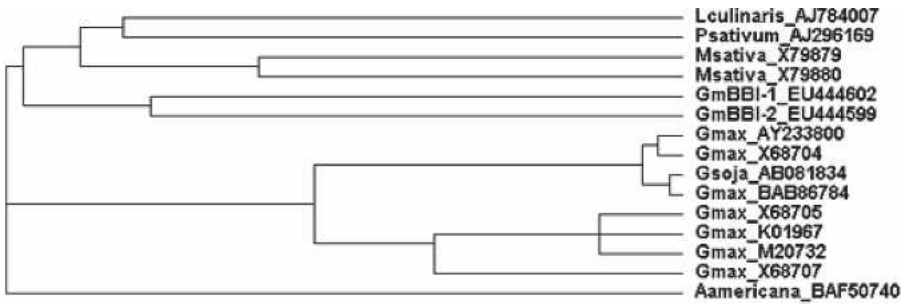

FIG. 2. Relationship of *G. max* serine protease inhibitors *GmBBI-1* and *GmBBI-2* with other Bowman-Birk serine protease inhibitors from *G. max* (AY233800, BAB86784, X68704, X68705, K01967,M20732, X68707); *G. soja* (AB081834); *M. sativa* (X79879, X79880); *Lens culinus* (lentil; AJ784007); *Pisum sativum* (AJ296169); and *Apios Americana* (BAF50740).

TABLE 2. Identification and characterization of soybean clones encoding protease inhibitors.

Gene name	Accession number	Protein size (aa)	Type inhibitor	Most closely related sequence[a]	Plant species origin	Blastp e-value
GmBBI-1	EU444602	117	Bowman-Birk	BAF507040.1	*Apios americana*	$2e^{-32}$
GmBBI-2	EU444599	118	Bowman-Birk	BAF507040.1	*A. americana*	$2e^{-35}$
GmKTI-1	EU444598	205	Kunitz P20-1	BAA82254	*Glycine max*	$3e^{-109}$
GmKTI-2	EU444603	221	Kunitz	AAF87095.1	*G. max*	$1e^{-84}$
GmKTI-3	EU444601	214	Kunitz PIpi1	CAD29731.1	*Sesabania rostrata*	$4e^{-87}$
GmPPI	EU444600	70	Potato PI	ABE83038.1	*Medicago truncatula*	$1e^{-22}$

[a] Nucleotide sequences were translated to amino acid sequences using ExPASy translate tool http://us.expasy.org/tools/dna.html. Protein sequences were compared using blastp http:///www.ncbi.nlm.hih.gov/BLAST/

soybean roots infected with SCN and compared with transcript levels of uninoculated roots. Transcript levels of *GmBBI-1* (Fig. 4A) were almost 4.5-fold increased at 0.5 dpi during the susceptible interaction, compared to no induction during the resistant interaction. Transcript levels were also elevated at 2 and 8 dpi in roots of the susceptible interactions, compared to the resistant. Transcript levels of this gene at the other time points were similar in susceptible and resistant roots. Transcript levels of *GmBBI-2* (Fig. 4B) during the resistant and susceptible interactions were similar throughout the time period (note the y-axis scale) except at 2 dpi, where transcripts of *GmBBI-2* were higher in the resistant interaction compared to the susceptible interaction. Transcripts were not detectable in either resistant or susceptible roots at 8 dpi.

In general, transcript levels of genes *GmKTI-1*, *GmKTI-2* and *GmPPI* were higher in susceptible roots than in resistant roots for most time points tested (Fig. 4C-E). Furthermore, transcript levels of these genes were generally higher in susceptible roots at 0.5 and 1 dpi than at later time points. Transcripts of *GmKTI-1* (Fig. 4C) increased over 27-fold at 0.5 dpi and 26-fold at 1 dpi, subsiding to 8-fold at 2 and 4 dpi in susceptible roots, followed by a 12-fold increase by 8 dpi. In the resistant interaction, transcript levels of *GmKTI-1* (Fig. 4C) increased 7-fold at 0.5 dpi and 12.7-fold by 1 dpi, then subsided before increasing again 6.6-fold by 8 dpi. Transcript levels of *GmKTI-2* (Fig. 4D) increased 37-fold at 0.5 dpi, 25-fold at 1 dpi, and 9-fold at 2 dpi in the susceptible interaction, whereas transcript level increases remained below 5-fold in the resistant interaction. Transcripts of *GmKTI-3* (Fig. 4E) did not accumulate beyond 2.5-fold at any time point in either interactions. The level of *GmPPI* transcripts (Fig. 4F) increased and was consistently higher in susceptible roots compared to resistant roots throughout the tested time period, with the most dramatic differences occurring at

FIG. 3. Amino acid alignment of *GmKTI-2* with the homologous protease inhibitor genes from *P. sativum* (Pisum), *M. truncatula* (Medicago), *C. arietinum* (Cicer) and *G. max*. The *G. max* sequence is shown without (AF128268 *G. max*) and with (AF128268 *G. max* +1) a frame shift to show the upstream alignment resulting from the addition of the one extra nucleotide found in *GmKTI-2*. The asterisk (*) represents amino acids conserved between the two sequences. The colon (:) represents substitution of similar amino acids.

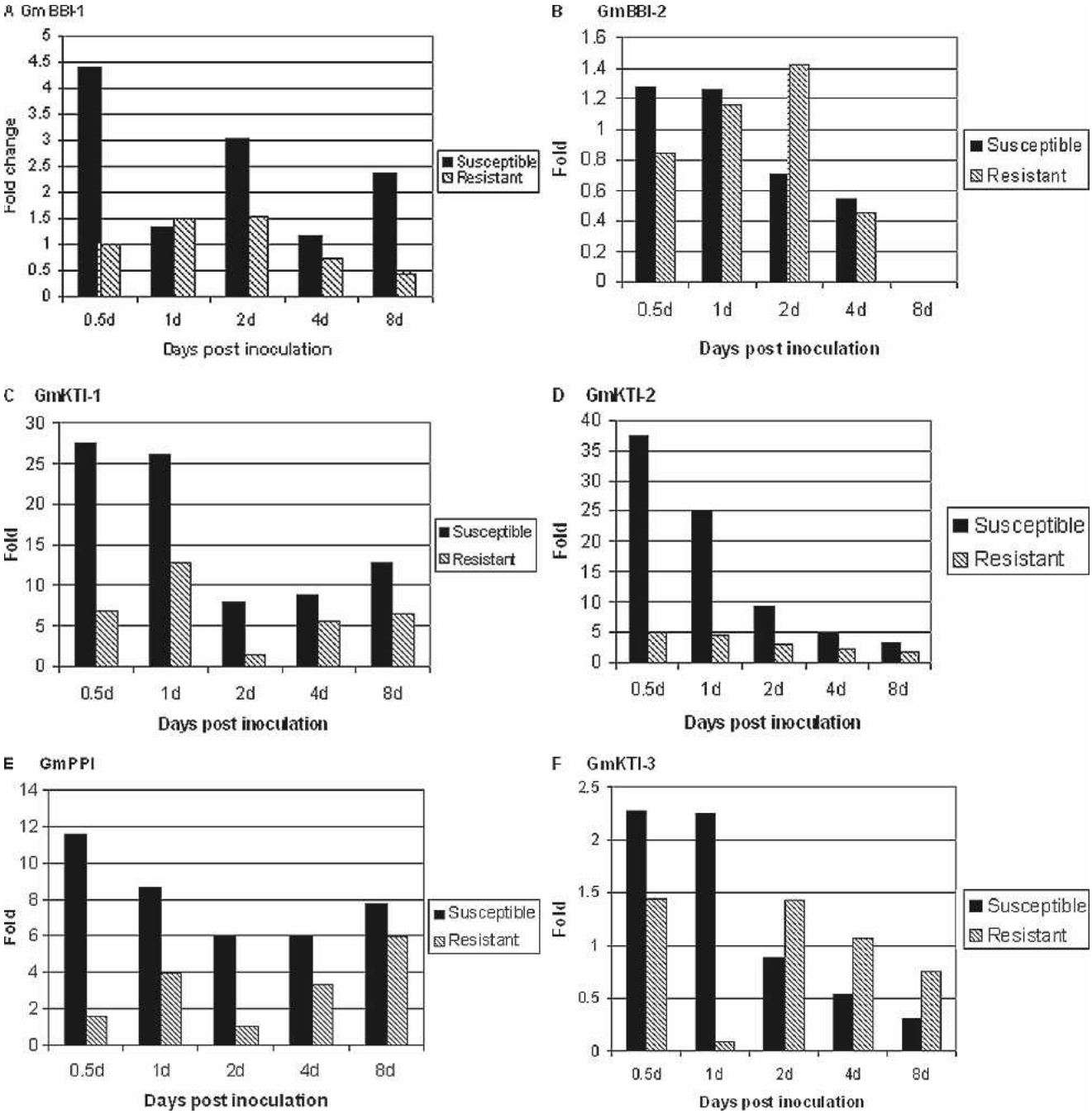

FIG. 4. Expression of PI genes in *G. max* cv. Peking roots displaying the susceptible (solid; SCN NL1-RHg) and resistant (striped; SCN TN8) interactions from 0.5 to 8 dpi. *GmBBI-1* (panel A), *GmBBI-2* (panel B), *GmKTI-1* (panel C), *GmKTI-2* (panel D), *GmPPI* (panel E), *GmKTI-3* (panel F). Fold expression is relative to the level of expression in control, non-infected roots.

0.5 dpi and 1 dpi, with transcript level increases of 11.5- and 8.6-fold, respectively.

DISCUSSION

Protease inhibitors are part of the plant's natural mechanisms for modulating protease activity within the cell. Proteases are generally highly compartmentalized to keep them from digesting needed proteins. Furthermore, protease inhibitors play an important role in the plant defense response to mechanical wounding, such as occurs during herbivorous insect feeding (Johnson et al., 1989; Boulter et al., 1990; Farmer et al., 1992; Botella et al., 1996; Koiwa et al., 1997). Protease inhibitors are present in seeds, flowers, leaves and roots and are differentially expressed in tissues over time (Jofuku and Goldberg, 1989; McGurl et al., 1995). There are several families of protease inhibitors, including those with Kunitz trypsin inhibitor domains and Bowman-Birk inhibitor domains. Kunitz trypsin-type inhibitors are the major trypsin inhibitors found in soybean seed and have one active site. Kunitz trypsin inhibitor gene family members are expressed at different levels during soybean embryogenesis and in leaf, root and stem

(Jofuku and Goldberg, 1989). Several of the protease inhibitors reported here are novel or are variations of previously reported protease inhibitors, probably because less molecular work has been done on roots and even less on wounded or infected plant roots. The two genes encoding Kunitz trypsin inhibitors reported here, clones *GmKTI-1* and *GmKTI-2*, were expressed in SCN-infected roots and are most closely related to Kunitz trypsin inhibitor p20–1 that is expressed in roots, stems, leaves and cultured soybean cells (Ashida et al., 2000). Transcripts of p20–1 are expressed at higher levels in roots compared to stems and leaves. TI p20–1 has trypsin inhibitor activity and GTP-binding activity (Hirata et al., 1999). Thus, these new members of the p20–1 gene family, *GmKTI-1* and *GmKTI-2*, may be multifunctional proteins.

Bowman-Birk inhibitors have two active sites targeted to a wide range of trypsin or alpha-chymotrypsin proteinases (Wilson, 1987). The two Bowman-Birk inhibitors we cloned from soybean and examined are novel and are closely related to ground nut (*A. americana*) serine protease inhibitor. They have similarity to two proteinase inhibitors from alfalfa that are expressed in flower parts and roots and are wound-inducible in roots, stems and leaves (McGurl et al., 1995). Furthermore, infection of the roots with *Pseudomonas putida* induced expression of these Bowman-Birk inhibitors in alfalfa roots.

Clone *GmKTI-3* encodes a novel Kunitz trypsin inhibitor most closely related to *S. rostrata* protease inhibitor pi1, a nodule-specific protease inhibitor CAD29731 (Lievens, Goormachtig, and Holsters, GenBank entry). It is also highly related to chickpea trypsin inhibitor 3, CAH61462, found in epicotyls of five-day-old seedlings (Dopico, Martin and Labrador, GenBank entry). Clone *GmPPI* is also novel and has no close matches to any reported *G. max* sequences, although it does contain a conserved potato (*Solanum tuberosum*) inhibitor I family domain (Heibges et al., 2003).

Analysis of cDNA microarray data using RNA extracted from susceptible soybean roots at different time points after inoculation indicated that transcript levels of several soybean protease inhibitors changed as early as 6 hpi when infected with SCN population TN8 (Alkharouf et al., 2006). Klink et al. (2007b) identified changes in transcript levels of numerous genes, including protease inhibitors in soybean cv. Peking roots 0.5 dpi when inoculated with TN8 and with NL1-RHg (resistant interaction). Using RT-PCR, we found that clones *GmKTI-1, GmKTI-2* and *GmPPI* were highly induced within 0.5 dpi, indicating a rapid response of soybean roots to SCN infection. Both clones *GmKTI-1* and *GmKTI-2*, encoding Kunitz trypsin inhibitors, were much more highly induced 0.5 and 1 dpi in the susceptible interaction than in the resistant interaction. Similarly, *GmPPI*, containing the potato inhibitor I domain, was much more highly expressed at 0.5 and 1 dpi in the susceptible interaction than in the resistant interaction. This implies that important interactions occur between the root and the nematode within 0.5 dpi which promote expression of soybean genes in response to the nematode. Furthermore, there appear to be gene expression differences in soybean roots within 0.5 dpi that are dependent upon the infecting nematode's virulence. These gene expression differences may be triggered due to mechanical wounding and by differences between nematode populations. Using microarrays, Kink et al. (2007b) demonstrated that there were expression differences for numerous genes at 0.5 dpi between soybean roots displaying a resistant interaction compared to a susceptible interaction. Transcripts of several peroxidases and members of the jasmonic acid biosynthesis pathway appeared to be more highly elevated in the susceptible interaction than in the resistant interaction. Perhaps different nematode populations secrete different cellulases or provide other signals that alter soybean gene expression. More work is required to determine why this occurs.

Transcripts of *GmBBI-1, GmKTI-1* and *GmPPI* were elevated in expression at 6 and 8 dpi. There may be more wounding in susceptible interactions because the nematode continues to inject secretions and withdraw food. In this period, the syncytium continues to grow by dissolution of walls of adjacent cells. Therefore, the induction of the genes may be maintained during the susceptible interaction in an effort to fight the nematode; whereas in the resistant interaction, the nematode is no longer feeding at 8 dpi, and the syncytium is not functional. Thus damage to the plant roots is less, leading to reduction in defense-related proteins.

Although feeding and initiation of syncytia by SCN in soybean roots has not been documented at 12 hr, the nematode is burrowing into the root and damaging root cells at this time. Thus, induction of typical wound-inducible genes such as protease inhibitors is not surprising (Endo 1991) and Endo and Veech (1970) reported a slight enlargement of cells adjacent to the initial syncytial cell and increase in cytoplasmic density 18 hpi, indicating that syncytia were being induced as early as 18 hpi. At about 2 dpi, syncytia develop, as evidenced by hypertrophy of cells selected for feeding, cell wall perforations, proliferated endoplasmic reticulum and increased cytoplasmic density (Gipson et al. 1971; Riggs et al., 1973; Mahalingam and Skorupska, 1996).

Protease inhibitors have known inhibitory effects on herbivorous insects, so transgenic plants expressing protease inhibitors have been made and tested for their ability to inhibit feeding or development of specific insects (for review see Jouanin et al., 1998; O'Callaghan et al., 2005). For example, soybean Kunitz trypsin inhibitor has been used to confer different levels of insect resistance to transgenic tobacco (*Nicotiana tabacum*), potato (Marchetti et al., 2000) and rice (*Oryza sativa*) (Lee et al., 1999). Proteinase inhibitor II from potato

has also been shown in transgenic rice to confer resistance to insects (Duan et al., 1996). Transgenic tomato (*Solanum lycopersicum*) containing two protease inhibitors, potato PI-II and carboxypeptidase inhibitor, exhibited increased resistance to larvae of the moth *Heliothis obsoleta* and the American serpentine leaf miner *Liriomyza trifolii* (Abdeen et al., 2005).

Many nematodes and insects possess dietary serine-threonine proteases. Over-expression of serine-threonine protease inhibitors in plant roots may be detrimental to the growth and development of these pests by inhibiting the pest's dietary serine-threonine proteases, thus decreasing their ability to digest plant proteins. Over-expression of protease inhibitors in roots of transgenic plants has resulted in reduction in the cross-sectional area of plant-parasitic nematodes. Expression of Oryzacystatin-I in transgenic hairy roots of tomato leads to reduction in the cross-sectional size of *Globodera pallida* (Urwin et al., 1995). The linear relationship of number of eggs per cyst to the cross-sectional area of the cyst predicted that a cyst of smaller cross-sectional area would contain fewer eggs. Simultaneous over-expression of cystein and serine protease inhibitors in transgenic *Arabidopsis thaliana* resulted in reduced infection by *Heterodera schachtii*. The simultaneous over-expression of the cysteine and serine protease inhibitors had an additive effect on reducing nematodes compared to either inhibitor alone. Similarly, using an engineered transgenic rice with cysteine proteinase inhibitor (oryzacystatin-IΔD86), Vain et al. (1998) reported 55% reduction in *M. incognita* infection. Expression of oryzacystatin-I and -II in alfalfa reduced infection by *Pratylenchus penetrans* (root-lesion nematode) of alfalfa roots by 29 and 32%, respectively (Samac and Smigocki, 2003).

Although protease inhibitors can be used to improve resistance of plants to nematodes (Urwin et al., 1995, 1998), our results demonstrating the relatively high expression of transcripts of *GmKTI-1*, *GmKTI-2* and *GmPPI* in the susceptible interaction compared to the resistant interaction suggest that these particular protease inhibitors are not playing a key role in the defense of soybean proteins against SCN.

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