Fragments of ATP synthase mediate plant perception of insect attack

Eric A. Schmelz*, Mark J. Carroll, Sherry LeClere, Stephen M. Phipps, Julia Meredith, Prem S. Chourey, Hans T. Alborn, and Peter E. A. Teal

Center of Medical, Agricultural, and Veterinary Entomology, Agricultural Research Service, Chemistry Research Unit, U.S. Department of Agriculture, 1600 Southwest 23rd Drive, Gainesville, FL 32608

Edited by May R. Berenbaum, University of Illinois at Urbana–Champaign, Urbana, IL, and approved April 21, 2006 (received for review March 22, 2006)

Plants can perceive a wide range of biotic attackers and respond with targeted induced defenses. Specificity in plant non-self-recognition occurs either directly by perception of pest-derived elicitors or indirectly through resistance protein recognition of host targets that are inappropriately proteolyzed. Indirect plant perception can occur during interactions with pathogens, yet evidence for analogous events mediating the detection of insect herbivores remains elusive. Here we report indirect perception of herbivory in cowpea (Vigna unguiculata) plants attacked by fall armyworm (Spodoptera frugiperda) larvae. We isolated and identified a disulfide-bridged peptide ("ICIDINGVCVDA"), termed inceptin, from S. frugiperda larval oral secretions that promotes cowpea ethylene production at 1 fmol leaf−1 and triggers increases in the resistance-related phytohormones salicylic acid and jasmonic acid. Inceptins are proteolytic fragments of chloroplastic ATP synthase γ-subunit regulatory regions that mediate plant perception of herbivory through the induction of volatile, phenylpropanoid, and protease inhibitor defenses. Only S. frugiperda larvae that previously ingested chloroplastic ATP synthase γ-subunit proteins and produced inceptins significantly induced cowpea defenses after herbivory. Digestive fragments of an ancient and essential plant enzyme, inceptin functions as a potent indirect signal initiating specific plant responses to insect attack.

Results and Discussion

Outside of a few experimental systems, how many plants specifically recognize insect attack is largely unknown (1). Using cowpea plants, we confirmed the inactivity of established FAC elicitors yet consistently detected responses from S. frugiperda OS (see Fig. 6, which is published as supporting information on the PNAS web site). This result was unexpected given that FAC elicitors were first described in the genus Spodoptera (1, 12). We collected 100-ml samples of OS from S. frugiperda that had fed on either cowpea or maize (Z. mays), respectively, and used a cowpea leaf ethylene (E) induction assay to drive the fractionation of biological activity. Biotic attack often induces production of E and provides a useful marker for exploring plant non-self-perception (1, 16). Crude OS was acidified, partitioned with MeCl2, and centrifuged. The aqueous phase was subjected to a series of HPLC separations including strong cation exchange (Fig. 1A and B), RP-C18 (Fig. 1C and D), gel filtration...
Cowpea OS

Maize OS

(A)

(B)

(C)

(D)

(E)

(F)

(G)

(H)

% E induction compared to S. frugiperda OS

HPLC Retention Time (min)

ICDINGVCVDA

ICDNGVCVDA

Fig. 1. HPLC purification of inceptins from maize- and cowpea-derived S. frugiperda OS. Fractions inducing E production in cowpea leaves, denoted by an asterisk, were isolated by a series of strong cation exchange (A and B), RP-C18 (C and D), gel filtration (E and F), and normal-phase chromatography (G and H). UV traces (λ = 200 Å) are overlaid on an arbitrary scale. Fractions were sequentially collected, desalted, evaporated, and resolubilized in H2O for leaf bioassays. Final purification resulted in single fractions (*) used for MS, Edman N-terminal sequencing, and confirmation with synthetic peptides.

Fig. 2. LC-MS confirmation of natural and synthetic inceptins. From left to right, MS fragment ions, predominant positive m/z [M+H]+ ions, and LC retention time (RT) of parent ion peak of isolated natural product inceptin from cowpea-derived S. frugiperda OS (A), synthetic cowpea inceptin (B), isolated natural product inceptin from maize-derived S. frugiperda OS (C), and synthetic maize inceptin (D).

Fig. 3. Inceptins are potent inducers of E production in cowpea leaves. Average (n = 6; ±SEM) E production of damaged cowpea leaves treated with synthetic cowpea inceptin ranging from 0.45 to 4.50 fmol (A) and 45 to 4,500 fmol (B). Damaged leaves treated with H2O only (filled squares) were arbitrarily placed at 0.2 and 2.0 on the x axes of A and B, respectively. Filled bars represent leaf responses to 1 μl of cowpea-derived S. frugiperda OS and 450 fmol of maize inceptin (Maize-I). Different letters (a–d) represent significant differences. (All ANOVA P values were < 0.0001. Tukey test corrections for multiple comparisons: P < 0.05.)

To confirm activity, wounded cowpea leaves were treated with synthetic inceptins. Plant responses to cowpea inceptin began at 1 fmol leaf⁻¹ and proved highly linear up to 4,500 fmol leaf⁻¹ (Fig. 3). Treatment of wounded leaves with 1 μl of cowpea-derived S. frugiperda OS produced an equivalent response to 450 fmol of either cowpea- or maize-derived inceptin (Fig. 3B). The independent isolation of nearly identical active OS peptides from S. frugiperda on diverse host plants demonstrates that this activity is highly specific to the inceptin sequence. Moreover, the exquisitely low level of inceptin required is consistent with the activity of established peptide signals with known ligand–receptor interactions (17–20).

To test the hypothesis that inceptin is the primary elicitor of plant responses in this system, we compared the activity of S. frugiperda OS and inceptin on the induced accumulation of jasmonic acid (JA) and salicylic acid (SA), which are generally associated with wound and pathogen defense signaling, respectively (6, 7). Both 1 μl of cowpea-derived OS and inceptin (450 fmol) induced quantitatively similar levels of E, SA, and JA that were significantly greater than damage plus H2O alone (Fig. 4 A–C). Through multiple synergistic and antagonistic crosstalk...
interactions E, JA, and SA are believed to mediate the specificity of induced plant defenses to biotic attack (21).

To estimate activation of both indirect and direct defenses, we quantified the volatile homoterpine (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and cinnamic acid and transcript levels of the protease inhibitor cystatin in leaves. *S. frugiperda* OS and inceptin induced equivalent leaf DMNT levels (Fig. 4D) and emission of volatiles including DMNT, (E)-β-ocimene, methyl salicylate, indole, (E)-β-farnesene, (E,E)-α-farnesene, and (3E,7E)-4,8,12-trimethyl-1,3,7,11-tetracatetraene (see Fig. 7, which is published as supporting information on the PNAS web site). These insect-inducible plant volatiles have established roles in the positive associative learning and attraction of natural enemies (22, 23). Inceptin and OS also increased cinnamic acid levels, a product of L-phenylalanine ammonia-lyase and precursor to phenylpropanoid defenses induced by biotic attack (Fig. 4E) (24). Transcripts for cowpea protease inhibitor cystatin were also equally induced in the leaves by *S. frugiperda* OS and inceptin (Fig. 4F). Wounding and insect herbivory often induce protease inhibitor production, which can function as an antinutritive defense by interfering with insect digestion (25).

To test the effect of inceptins on induced direct defenses, cowpea foliage-reared sixth-instar *S. frugiperda* were caged for 12 h on damaged leaves of intact plants treated 24 h previously with either H$_2$O or 450 fmol inceptin. Larvae demonstrated a mass gain of 51.2 ± 2.6% and 42.2 ± 2.1%, respectively, with H$_2$O and inceptin, respectively, demonstrating that inceptin treatment resulted in a significant (n = 35; paired Student’s t-test, t = −2.572, P = 0.012) reduction in larval biomass accumulation. Combined, these results demonstrate that inceptin activates plant defense in cowpea.

A BLAST (www.ncbi.nlm.nih.gov/blast) search using the amino acid sequences of cowpea and maize inceptin revealed high homology to chloroplastic ATP synthase 1-subunit (*cATPC*) 1 encoded by *atpC1* from rice (GenBank accession no. XM.478377). A BLAST search using rice *cATPC1* against all translated GenBank sequences revealed a nonannotated maize mRNA (GenBank accession no. AY108268) containing an identical match to the maize-derived inceptin sequence (‘ICD-VNGCVDAG’—Fig. 5A). To obtain the *cATPC* sequence for cowpea, which was not present in the database, primers were designed to conserved regions of *cATPC*, and PCR product was amplified from cowpea leaf cDNA, cloned, and sequenced. The predicted amino acid sequence from the cowpea *cATPC* homologue (GenBank accession no. DQ312300) contained an exact match to the inceptin peptide isolated from cowpea-derived *S. frugiperda* OS (Fig. 5A). Alignments of the translated *cATPC* genes from multiple plants demonstrate a high degree of conservation in the amino acid sequence that corresponds to the predicted source of inceptins (see Fig. 8, which is published as supporting information on the PNAS web site).

ATP synthase is a highly conserved enzyme catalyzing the synthesis of ATP from ADP and phosphate by means of a flux of protons over an electrochemical gradient. Chloroplast ATP synthase consists of a CF1CF0 complex composed of nine different subunits encoded by the genes *atp* through *atpF* (26). The *cATPC* protein resides in CF1 and contains an extra domain, which is absent from mitochondrial ATPC, spanning the inceptin peptide sequence that corresponds to the inhibitory e-subunit (27). Thus, inceptin fragments are derived from a regulatory domain unique to *cATPC* synthases.

To test the hypothesis that inceptins are proteolytic fragments of *cATPC* that mediate plant defense during herbivore attack, we collected OS from *S. frugiperda* larvae fed an artificial diet, cowpea shoots, or roots mixed with either H$_2$O or 60 µg of purified *E. coli*-expressed protein containing either GST or a partial *cATPC*-GST fusion protein. After 12 h of feeding, only
OS from *S. frugiperda* on diets containing shoots or roots mixed with cATPC-GST contained inceptin (Fig. 5B). No inceptin was found in larval OS from artificial diet, roots, or roots plus GST protein. Similarly, only OS containing inceptin promoted E production (Fig. 5C). Purified cATPC-GST protein alone is not biologically active (see Fig. 9, which is published as supporting information on the PNAS web site). Importantly, cowpea leaf-derived OS contained 530 fmol μl⁻¹ inceptin and accounts for the matching activity of synthetic inceptins (450 fmol μl⁻¹) and crude OS (Fig. 3B). Cowpea responses to herbivory by sixth-instar *S. frugiperda* previously fed roots, shoots, or artificial diet with 9 μg of purified GST or cATPC-GST protein follow the same predicted patterns of DMNT (Fig. 5 D and E) and phytohormone (see Fig. 10, which is published as supporting information on the PNAS web site) production, demonstrating that inceptins regulate plant perception during insect attack. To examine inceptin perception in other species, we tested the synthetic predicted elicitor “ICDVNGVCIDA” from bean (*P. vulgaris*) on bean leaves at 1 pmol leaf⁻¹ and found similar E- and volatile-inducing activity (see Fig. 11, which is published as supporting information on the PNAS web site). Preliminary experiments failed to detect similar biological activity of inceptins in maize and tobacco (data not shown).

*S. frugiperda* larvae assimilate the nitrogen in plant tissue through the proteolytic action of endopeptidases such as trypsin and exopeptidases like aminopeptidases and carboxypeptidases (28). A combination of these midgut enzyme activities is likely involved in the proteolysis of cATPC and production of inceptin. Curiously, the cATPC precursor to inceptin is predicted to be present only in chloroplasts and may enable pod-, stem-, and root-feeding insects to avoid elicitor generation. With processing external to plant tissues, inceptin signaling could offer a conceptual advantage in engineering plant resistance. Transgenic plants expressing recombinant proteins, each harboring multiple copies of inceptin, should preserve plants in an uninduced state yet increase perception and response during herbivore attack. Given the sequence conservation and similar response to corn- and cowpea-derived inceptins, *S. frugiperda* larvae that move onto cowpea plants are envisioned to be rapidly perceived through cATPC fragments originating from previously fed-on hosts.

Selected examples of defense-related peptide signaling in plants include systemin and bacterial-derived Flg22. Systemin is an 18-aa peptide produced upon wounding by plant proteolysis of prosystemin, which then binds the SR160/BR1 receptor, a leucine-rich repeat receptor-like kinase, and is responsible for a broad array of jasmonate-mediated defense responses (19). Derived from the flagellin protein of phytopathogenic bacteria, the peptide fragment Flg22 interacts with the FLS2 receptor, also a leucine-rich repeat receptor-like kinase, and initiates disease-resistance responses (20). Flg22 is specific to bacterial invasion, highly conserved among classes of bacteria, and widely perceived by plants. Inceptin shares a number of similarities as well as differences with systemin and Flg22. Like systemin, inceptins are plant-derived yet are similar to Flg22 in their NH4CH3COOH (pH 5) and eluting activity with the same buffer

### Materials and Methods

#### Plant and Insect Material

*S. frugiperda* larvae were obtained from R. Meagher (Center of Medical, Agricultural, and Veterinary Entomology, Agricultural Research Service, U.S. Department of Agriculture) and reared on a pinto bean-based diet (31). Cowpea (*Vigna unguiculata* var. California Blackeye) no. 5, The Wax Company, Amory, MS), maize (*Z. mays* var. Golden Queen), and black bean (*P. vulgaris*) were germinated in a professional grower’s soil mix (Piedmont Pacific, Statham, GA) supplemented with 14–14–14 Osmocote (Scotts, Marysville, OH). All plants were maintained in a greenhouse with a 12-h photoperiod, a minimum of 300 μmol m⁻² s⁻¹ of photosynthetically active radiation supplied by supplemental lighting, 70% relative humidity, and a temperature cycle of 24°C/28°C (night/day). Hydroponic plants, for harvesting of clean root material, were grown as previously described (32).

#### Cowpea Leaf Bioassays

All experiments used 2- to 3-week-old plants containing two fully expanded pairs of trifoliate leaves. For all induction assays, the adaxial sides of new fully expanded leaves were superficially scratched with a razor in three areas, removing ~5% of the total waxy cuticle. The damage sites (2 cm² each) included the central leaf tip spanning both sides of the midrib and two midbasal sections on opposite sides of the midrib. Test solutions in 5 μl of H₂O were immediately applied and dispersed over the damage sites. Leaves remained on the intact plants for 1, 4, and 5 h before E, leaf metabolite/mRNA, and volatile emission sampling, respectively.

#### Elicitor Isolation

Two 100-ml samples of OS from sixth-instar *S. frugiperda* larvae, fed separately on either cowpea or maize for at least 12 h, were collected (33). Crude OS was acidified with HCl to pH 1, partitioned with an equal volume of CH₂Cl₂, and dried over anhydrous Na₂SO₄. The resulting material was passed over a plug of Al₂O₃ and eluted with 30% CH₂Cl₂:CH₃OH. The appropriate fraction of inceptin was then co-crystallized with 1.0 M NaCl. Strong cation exchange HPLC used a Polysulfoethyl A column (250 × 9.4 mm, 5 μm, 300 Å; The Nest Group, Southboro, MA), a flow rate of 5 ml min⁻¹, and MP A and B, both containing 1:4 CH₃CN:H₂O (pH 3.0) and 25 mM KH₂PO₄ with the addition of 0.5 M KCl to MP B. Activity, between 3 and 6 min, was eluted with binary gradient of 100% A to 100% B over 20 min. Multiple 10-lg injections were pooled, with each repeated 1-min fraction collection combined for each shared time interval. Fractions with maximal activity (4–5 min) were...
subjected to RP-C18 by using a YMC ODS-AQ column (250 × 4.6 mm, S-5 μm, 20 mm; Waters, Milford, MA) heated to 60°C with a flow rate of 1 ml min⁻¹, with MP A and B containing 95:5 H₂O:CH₃CN and 9:1 CH₃CN:H₂O, respectively. Both solutions were buffered with 10 mM NH₄COOH. Activity was eluted with MP A isocratic for 2 min followed by a binary gradient of 100% A to 100% B over 18 min. Active fractions, eluting at 9–10 min, were separated by gel filtration by using a Tricorn Superdex Peptide 10/300 GL column (Amersham Pharmacia Biosciences) and an isotropic 1-ml min⁻¹ flow of H₂O containing 100 mM NH₄CH₃COOH. Activity eluting at 13 min was further fractionated by using an normal-phase carbamoyl-bonded TSKgel Amide-80 column (250 × 4.6 mm; Tosoh), a 1-ml min⁻¹ flow rate, with MP A and B containing 95:5 CH₃CN:H₂O and H₂O, respectively. Both solutions were buffered with 25 mM NH₄COOH. MP A was held isocratic for 2 min and followed by a linear binary gradient reaching 1:1 A:B over 28 min. E-inducing activity eluted between 21 and 24 min.

Phytohormone and Biochemical Analysis. GC-based quantification of elicitor-induced E production followed from Schmelz et al. (31) with modification. One hour after treatment, experimental leaves were excised and sealed in 13-ml tubes for an additional hour before headspace sampling. To estimate additional metabolites, cowpea leaves were left as undamaged controls or were damaged and treated with 5 μl of an aqueous solution containing H₂O only, 1 μl of cowpea-derived S. frugiperda OS, or 450 fmol of inceptin. At time 0, half of this application was applied on one-half of the midrib, with the second half of the leaf treated 2 h later. All leaves were harvested at 4 h in liquid N₂ for metabolite and transcript analyses. Isobutane chemical ionization GC-MS-based leaf tissue quantification of JA, SA, cinnamic metabolite and transcript analyses. Isobutane chemical ionization MS. RP-C18 columns, MP, and gradients were as described in the HPLC isolation. The 1-ml min⁻¹ flow was split, allowing 0.1 ml min⁻¹ to enter the ion source. N-terminal amino acids were protected by acetamidomethyl chemistry degradation on a Model 475 sequencer (Applied Biosystems). Inceptin sequence and activity were confirmed by sequencing was performed at the Institute of Biological Chemistry (Washington State University, Pullman) with Edmund sequencing was performed at the Institute of Biological Chemistry (Washington State University, Pullman) by using LC-MS as described. Quantification was based on peak retention times (10.0–10.1 min) and monitoring [M+H⁺]⁺ ions with a m/z of 1,119.5 and 1,125.5 for the natural and isotope-labeled inceptins, respectively. The identity of each sample was confirmed with MS daughter ion spectra.

RNA Isolation and Quantitative PCR of Cystatin. Total RNA was isolated from control and treated cowpea leaves (500 ng) and used to synthesize cDNA by using the SuperScript II First-Strand Synthesis Kit (Invitrogen) (35). First-strand reactions, run in triplicate, were subjected to quantitative PCR with primers TTGAGATCGATAGTTTAGCTGC and TAAATACAC-TATGCAAGGTGCATC designed from cowpea cystatin (36). All reactions were done in triplicate. Molecule numbers per microgram of total RNA were calculated by using a standard curve technique (37).

Identification of CATPC Sequences. Sequences of atpC homologs were identified by BLAST analysis of the purified inceptin sequences against all translated GenBank sequences. Significant homology was first found with the rice (Oryza sativa) atpC mRNA, accession number XM.478377. Searches in maize returned mRNA sequences for chloroplastic and mitochondrial atpC with the GenBank accession nos. YA102868 and YA108441, respectively. Additional sequences located in the BLAST and The Institute for Genomic Research databases included Arabidopsis thaliana, pea (Pisum sativum), tomato (Lycopersicon esculentum), tobacco (Nicotiana tabacum), potato (Solanum tuberosum), medicago (Medicago truncatula), wheat (Triticum aestivum), and spinach (Spinacia oleracea), with accession nos. NM.116702, X63604, BT012794, X63606, BQ045978, TC94286, TC232682, and CAA68727, respectively. Primers ATPC-F (GGATCCGCTCTACACCAAGTTCGTAATGA and ATPC-R (GCGGCCGCATTGCTCATGGCACTTATC) were designed against conserved regions of the DNA sequence and contain BamHl and NotI sites for subcloning, respectively.

Cloning of Other Legume ATPC Sequences. DNA and cDNA were made from leaf tissues as described above. Primers ATPC-F and ATPC-R were used to amplify sequences from cowpea, P. vulgaris varieties navy bean, kidney bean, and black bean; lima bean (P. lunatus); and peanut (Arachis hypogaea). Primers ATPC-F2 (GGATCCGCTCTACACCAAGTTCGTAATGA) and ATPC-R2 (GCGGCCGCATTGCTCATGGCACTTATC) were designed against conserved regions of the DNA sequence and contain BamHl and NotI sites for subcloning, respectively.

Inceptin Characterization and Synthesis. Selected samples were analyzed with an LC, identical to the purification system, coupled to an LCQ Deca XP/Max (Thermo Electron, San Jose, CA) ion trap MS. RP-C18 columns, MP, and gradients were as described in the HPLC isolation. The 1-ml min⁻¹ flow was split, allowing 0.1 ml min⁻¹ to enter the ion source. N-terminal sequencing was performed at the Institute of Biological Chemistry (Washington State University, Pullman) with Edmund chemistry degradation on a Model 475 sequencer (Applied Biosystems). Inceptin sequence and activity were confirmed by solid-phase peptide synthesis at the Protein Core Chemistry Facility (University of Florida, Gainesville) by using N-(9-fluorenylmethoxycarbonyl)-protected amino acids on an 432A Peptide Synthesizer (Applied Biosystems). Cystine side chains and N-terminal amino acids were protected by acetylmethyl and t-butyloxycarbonyl groups, respectively. The disulfide bond formation was performed on p-methyl benzhydrylamine resin by iodine oxidation, and the peptides were cleaved from the resin with modified reagent K. All peptides were HPLC-purified.

Inceptin Quantification. Internal standard-based quantification of inceptins from S. frugiperda OS was achieved by using ubiquitously labeled ¹³C and ¹⁵N valine-N-(9-fluorenylmethoxycarbonyl) (V*; Cambridge Isotope Laboratories, Andover, MA) incorporated into the synthetic peptide "ICDING-V*-CVDA." Aliquots of crude OS, typically 50 μl, were sequentially spiked with 50 ng of the internal standard-based peptide, 5 μl of HCl, vortexed, and centrifuged 12,000 × g for 5 min. The aqueous phase was mixed with an equal volume of EtOH, stored at −70°C for 30 min, and centrifuged at 12,000 × g for 2 min. Samples were diluted to 5% EtOH, loaded on 100-μg RP-C18 SPE columns, washed with 2 ml of H₂O, and eluted with 9:1 CH₃CN:H₂O. Samples were then concentrated to dryness under vacuum and brought up in 50 μl of 5:95 CH₃CN:H₂O containing 10 mM NH₄COOH, and 10 μl was analyzed by LC-MS as described.
**Bacterial Expression of cATPC Fragment.** The cowpea cATPC PCR fragment, corresponding to bases 716-1053 compared with the maize mRNA, was excised from the TOPO 2.1 vector with BamHI and NotI and was ligated into pET41b+ (Novagen, Darmstadt, Germany) cut with the same enzymes. Empty-vector GST controls and cATPC-GST fusion constructs were expressed in BL21(DE3)pLysS E. coli (Novagen) by induction with 1 mM IPTG for 16 h at 25°C and were purified by using GST-Bind Resin (Novagen).

**S. frugiperda Feeding Studies.** Sixth-instar *S. frugiperda* larvae were isolated and allowed to feed for 12 h on 1 g of artificial diet, cowpea shoots, or roots spiked with 50 µl of either H2O only or 60 µg of bacterially expressed GST protein or cATPC-GST fusion protein. The OS was collected and pooled from a subset of six larvae (*n* = 4) for inceptin quantification and cowpea leaf E bioassays. Similarly, for *S. frugiperda* herbivory experiments larvae (*n* = 12) were allowed to feed on cowpea shoots or roots overnight. To avoid the presence of plant enzymes and moderate inceptin production, additional larvae (*n* = 12) were fed 0.1 g of artificial diet overnight containing either 9 µg of GST or cATPC-GST fusion protein. The OS was collected and pooled from a subset of six larvae (*n* = 1) for inceptin quantification. In these two experiments, larvae were carefully placed on paired cowpea leaves and covered with ultra-lightweight clear plastic domes (2.5-cm diameter) to partially restrict movement. Within 15 min, larvae typically initiated a single feeding bout, exited to the leaf underside through a hole (∼25 mm²) created by feeding, and were removed. Only leaf pairs with comparable physical damage were analyzed (*n* = 6). Purified GST and cATPC-GST proteins were estimated by using Coomassie blue staining of SDS/PAGE gels with BSA standards of 0.1, 0.5, 1.0, 2.5, and 5.0 µg and previously purified GST and cATPC-GST.

We thank R. L. Meagher, N. Lowman, and C. Dillard for supplying *S. frugiperda* larvae; A. Y. Chung, S. Stevens, and S. H. McClung for assistance in peptide analysis and synthesis; C. A. Ryan, G. Pearce, and G. Munske for facilitating amino acid sequencing; and H. J. Klee and A. R. Zangerl for useful editorial feedback. E.A.S. thanks previous mentors I. T. Baldwin, W. S. Bowers, and J. H. Tumlinson for years of guidance that enabled this work. This work was supported by U.S. Department of Agriculture–Agricultural Research Service base funds and U.S. Department of Agriculture–National Research Initiative Competitive Grants Program Grant 2002-35302-12375 subaward 2394- USDA-USDA-2375.