

# Cowpea Chloroplastic ATP Synthase Is the Source of Multiple Plant Defense Elicitors during Insect Herbivory<sup>1,2[W][OA]</sup>

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In cowpea (*Vigna unguiculata*), fall armyworm (*Spodoptera frugiperda*) herbivory and oral secretions (OS) elicit phytohormone production and volatile emission due to inceptin [*Vu*-In; <sup>+</sup>ICDINGVCVDA<sup>-</sup>], a peptide derived from chloroplastic ATP synthase  $\gamma$ -subunit (cATPC) proteins. Elicitor-induced plant volatiles can function as attractants for natural enemies of insect herbivores. We hypothesized that inceptins are gut proteolysis products and that larval OS should contain a mixture of related peptides. In this study, we identified three additional cATPC fragments, namely *Vu*-GE<sup>+</sup>In [<sup>+</sup>GEICDINGVCVDA<sup>-</sup>], *Vu*-E<sup>+</sup>In [<sup>+</sup>EICDINGVCVDA<sup>-</sup>], and *Vu*-In<sup>-A</sup> [<sup>+</sup>ICDINGVCVD<sup>-</sup>]. Leaf bioassays for induced ethylene (E) production demonstrated similar effective concentration<sub>50</sub> values of 68, 45, and 87 fmol leaf<sup>-1</sup> for *Vu*-In, *Vu*-E<sup>+</sup>In, and *Vu*-GE<sup>+</sup>In, respectively; however, *Vu*-In<sup>-A</sup> proved inactive. Shortly following ingestion of recombinant proteins harboring cATPC sequences, larval OS revealed similar concentrations of the three elicitors with 80% of the potential inceptin-related peptides recovered. Rapidly shifting peptide ratios over time were consistent with continued proteolysis and preferential stability of inceptin. Likewise, larvae ingesting host plants with inceptin precursors containing an internal trypsin cleavage site rapidly lost OS-based elicitor activity. OS containing inceptin elicited a rapid and sequential induction of defense-related phytohormones jasmonic acid, E, and salicylic acid at 30, 120, and 240 min, respectively, and also the volatile (E)-4,8-dimethyl-1,3,7-nonatriene. Similar to established peptide signals such as systemin and flg22, amino acid substitutions of *Vu*-In demonstrate an essential role for aspartic acid residues and an unaltered C terminus. In cowpea, insect gut proteolysis following herbivory generates inappropriate fragments of an essential metabolic enzyme enabling plant non-self-recognition.

Plant responses to biotic and abiotic perturbation often reduce the severity of future damage through complex changes, including increased cell wall lignification, hypersensitive responses that limit pathogen mobility, production of direct defenses toxic to the offending organism, or volatile emissions that act indirectly in the attraction of natural enemies of the pests (Hiraga et al., 2001; Kessler and Baldwin, 2002; Greenberg and Yao, 2004). The phytohormones ethylene (E) and jasmonic acid (JA) are known to be

important regulators in plant responses to mechanical damage, insect herbivory, and pathogens (Howe, 2004; Zhao et al., 2005). Phytohormone-mediated plant responses are initiated by complex interactions of exogenous and endogenous signals, many of which have been identified as peptides (Boller, 2005; Matsubayashi and Sakagami, 2006). For example, in tomato species (*Solanum lycopersicum* and *Solanum peruvianum*), damage-induced E and JA-mediated plant responses are amplified by the action of an endogenous 18-amino acid peptide termed systemin (Pearce et al., 1991; Felix and Boller, 1995; Howe et al., 1996). Cellular damage initiates proteolysis or release of the 200-amino acid prosystemin protein, systemin production, ligand binding to the membrane-bound Leu-rich repeat (LRR) receptor kinase (RK) SR160, mitogen-activated protein kinase cascades, and ultimately increased JA biosynthesis leading to the production of defensive proteins (Pearce et al., 1991; Howe et al., 1996; Stratmann and Ryan, 1997; Dombrowski et al., 1999; Scheer and Ryan, 2002). Endogenous wound-related Hyp-rich peptides serving similar functions are also liberated from cell wall proteins in tobacco (*Nicotiana tabacum*) and tomato (Pearce et al., 2001; Pearce and Ryan, 2003). These functionally defined systemins (Ryan and Pearce, 2003) promote plant defenses in response to insect herbivory, yet are not known to enable specific discrimination between biotic and abiotic damage.

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Mechanistic knowledge detailing how plants specifically recognize biotic attack comes primarily from pathology research and includes the established role of small peptides, derived from both plants and pathogens, as critical signals. In *Arabidopsis thaliana*, the isolated 23-amino acid peptide *AtPep1* is produced from the 92-amino acid PROPEP1 protein and is both up-regulated by JA/E and requires functional JA/E signaling for defense gene (*PDF1.2*) expression (Huffaker et al., 2006). Constitutive expression of PROPEP1 in *Arabidopsis* results in enhanced resistance to the fungal root pathogen *Pythium irregulare*, and this response is initiated by the membrane-associated 170-kD LRR-RK protein PEPR1 (Huffaker et al., 2006; Yamaguchi et al., 2006). In bacteria, the *flg22* peptide is derived from a highly conserved amino acid sequence within the bacterial flagellin motor protein and constitutes a pathogen-associated molecular pattern (PAMP) broadly recognized by plants (Felix et al., 1999). Innate immunity in plants is promoted by *flg22* binding to the LRR-RK FLS2 followed by a subsequent cascade of defense responses that limit bacterial growth (Gomez-Gomez et al., 2001; Zipfel et al., 2004). Curiously, while *flg22* is believed to activate the E, JA, and salicylic acid (SA) pathways, signaling processes independent of these phytohormones are thought to be significant for bacterial resistance (Zipfel et al., 2004). Plant detection of pathogen-derived signals should offer greater specificity in the activation of defenses compared to those promoted by wounding alone; however, this specificity may come at a cost. For example, *Agrobacterium tumefaciens* produces a highly divergent sequence spanning the *flg22* active core that does not elicit pathogen defense responses (Felix et al., 1999). Similarly, single amino acid sequence polymorphisms within the flagellin of *Xanthomonas campestris* pv *campestris* alter elicitation and FLS2-mediated defenses (Sun et al., 2006).

Despite advances in our understanding of pathogen-derived and endogenous peptide signals, the production of bioactive peptides specifically during insect herbivory has only recently been demonstrated. We utilized an induced E production bioassay in cowpea (*Vigna unguiculata*) leaves to guide the biochemical fractionation of elicitors from oral secretions (OS) of fall armyworm (*Spodoptera frugiperda*) larvae and isolated an 11-amino acid protein fragment. This peptide, termed inceptin, is derived from the proteolytic cleavage of chloroplastic ATP synthase  $\gamma$ -subunits (cATPC) and accentuates plant defense responses in cowpea during herbivory (Schmelz et al., 2006). Following contact with wounded cowpea leaves, inceptin elicits the increased production of JA, E, SA, and volatiles commonly associated with the attraction of arthropod predators and parasitoids, including methyl salicylate (MeSA) and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT; James, 2005; Kappers et al., 2005; Schmelz et al., 2006). Conceptually, inceptin appears intermediate between endogenous peptides and PAMPs represented by the well-described systemin and *flg22* models. On the one

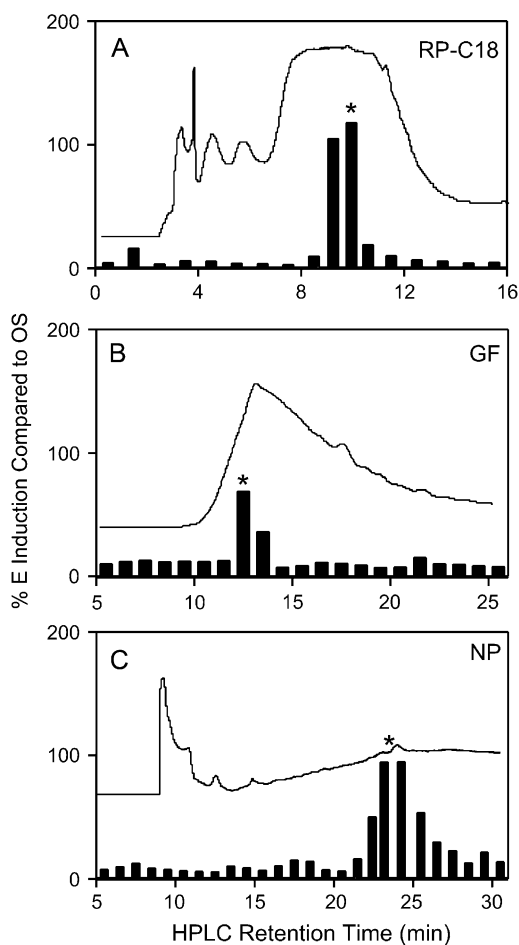
hand, inceptin is similar to systemin in that it originates from a plant protein yet is different in that wounding alone does not readily trigger responses. On the other hand, like *flg22*, inceptin originates from a highly conserved essential protein and requires a biotic attacker for the elicitor to be present. Although small plant-derived peptide elicitors are not yet known to interact with plant resistance (R) proteins, inceptin activity is consistent with the guard hypothesis model in which proteases from the biotic attacker mediate indirect perception through R-protein detection of inappropriately cleaved plant proteins (Dangl and Jones, 2001).

To better understand how legumes recognize insect herbivores via peptide signals derived from plant metabolic proteins, we further examined components of armyworm larval OS and their role in eliciting cowpea responses. In this study, we investigated: (1) the range of inceptin-related peptides present in larval OS and their comparative biological activity; (2) the progressive pattern of peptide abundance and activity in larval OS following ingestion of proteins containing cATPC sequences; (3) the importance of leaf-derived OS containing inceptin-related peptides in triggering the rapid and dynamic production of phytohormone and volatile metabolites; and (4) structural features and amino acid residues of inceptin required for biological activity in cowpea, with a focus on induced E, SA, and DMNT volatile production. Consistent with the predicted proteolytic complexity of insect guts, we identify three additional inceptin-related peptides present in larval OS. We then demonstrate that peptides with additional N-terminal amino acids retain full elicitor activity, while C-terminal deletions abolish activity. Additionally, we show that inceptin-related peptides are rapidly generated in OS upon larval consumption of cATPC proteins and, consistent with gut proteolysis, exhibit preferential losses of larger peptide elicitors over time. Furthermore, we show that 1  $\mu$ L of shoot-derived larval OS containing inceptin-related peptides initiates a rapid, sequential, and maximal induction of JA, E, and SA/DMNT in cowpea leaves at 30, 120, and 240 min, respectively. Comparatively, wounding plus cowpea root-derived OS lacking inceptins promotes only moderate JA increases. Finally, we demonstrate that the activity of modified inceptins in cowpea can vary slightly depending upon the induced biochemical marker selected but overall is highly sensitive to select modifications near the C terminus and relatively tolerant to numerous changes at the N terminus.

## RESULTS

### cATPC Is the Source of Multiple Active Elicitors

Lepidoptera larvae contain a diverse array of digestive proteases capable of converting plant proteins into complex peptide mixtures (Bown et al., 1997). To



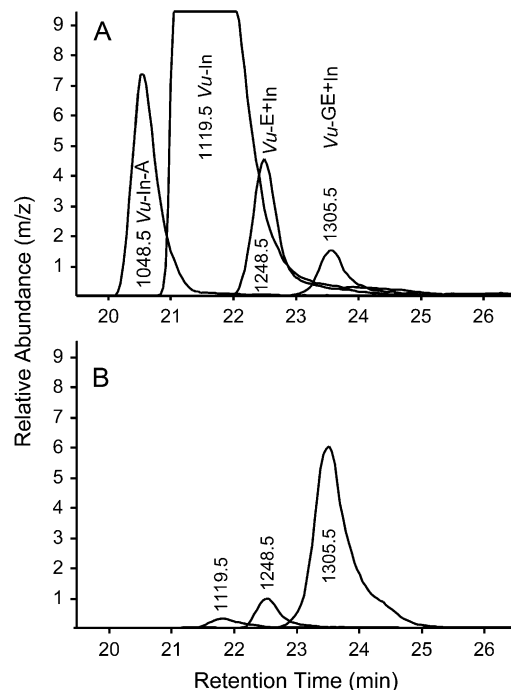
**Figure 1.** HPLC purification of  $Vu-GE^+In$  from cowpea-derived armyworm OS. A previously uninvestigated SCX HPLC fraction (3–4 min; Schmelz et al., 2006) inducing E production in cowpea leaves was sequentially purified by: A, RP-C18; B, GF; and C, NP chromatography. UV traces ( $\lambda = 200 \text{ \AA}$ ) are overlaid on an arbitrary scale. Active fractions (\*) were sequentially collected, desalted, evaporated, and resolubilized in water for bioassays. C, Final purification resulted in fractions (\*) used for MS, Edmund N-terminal sequencing, and confirmation by comparison with synthetic peptides.

confirm the predicted existence of multiple inceptin-related peptides, a previously uninvestigated strong cation exchange (SCX) HPLC fraction, derived from a 100-mL sample of cowpea-based armyworm OS, eluting between 3 to 4 min, and displaying significant E-inducing activity (Schmelz et al., 2006), was utilized in this study. This fraction was progressively purified in repeated rounds of reverse phase (RP)-C18, gel filtration (GF), and normal phase (NP) chromatography by selecting single fractions with maximal bioassay activity for further purification (Fig. 1, A–C). The final NP-HPLC purification step yielded an active fraction exhibiting a greater retention time compared to that previously reported for  $Vu-In$  (Fig. 1C). Edmund chemical N-terminal sequencing of this peptide revealed the  $Vu-In$  core with an additional Gly-Glu at the N terminus [ $Vu-GE^+In; ^+GEICDINGVCVDA^-$ ] match-

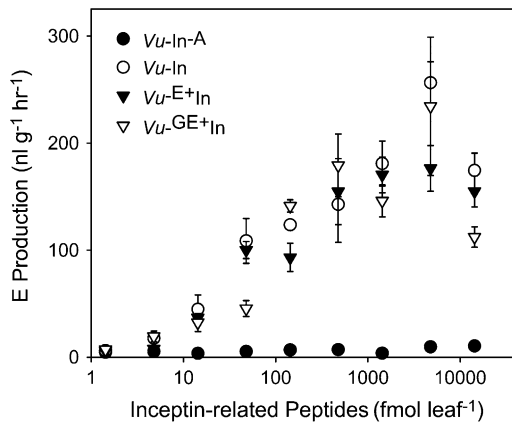
ing the corresponding predicted cATPC peptide sequence for cowpea. Liquid chromatography (LC)/mass spectrometry (MS) analysis of the original  $Vu-In$  purified sample revealed trace levels of additional peptides with  $[M + H]^+$  mass-to-charge ratios ( $m/z$ ) of 1,048.5, 1,248.5, and 1,305.5 (Fig. 2A). Likewise, LC/MS analysis of the sample used to sequence  $Vu-GE^+In$  indicated low levels of  $[M + H]^+$   $m/z$  1,119.5 and 1,248.5 in addition to the predominant 1,305.5 ion (Fig. 2B). MS/MS fragmentation patterns of the  $[M + H]^+$   $m/z$  1,048.5 and 1,248.5 peptides were consistent with a C-terminal Ala loss [ $Vu-In^{-A}; ^+ICDINGVCVD^-$ ] and an N-terminal Glu addition [ $Vu-E^+In; ^+EICDINGVCVDA^-$ ], respectively. MS/MS fragmentation of the natural and synthetic disulfide-bridged peptides confirmed the assignments of  $[M + H]^+$   $m/z$  of 1,048.5, 1,248.5, and 1,305.5 to  $Vu-In^{-A}$ ,  $Vu-E^+In$ , and  $Vu-GE^+In$ , respectively (Supplemental Fig. S1). These results demonstrate that cowpea-derived larval OS contain a complex mixture of inceptin-related peptides.

### The Inceptin C Terminus Is Required for Elicitor Activity

To address how the differential proteolysis of inceptin-related peptides influences activity, we compared the



**Figure 2.** Purified OS contain a mixture of proteolysis products related to inceptin. LC/MS selected  $[M + H]^+$   $m/z$  ion traces of inceptin-related peptides copurified with  $Vu-In$  and  $Vu-GE^+In$  from armyworm OS. A, NP-LC/MS of the isolated  $Vu-In$  natural product reveals the predominant  $m/z$   $[M + H]^+$  ion 1,119.5 ( $Vu-In$ ) and trace amounts of 1,048.5, 1,248.5, and 1,305.5, representing  $Vu-In^{-A}$ ,  $Vu-E^+In$ , and  $Vu-GE^+In$ , respectively. B, Analysis of purified  $Vu-GE^+In$  demonstrates the primary  $m/z$   $[M + H]^+$  ion 1,305.5 and trace amounts of 1,248.5 ( $Vu-E^+In$ ) and 1,119.5 ( $Vu-In$ ).



**Figure 3.** Activity of inceptin-related peptides requires conservation of the C terminus. Average ( $n = 4$ ,  $\pm$ SEM) dose responses of induced E production in cowpea leaves stimulated by the four biochemically characterized inceptin-related peptides  $Vu\text{-In}^{-A}$ ,  $Vu\text{-In}$ ,  $Vu\text{-E}^{+}\text{In}$ , and  $Vu\text{-GE}^{+}\text{In}$ .  $EC_{50}$ s of  $Vu\text{-In}$ ,  $Vu\text{-E}^{+}\text{In}$ , and  $Vu\text{-GE}^{+}\text{In}$  were calculated to be 68, 45, and 87  $\text{fmol leaf}^{-1}$ , respectively. Symbols for treatments are denoted in the legend.

$Vu\text{-In}$ -induced E responses of cowpea leaves to the peptides containing additional N-terminal ( $Vu\text{-E}^{+}\text{In}$ ,  $Vu\text{-GE}^{+}\text{In}$ ) and cleaved C-terminal amino acids ( $Vu\text{-In}^{-A}$ ). Peptides sharing the same C terminus, namely  $Vu\text{-In}$ ,  $Vu\text{-E}^{+}\text{In}$ , and  $Vu\text{-GE}^{+}\text{In}$ , displayed similar effective concentration ( $EC$ )<sub>50</sub> values for induced E production in cowpea leaves of 68, 45, and 87  $\text{fmol leaf}^{-1}$ , respectively (Fig. 3). In contrast,  $Vu\text{-In}^{-A}$  proved inactive at all concentrations tested (Fig. 3). These results demonstrate the occurrence of at least three active peptide elicitors in the OS of armyworm and also identify proteolytic sites resulting in peptide inactivation.

#### Time-Dependent Changes in the Composition of Active Elicitors

Cowpea-derived armyworm OS contain a mixture of active and inactive inceptin-related peptides, yet it was unknown how the levels of these peptides vary over time following larval ingestion of cATPC. To quantify the production and dynamics of inceptin-related peptides, we generated an *Escherichia coli*-expressed recombinant protein derived from eight repeated sequences of the chloroplast-specific region of soybean (*Glycine max*) *atpC* (termed glutathione S-transferase [GST]-*GmIn8H*), fed this protein to larvae, and collected OS either 2 or 6 h after the initiation of feeding. In the 2-h sample, the final NP-HPLC purification step resulted in two distinct regions of strong E-inducing activity eluting between 20 and 24 min (Fig. 4A). In contrast, the 6-h sample produced only a single fraction with predominant E-inducing activity (Fig. 4C). In both samples, early (20.5 min) and late (22–24 min) eluting activity closely corresponded with the relative amounts of  $[M + H]^{+} m/z$  1,105.5

( $Gm\text{-In}$ ;  $^{+}\text{ICDVNGVCVDA}^{-}$ ), 1,234.5 ( $Gm\text{-E}^{+}\text{In}$ ), and 1,291.5 ( $Gm\text{-GE}^{+}\text{In}$ ; Fig. 4, B and D). The relatively poor elution behavior and fmol activity level of the inceptin-related peptides harboring an additional acidic group ( $E^{+}\text{In}$  and  $GE^{+}\text{In}$ ) resulted in chromatographic tailing and a broad range of HPLC fractions promoting E induction (Figs. 1C and 4, A and C). These results indicate that levels and ratios of inceptin-related peptides in OS change over time following ingestion of cATPC.

#### OS Levels of Inceptin Display Preferential Stability over Related Peptides

To better understand the dynamics and relative contribution of inceptin-related peptides to OS elicitor activity after ingestion of cATPC, we quantitatively analyzed a time course of selected peptides present in armyworm OS. Within 15 min of complete GST-*GmIn8H* protein consumption by larvae, defined here as time zero,  $Gm\text{-In}$ ,  $Gm\text{-E}^{+}\text{In}$ , and  $Gm\text{-GE}^{+}\text{In}$  constituted a nearly equal mixture representing 36%, 38%, and 26% of the total active peptides, respectively (Fig. 5). Two hours after consumption of the GST-*GmIn8H* protein, the relative abundance of active peptides  $Gm\text{-In}$ ,  $Gm\text{-E}^{+}\text{In}$ , and  $Gm\text{-GE}^{+}\text{In}$  shifted dramatically to 80%, 17%, and 3%, respectively. Rapid loss of the larger peptides  $Gm\text{-GE}^{+}\text{In}$  and  $Gm\text{-E}^{+}\text{In}$  follows predictions of continued gut proteolysis over time. Despite trace abundance, levels of  $Gm\text{-In}^{-A}$  increased 3.6-fold from  $3.1 \pm 0.6$  to  $11.3 \pm 1.8$   $\text{pmol larvae}^{-1}$  between 0 and 2 h, then declined back to  $3.7 \pm 1.1$   $\text{pmol larvae}^{-1}$  at 6 h. While  $Gm\text{-In}^{-A}$  levels do not quantitatively account for the loss of active elicitors, this pattern is consistent with catabolism concordant with the dramatic loss of  $Gm\text{-GE}^{+}\text{In}$  and  $Gm\text{-E}^{+}\text{In}$ . Of the 492  $\text{pmol}$  of inceptin-related peptides contained within the GST-*GmIn8H* protein consumed by each larva,  $80.8\% \pm 1.9\%$ ,  $42.2\% \pm 3.0\%$ ,  $24.5\% \pm 5.5\%$ ,  $11.1\% \pm 1.4\%$ , and  $4.9\% \pm 1.3\%$  was recovered in the OS at times designated as 0, 2, 4, 6, and 8 h, respectively. These results demonstrate: (1) the rapid and efficient proteolysis of cATPC proteins into inceptin-related peptides; (2) the preferential stability of inceptin; and (3) the gradual loss of all inceptin-related peptides over time.

#### A Trypsin Cleavage Site within Inceptin Promotes the Loss of OS Elicitor Activity

The composition of armyworm OS during feeding is a rich combination of both plant and insect biochemicals and their reciprocal postingestive enzymatic products (Paulillo et al., 2000; Chen et al., 2005; Mohan et al., 2006). To examine whether or not inceptin-related fragments in OS are the primary elicitors of cowpea, we fed larvae either cowpea or spinach (*Spinacea oleracea*), removed larvae from the host plants, and collected OS over a 4-h time course. Spinach is relatively unique in harboring a Lys within the inceptin

amino acid sequence [ $^1$ ICDINGKCVDA $^-$ ] (Miki et al., 1988; Schmelz et al., 2006) that is readily cleaved by trypsin (Hightower and McCarty, 1996), a predominant digestive enzyme in armyworm larvae (Paulillo et al., 2000). The E-inducing activity of OS collected at time zero from actively feeding larvae, bioassayed on cowpea leaves, was not significantly different between cowpea- and spinach-fed larvae (Fig. 6). However, 1 h later, OS derived from spinach-fed larvae displayed no E-inducing activity, whereas the cowpea-derived OS remained active and unchanged (Fig. 6). These results support the proteolytic stability of *Vu*-In/*Gm*-In compared to other amino acid sequences and also the relative importance of inceptin-related peptides within crude OS in mediating the induced responses in cowpea.

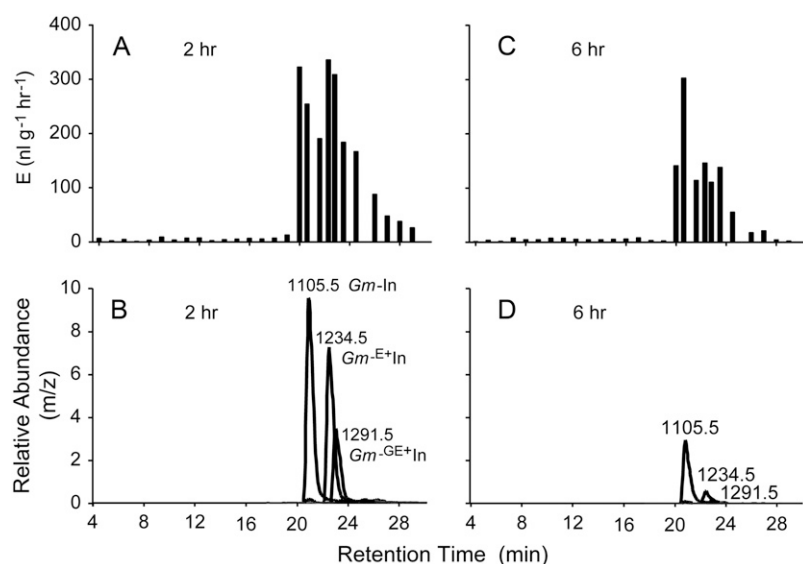
### Inceptins in Larvae OS Induce Sequential Increases in JA, E, SA, and Volatiles

We previously demonstrated that repeated applications of synthetic *Vu*-In promote increases in E, JA, SA, and volatile pools of DMNT in cowpea leaves at a single sampling time point; however, the temporal coordination of these events was unknown. To address the phytohormone dynamics elicited by a natural mixture of inceptin-related peptides, we analyzed and bioassayed OS collected from larvae actively feeding on either cowpea roots or shoots. Root-derived OS lacked inceptins ( $<10$  fmol  $\mu\text{L}^{-1}$ ), while shoot-derived OS contained *Vu*-In, *Vu*- $^{\text{E}+}$ In, and *Vu*- $^{\text{GE}+}$ In at concentrations of  $616 \pm 59$ ,  $170 \pm 17$ , and  $150 \pm 15$  fmol  $\mu\text{L}^{-1}$ , respectively ( $n = 4$ ,  $\pm$ SEM). Wounding plus root-derived OS-induced increases in JA levels above unwounded control leaves at 30 min (Fig. 7A); however, shoot-derived OS induced the highest JA accumulations at 30, 60, and 90 min. Root-derived OS treatments failed to result in significant increases in E, SA, or DMNT (Fig. 7, B–D), while shoot-derived OS induced a sequential maximal production of JA, E, and

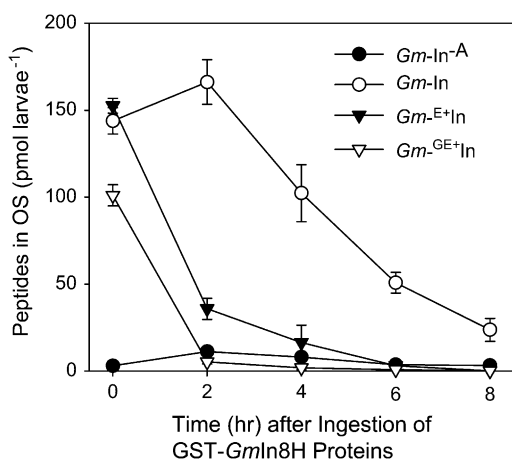
SA at 30, 120, and 240 min, respectively (Fig. 7, A–C). A similar yet delayed sequential activation of phytohormones has been described during *Xanthomonas* pathogen infection of tomato (O'Donnell et al., 2003). Increases in JA and E both preceded the accumulation of significant DMNT pools and exist as potential regulators of induced volatile production (Fig. 7, C and D). These results demonstrate that OS containing inceptin-related peptides rapidly promote sequential increases in a series of defense-related phytohormones and volatile pools.

### Ala Substitution of Inceptin Reveals Roles for Asp, Cys, and the C Terminus

Of all the potential peptides present in the OS of actively feeding larvae, inceptin-related fragments are hypothesized to possess a unique sequence that promotes activity. Here, we consider the cyclic structure, the role of individual *Vu*-In residues via Ala substitutions, and flexibility (via additions and deletions) of both termini in the elicitation of E, SA, and DMNT as different markers for activity. Peptides and their abbreviations considered in this experiment are detailed in Figure 8. In the E bioassay, replacement of the penultimate C-terminal Asp (*Vu*-In-A10) completely abolished activity (Fig. 8A). Surprisingly, replacement of the Cys proximal to the N terminus (*Vu*-In-A2) resulted in statistically insignificant reduction of activity, suggesting a nonessential role of the disulfide bridge and cyclized peptide. Significant reductions in E-inducing activity were detected in *Vu*-In-A3, *Vu*-In-A8, and *Vu*-In-A10, demonstrating important functions for both Asp residues and the Cys proximal to the C terminus. Both removal (*Vu*-In $^{-\text{A}}$ ) and addition (*Vu*-In $^{+\text{A}}$ ) of Ala to the C terminus significantly reduced activity (Fig. 8A), whereas additions (*Vu*- $^{\text{E}+}$ In; Fig. 3) and truncations (*Vu*- $^{\text{I}-}$ In; Fig. 8A) at the N terminus had no significant effect on E production. The only



**Figure 4.** Activity and elicitor abundance in HPLC-purified OS from armyworm larvae collected at different times after consuming GST-*GmIn8H* precursor proteins. A and C, Induced E production in cowpea leaves stimulated by fractions of NP-HPLC-purified OS originally collected at 2 and 6 h after the initiation of larval feeding on GST-*GmIn8H*. B and D, LC/MS analysis of  $m/z$   $[M + H]^+$  ion relative abundance of 1,105.5 (*Gm*-In), 1,234.5 (*Gm*- $^{\text{E}+}$ In), and 1,291.5 (*Gm*- $^{\text{GE}+}$ In) in samples corresponding to A and C, respectively.



**Figure 5.** Inceptin exhibits preferential persistence in armyworm larvae OS. Average ( $n = 4$ ,  $\pm$ SEM) picomoles per larvae of inceptin-related fragments *Gm-In*, *Gm-E*<sup>+</sup>*In*, *Gm-GE*<sup>+</sup>*In*, and *Gm-In*<sup>-A</sup> present in OS collected at times designated as 0, 2, 4, 6, and 8 h following the complete consumption of GST-*Gmln8H* proteins (4.8  $\mu$ g). Symbols for individual peptides analyzed are denoted in the legend.

substitutions, additions, or truncations that abolished induced E activity were within the last four amino acids of the C terminus.

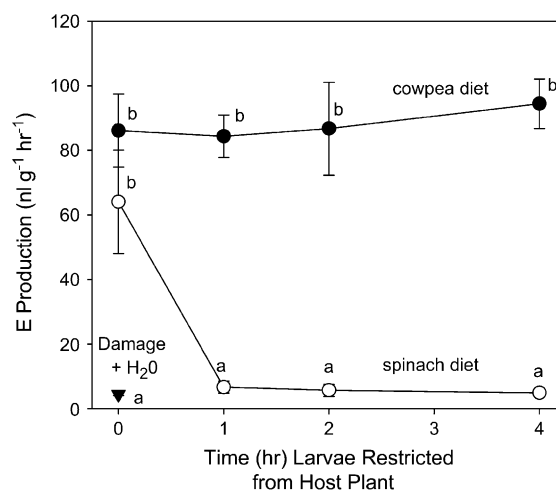
Similar to the E responses, SA production was significantly lower in response to modifications at the C terminus, including *Vu-In*-A10, *Vu-In*<sup>-A</sup>, and *Vu-In*<sup>+A</sup> (Fig. 8B). However, amino acid sequence modifications proximal to the N terminus (*Vu-In*-A3) and substitution of either Cys residue (*Vu-In*-A2 and *Vu-In*-A8) failed to significantly reduce SA accumulation compared to *Vu-In* (Fig. 8B). This contrasts the pattern established for E production and suggests that the SA responses may occur at either lower elicitor concentrations or have different ligand-binding requirements. Accumulation of tissue levels of DMNT more closely mirrored elicitor-induced changes in E production. Substitution of both Asp residues (*Vu-In*-A3 and *Vu-In*-A10) significantly reduced DMNT accumulation compared to *Vu-In* (Fig. 8C). Likewise, removal or addition of Ala to the C terminus in peptides *Vu-In*<sup>-A</sup> and *Vu-In*<sup>+A</sup> also suppressed DMNT levels. Substitution of either Cys (*Vu-In*-A2 and *Vu-In*-A8) produced weak intermediate DMNT responses not statistically different from numerous active and inactive peptides. JA levels were also measured at 4 h but, as expected, did not display strong patterns, in part due to the relaxation of the response at this time point (Fig. 7A; E.A. Schmelz, unpublished data). These activity assays demonstrate a significant role for Asp residues, relative flexibility of additions or truncations at the N but not at the C terminus, and a nonessential role of the *Vu-In* disulfide bridge for phytohormone activity.

## DISCUSSION

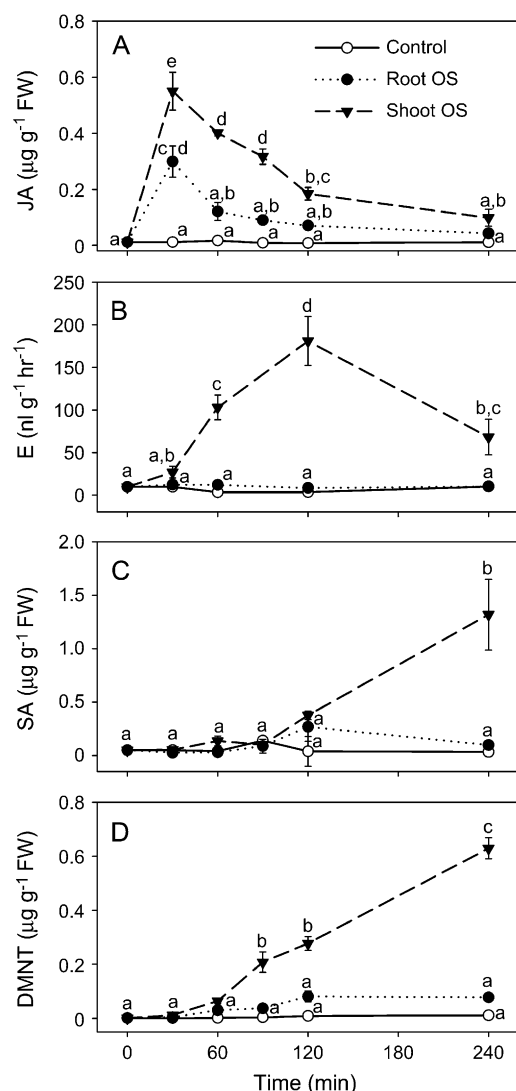
As a model, we propose that herbivory by armyworm larvae on cowpea leaves generates three active

peptide elicitors, namely *Vu-In*, *Vu-E*<sup>+</sup>*In*, and *Vu-GE*<sup>+</sup>*In*, during gut proteolysis of ingested cATPC proteins (Fig. 9). During herbivory, larval OS contact the feeding sites (Truitt and Pare, 2004) and promote inceptin-mediated responses (Schmelz et al., 2006). Larvae that have previously fed on nonphotosynthetic tissue or have moved between alternate host plants may initially lack these elicitors. Inceptin triggers the sequential induction of defense-related phytohormones JA, E, and SA in addition to the induction of volatile biochemical defenses, including DMNT, (*E*)- $\beta$ -ocimene, MeSA, indole, (*E*)- $\beta$ -farnesene, (*E,E*)- $\alpha$ -farnesene, and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Schmelz et al., 2006). These elicitor-induced volatiles have established roles in the attraction of predators and parasitoids to arthropod herbivores (Kessler and Baldwin, 2002; Degenhardt et al., 2003; De Boer and Dicke, 2004; Kappers et al., 2005). Inceptin-related peptides are active in the low femtomole range, exhibit essential Asp residues, and require a conserved C terminus consistent with systemin and flg22 receptor-ligand models (Pearce et al., 1993; Felix et al., 1999). Proteolytic cATPC fragments represent the first identified class of small peptide elicitors that are specifically generated by and mediate the interaction between insects and plants.

Peptides regulate numerous processes, including rapid responses to biotic and abiotic stress. Biochemically characterized endogenous peptides include systemins, *AtPep1*, phytosulfokine, *S*-locus Cys-rich protein, and the rapid alkalization factor, which are involved in wound-induced defenses, *Pythium* resistance, somatic embryogenesis, self-incompatibility, and



**Figure 6.** OS from spinach-fed larvae rapidly loses E-inducing activity. Average ( $n = 4$ ,  $\pm$ SEM) induced E production in cowpea leaves treated with OS collected from larvae 0, 1, 2, or 4 h after being removed from cowpea and spinach plants. Unlike cowpea, spinach harbors a trypsin-sensitive Lys (K) within the predicted inceptin sequence [<sup>+</sup>ICDINGKCVDA<sup>-</sup>]. A damage plus water-only treatment was included as a negative control. Different letters (a and b) represent significant differences (all ANOVA  $P$ s < 0.001, Tukey's test corrections for multiple comparisons [ $P < 0.05$ ]).



**Figure 7.** Sequential induction of phytohormones and volatiles induced by cowpea-derived armyworm OS. Average ( $n = 5$ ,  $\pm$ SEM). A, JA. B, E. C, SA. D, DMNT levels in undamaged control cowpea leaves or those damaged and treated with  $1 \mu\text{L}$  armyworm OS collected from larvae feeding on either cowpea roots or shoots. Symbols for treatments are denoted in the legend. Shoot-derived OS contained  $937 \pm 54 \text{ fmol } \mu\text{L}^{-1}$  ( $n = 4$ ,  $\pm$ SEM) of total active inceptin-related peptides ( $Vu\text{-In}$ ,  $Vu\text{-E}^+\text{In}$ , and  $Vu\text{-GE}^+\text{In}$ ), while corresponding root-derived OS peptides were undetectable ( $<10 \text{ fmol } \mu\text{L}^{-1}$ ). Within figures, different letters (a–e) represent significant differences (all ANOVA  $P$ s  $< 0.001$ , Tukey's test corrections for multiple comparisons [ $P < 0.05$ ]).

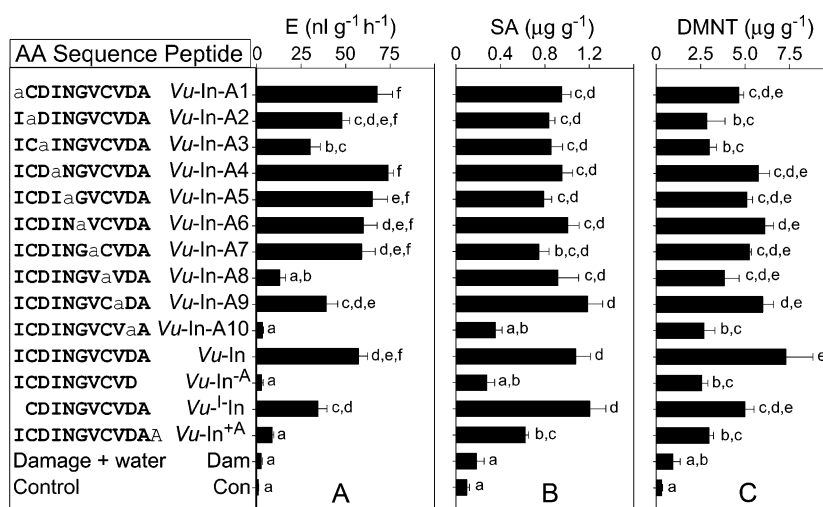
root growth inhibition, respectively (Huffaker et al., 2006; Matsubayashi and Sakagami, 2006). Many of these peptides are active in the femtomolar to picomolar range and are derived from single or multiple homologous genes encoding precursor proteins. Bacterial peptides flg22 and elf18 (derived from elongation factor Tu) exhibit similar activity ranges and elicit resistance in *Arabidopsis* to *Pseudomonas syringae* and *A. tumefaciens* (Zipfel et al., 2004, 2006). In general,

these PAMPs are derived from essential proteins that display infrequent mutations, whereas endogenous plant peptides are more variable between plant families.

In contrast to previously described plant peptide signals, prior to proteolysis, inceptin functions in planta as a critical dithiol region and redox switch that controls ATPase activity (Samra et al., 2006). While not pathogen associated, this molecular pattern exhibits high levels of amino acid sequence conservation between plants (Schmelz et al., 2006) and shares characteristics of PAMP and endogenous peptide signals. Overall, inceptin activity is consistent with the guard hypothesis (Dangl and Jones, 2001; Jones and Dangl, 2006), which is supported by studies of bacterial proteases secreted into the cytoplasm that cleave host proteins, resulting in ligands that bind cytoplasmic nucleotide binding site (NBS)-LRR R proteins (Axtell and Staskawicz, 2003; Mackey et al., 2003; Shao et al., 2003). However, not all R-protein ligands are plant derived or intracellular. For example, in flax (*Linum usitatissimum*), the *L* locus consists of multiple alleles encoding cytoplasmic NBS-LRR proteins that interact directly with AvrL proteins produced by the flax rust fungus (Dodds et al., 2006). Likewise, rice *Xa21* and *Lycopersicon pimpinellifolium* Cf-7 R proteins contain extracellular receptor domains conferring resistance against *Xanthomonas oryzae* pv *oryzae* and *Cladosporium fulvum*, respectively (Jones et al., 1994; Song et al., 1995). We speculate that cATPC is a guarded protein and that inceptin-related fragments interact with extracellular regions of a membrane-bound receptor after recontacting wounded plant cells (Fig. 9). In a similar arrangement, systemin, AtPep1, and flg22 responses are all mediated by membrane-bound extracellular LRR-RKs (Gomez-Gomez et al., 2001; Scheer and Ryan, 2002; Yamaguchi et al., 2006). Areas of overlap in receptor-mediated Avr protein, PAMP, and endogenous peptide signaling are becoming increasingly appreciated (Navarro et al., 2004; Boller, 2005; Abramovitch et al., 2006; Sun et al., 2006).

Unlike pathogens, receptor-ligand pairs mediating specific nematode and insect resistance in plants remain unknown. Over a dozen nematode and insect R genes have been either cloned or mapped in close association with NBS-LRR gene clusters, yet no corresponding ligands have been identified (Rossi et al., 1998; Brotman et al., 2002; Klingler et al., 2005; Williamson and Kumar, 2006). In legumes, insect elicitors and potential ligands also include 3-hydroxypropanoate esters of long-chain  $\alpha,\omega$ -diols, termed bruchins, present in pea (*Pisum sativum*) weevil oviposition fluid and the fatty acid amino acid conjugates (FACs) from lepidopteran OS (Doss et al., 2000; Gomez et al., 2005). On pea pods, fmol amounts of bruchins induce rapid tumor-like neoplastic cellular growths and isoflavone phytoalexins that act to inhibit weevil larvae establishment (Doss et al., 2000; Cooper et al., 2005). In *Medicago truncatula* and lima bean (*Phaseolus lunatus*), FACs are known to elicit terpene synthase transcription and

**Figure 8.** Ala substitution of inceptin confirms essential role of the C terminus for activity. *Vu*-In was sequentially substituted with Ala from the N terminus (A1) to the penultimate C-terminal residue (A10). C and N termini of *Vu*-In were also examined by either adding (*Vu*-In<sup>+A</sup>) or removing residues (*Vu*-In<sup>-A</sup>, *Vu*-I<sup>-In</sup>). Average ( $n = 4$ ,  $\pm$ SEM) induced leaf production of: A, E (1 h); B, SA (4 h); and C, DMNT (4 h) in cowpea leaves treated as either undamaged controls (Con), damage + 5  $\mu$ L water (Dam), or damage plus 4 pmol of peptide in 5  $\mu$ L water. Within figures, different letters (a–f) represent significant differences (all ANOVA  $P$ s < 0.001, Tukey's test corrections for multiple comparisons [ $P < 0.05$ ]).



depolarization of membrane potentials, respectively (Maffei et al., 2004; Gomez et al., 2005). Support for FAC receptors comes from kinetic investigations of plasma membrane binding in maize (*Zea mays*); however, these proteins remain unidentified (Truitt et al., 2004).

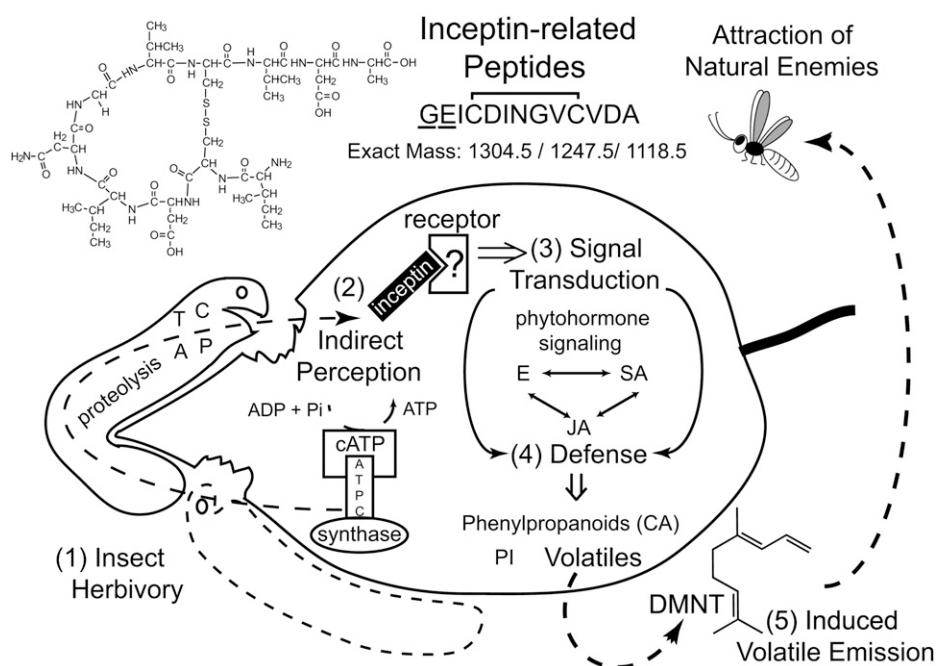
For elicitors derived from variable length polypeptides, understanding the exact size of predominant native ligands is often not trivial. For example, despite considerable research, the actual contribution to elicitation in planta caused by prosystemin (23 kD) and flagellin (33 kD) relative to their respective active core peptides remains unclear. Comparatively, both intact proteins display 100-fold lower EC<sub>50</sub>s for alkalization responses than the corresponding core peptides, yet still retain potent activity in the picomolar to nanomolar range (Dombrowski et al., 1999; Meindl et al., 2000). Unlike prosystemin and flagellin, assays of expressed proteins harboring cATPC sequences failed to demonstrate significant inceptin activity (Schmelz et al., 2006). Overall, the dose-response analyses of active peptides with variable N termini revealed similar EC<sub>50</sub>s (Fig. 3), yet displayed reduced E responses in cowpea when tested above 10 pmol leaf<sup>-1</sup>. Similar biphasic peptide hormone dose-response relationships are now recognized as common in biological systems (Calabrese and Baldwin, 2003). At moderate to high doses, *Vu*-E<sup>+</sup>In and *Vu*-GE<sup>+</sup>In produced variable E inductions that slightly deviated from a normal dose response. This variability is consistent with our experienced difficulty in both purifying these highly acidic natural peptides and in solubilization of the synthetic peptides.

To assess the production of bioactive peptides, we fed larvae recombinant GST-*Gm*In8H proteins derived from the soybean cATPC gene. This sequence encodes inceptin-related peptides identical to maize, established to have activity in cowpea equivalent to *Vu*-In (Schmelz et al., 2006), and should prove useful in the discrimination of elicitor origins in either mixed diets

or transgenic cowpea. Dependent upon the time of OS collection following GST-*Gm*In8H consumption, patterns of HPLC-separable peptides and E-inducing activity varied (Fig. 4, A–D). Consistent with protease digestion, our results demonstrate the rapid processing and gradual removal of inceptin-related peptides over time (Fig. 5). Within 15 min of completed GST-*Gm*In8H consumption by the larvae (defined as time zero), 80% of the total potential inceptin-like peptides present within the diet were recovered in the OS as active elicitors. Similarly, gut fluids of hornworm (*Manduca sexta*) larvae are known to efficiently proteolyze 100% of precipitable Rubisco within this same period (Martin et al., 1987). The midguts of armyworm harbor potent trypsin and chymotrypsin endopeptidase activities; thus, Lys and Phe cleavage sites near the termini of the *Gm*-In (<sup>+</sup>KGEICDVNGVCVDAAEDEF<sup>-</sup>) are predicted to facilitate this process (Keil, 1992; Paulillo et al., 2000). Spinach cATPC harbors an additional trypsin cleavage site within the disulfide bridge of inceptin (Miki et al., 1988; Hightower and McCarty, 1996) and predictably results in larval OS devoid of cowpea E-inducing activity within 1 h (Fig. 6). While supportive, larvae feeding studies with spinach cATPC coupled with E-inducing activity and OS peptide analysis will be required to confirm that inceptin degradation drives this loss of activity. Additional exopeptidase processing by amino- and carboxypeptidases have been partially investigated in armyworm (Ferreira et al., 1994) and merit further characterization. In general, cyclized peptides are known to exhibit greater stability and increased resistance to protease attack (Horton et al., 2000; Clark et al., 2005). It is tempting to speculate that the relative proteolytic resistance of inceptin within insect guts contributed to the reliability and evolved recognition of this peptide signal in plants.

Peptide signals are known to mediate complex reorganizations in transcription and metabolite production, in part through phytohormone signaling





**Figure 9.** Simplified proposed model for inceptin-related peptides in mediating interactions between armyworm larvae and cowpea. (1) Larvae consume cowpea leaves and produce digestive fragments of cATPC; (2) plants indirectly perceive attack when inceptin-related peptides recontact the wounded leaf surface and bind a putative receptor; (3) multiple signaling pathways are activated that include the phytohormones JA, E, and SA; (4) biochemical defense responses are induced, including protease inhibitor transcripts (PI), phenylpropanoids (cinnamic acid [CA]), and volatiles including MeSA and DMNT (Schmelz et al., 2006); and (5) insect-induced plant volatiles are released, providing reliable cues that can facilitate attraction of natural enemies.

pathways (Navarro et al., 2004). We previously demonstrated that inceptin promotes the production of JA, E, SA, and DMNT in cowpea (Schmelz et al., 2006) and now clarify their rapid sequential dynamics following treatment with root- and shoot-derived OS (Fig. 7, A–D). While root-based diets provide a useful comparative tool to manipulate OS elicitor content, armyworm are not ecologically relevant below-ground herbivores. However, it is curious that many significant natural insect pests of cowpea in Africa functionally avoid tissues rich in inceptin precursors by preferentially targeting root, stem, flowers, phloem, pod, and seed tissues (Alghali, 1991; Jackai et al., 1996; Bottenberg et al., 1998). Shoot-derived OS elicited the highest JA levels within 30 min; yet, leaf damage plus root-derived OS also induced statistically significant JA accumulation above unwounded control leaves (Fig. 7A). This increase likely reflects damage-induced JA; however, damage-only controls are required to rule out the possibility of additional OS factors that might contribute to JA production. Consistent with a role for JA in promoting plant volatile emission (Boland et al., 1995; Schmelz et al., 2003a), preliminary experiments indicate that exogenous JA promotes DMNT production in cowpea leaves (data not shown). In maize, FAC-induced JA also interacts synergistically with E in the elicitation of induced volatiles (Schmelz et al., 2003b, 2003c), yet it is currently unclear if E plays a similar role in cowpea. In lima bean, treatment of excised leaves with JA or continually repeated mechanical damage is sufficient to induce volatile emission of DMNT (Boland et al., 1995; Dicke et al., 1999; Mithofer et al., 2005). However, two-spotted spider mite (*Tetranychus urticae*) infestation of lima bean results in greater levels of DMNT and MeSA production than those induced by JA alone, suggest-

ing the presence of additional signals (Dicke et al., 1999). Importantly, these spider mite-induced volatiles are also preferentially attractive to carnivorous mites (De Boer and Dicke, 2004; Kappers et al., 2005). While it is unknown if spider mites produce inceptin, it is of interest to determine both the breadth of biotic attackers that produce these elicitors and likewise the range of responding plants.

Inceptin activity depends on a limited number of conserved amino acid residues. As with systemin and flg22 deletion studies (Pearce et al., 1993; Meindl et al., 2000), an analogous essential role for the C terminus was demonstrated by the inactivity of *Vu-In*<sup>-A</sup> (Fig. 3). Surprisingly, E-inducing activity of the Ala-substituted peptide *Vu-In*-A2 indicated a nonessential role of the Cys disulfide bridge (Fig. 8A), demonstrating that *Vu-In* can function as a linear peptide. Attempts to assay reduced linear forms of *Vu-In* were confounded by rapid and significant cyclization at biologically relevant pHs in aqueous solutions (E.A. Schmelz, unpublished data). Analogous to the 100-fold reductions in flg15 activity when Asp is substituted with flg15-Ala-7 (Felix et al., 1999), substitution of Asp in *Vu-In* for Ala either significantly lowered or abolished activity. Similar to systemin (Pearce et al., 1993), only three Ala-substituted *Vu-In* peptides displayed dramatic reductions in E-inducing activity. Increases in DMNT pools were more variable than induced E production but followed approximately the same patterns (Fig. 8C). For induced SA levels, only the loss of Asp in the *Vu-In*-A10 substitution significantly reduced the response compared to all other substituted peptides. While unexpected, this result suggests that either peptide concentration or essential amino acids required for SA and E induction may differ. The peptide *Vu-In*-A8 resulted in the most incongruent E

and SA response. Interestingly, this Cys is situated between the substitution-sensitive C terminus and more flexible N terminus (Fig. 8, A and B). Both systemin and flg22 exhibit two-step receptor interactions involving initial binding of the inactive peptide N-terminal sequences followed by activation via a limited number of essential C-terminal amino acids (Meindl et al., 1998, 2000). For example, in cut tomato seedlings, the systemin C-terminal tetrapeptide Met-Gln-Thr-Asp exhibits half-maximal protease inhibitor I-inducing activity at 4 nmol plant<sup>-1</sup>, while systemin requires only 12 fmol plant<sup>-1</sup> (Pearce et al., 1993). Curiously, Met-Gln-Thr-Asp induced neither E nor alkalization in *S. peruvianum* cell cultures at concentrations up to 30 μM (Meindl et al., 1998), suggesting that altered peptides may exhibit partial activities. Due to the laborious nature of testing amino acid substitution libraries, analyses are typically limited to single assays as opposed to broader targeted profiling (Pearce et al., 1993; Meindl et al., 2000). Detailed EC<sub>50</sub> analysis of both E and SA induction in cowpea leaves utilizing a multiple series of modified peptides will be required to pinpoint these requirements.

Through the overexpression of precursor proteins, plant-derived peptide signals have been used to elevate the expression of plant defenses and resistance to biotic attack (McGurl et al., 1994; Huffaker et al., 2006; Ren and Lu, 2006). Cowpea leaves already contain inceptin precursors, yet nonphotosynthetic tissues do not. Our future goal is to generate transgenic legumes displaying broad tissue distributions of recombinant proteins harboring multiple inceptin sequences. These plants should exhibit either faster or greater defense responses following insect herbivory. The nonexistent or greatly reduced activities of intact cATPC-GST fusion proteins (Schmelz et al., 2006) and Vu-In<sup>+</sup>A (Fig. 8) suggest that complete C-terminal inceptin processing is important for plant responses. This characteristic is not trivial, as the preservation of transgenic plants in an uninduced state prior to insect attack will be important for the maintenance of herbivore-induced volatiles as informative cues for natural enemies (Degenhardt et al., 2003). Ultimately, the future discovery of receptor-ligand pairs that regulate insect-induced defenses will prove valuable in revealing common themes of plant non-self-recognition.

## MATERIALS AND METHODS

### Plant and Insect Material

Armyworm (*Spodoptera frugiperda*) eggs were obtained from Dr. R. Meagher (U.S. Department of Agriculture-Agricultural Research Service, Gainesville, FL) and reared on a pinto bean (*Phaseolus vulgaris*) diet (Schmelz et al., 2003a). Cowpea (*Vigna unguiculata* var. California Blackeye no. 5; The Wax Company), spinach (*Spinacea oleracea* var. Avon; W. Atlee Burpee), and soybean (*Glycine max* var. 3880; Nobbe Seeds) were germinated in a soil mix (Jungle Growth, Piedmont Pacific Industries) supplemented with 14-14-14 Osmocote (Scotts Miracle-Gro). All plants were maintained in a greenhouse with a 12-h photoperiod, minimum of 300 μmol<sup>-2</sup> s<sup>-1</sup> of photosynthetically active radiation supplied by supplemental lighting, 70% relative humidity, and temperature cycle of 24°C/28°C (night/day).

### Cowpea Leaf Bioassays and Armyworm OS Collection

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 approximately 5% of the total waxy cuticle. The damage sites (2 cm<sup>2</sup> 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 water were immediately applied and dispersed over the damage sites. Leaves remained on the intact plants for specified lengths of time prior to E and leaf metabolite sampling. Large-scale collection of OS from the sixth instar larvae was as previously described (Turlings et al., 1993). For quantitative OS collection, larvae were additionally allowed to intermittently bite the edge of the capillary collection tube before directly placing head capsules into the opening of the capillary. For each larva, this process continued for 1 min after the last detectable trace of OS was released.

### Isolation of Multiple Elicitors

As previously reported, a 100-mL sample of cowpea-derived armyworm OS was initially fractionated using RP-C18 and strong anion exchange Discovery solid phase extraction columns (Supelco; Schmelz et al., 2006). HPLC fractionation of E-inducing activity utilized a P4000 pump, AS3000 autosampler, and UV6000LP detector (Thermo Separation Products). All HPLC samples were dissolved in the initial mobile phase (MP), and 1-min fractions were collected, desalted using RP-C18 solid-phase extraction (SPE) columns, bioassayed, and stored for further purification at -70°C. SCX HPLC utilized a polysulfoethyl A (250 × 9.4 mm, 5 μm, 300 Å; Nest Group) column at flow rate of 5 mL min<sup>-1</sup>, with MP A and B both containing 1:4 CH<sub>3</sub>CN:water, pH 3.0, 25 mM KH<sub>2</sub>PO<sub>4</sub> with the addition of 0.5 M KCl to MP B. Using a binary gradient of 100% A to 100% B over 20 min, active fractions were obtained from 3 to 6 min. In this study, further purification of the 3- to 4-min fraction, as reported in Schmelz et al. (2006), was undertaken. Fractions from multiple 10-mg injections were pooled at 1-min intervals. The combined sample was then subjected to RP-C18 using a YMC ODS-AQ (250 × 4.6 mm, S-5 μm, 20 nm; Waters) column heated to 60°C, using a flow rate of 1 mL min<sup>-1</sup>, with MP A and B containing 95:5 water:CH<sub>3</sub>CN and 9:1 CH<sub>3</sub>CN:water, respectively. Both solutions were buffered with 10 mM HCO<sub>2</sub>NH<sub>4</sub>. Activity was eluted with an isocratic flow MP A for 2 min, followed by a binary gradient of 100% A to 100% B over 18 min. The most active fraction, eluting between 9.6 and 10.1 min, was separated by GF using a Tricorn Superdex Peptide 10/300 GL (Amersham Biosciences) column and an isocratic 1-mL min<sup>-1</sup> flow of water containing 100 mM CH<sub>3</sub>CO<sub>2</sub>NH<sub>4</sub>. Samples with activity eluting at 12 to 13 min were further fractionated using a NP carbamoyl-bonded TSKgel Amide-80 (250 × 4.6 mm; Tosoh) column at 1-mL min<sup>-1</sup> flow rate, with MP A and B containing 95:5 CH<sub>3</sub>CN:water and water, respectively. Both solutions were buffered with 25 mM HCO<sub>2</sub>NH<sub>4</sub>. MP A was held isocratic for 2 min and followed by a linear binary gradient reaching 1:1 A:B over 28 min. Fractions containing peak E-inducing activity eluting between 22.5 and 24 min were used for further analysis.

### Characterization and Synthesis of Inceptin-Related Peptides

Edmund chemical N-terminal sequencing was performed as previously described (Schmelz et al., 2006). Samples of Vu-In and Vu-GE<sup>+</sup>In isolated from cowpea-derived OS were separated by NP-HPLC and analyzed by MS using a LCQ Deca XP MAX ion trap (Thermo Electron). TSKgel Amide-80 columns, MP, and gradients were as described in the HPLC isolation. The 1-mL min<sup>-1</sup> flow was split allowing 0.1 mL min<sup>-1</sup> to enter the ion source. Inceptin-related sequences Vu-GE<sup>+</sup>In, Vu-E<sup>+</sup>In, and Vu-In<sup>-A</sup> were synthesized and purified at the Protein Core Chemistry Facility (University of Florida, Gainesville, FL) as previously described for Vu-In (Schmelz et al., 2006). HPLC retention times, positive m/z [M + H]<sup>+</sup> ions, and MS fragment ions of natural and synthetic peptides were used to confirm identities.

### Activity of Four Naturally Occurring Inceptin-Related Peptides

Synthetic Vu-GE<sup>+</sup>In, Vu-E<sup>+</sup>In, Vu-In, and Vu-In<sup>-A</sup> peptides were HPLC purified, dissolved into water, and applied (*n* = 4) at 1.3, 4, 12, 45, 130, 430, 1,280, 4,300, and 12,800 fmol leaf<sup>-1</sup> to a total of 144 cowpea leaves. Immediately prior

to the construction of these dilutions, peptide concentrations in stock solutions were analyzed by LC/MS and adjusted. Compared to *Vu*-In, natural and synthetic *Vu*-<sup>GE+</sup>In and *Vu*-<sup>E+</sup>In consistently displayed greater losses of these peptides in solution suggesting lower aqueous solubility.

### Expression of GST-*GmIn8H* Protein in *Escherichia coli*

Primers CTCGAGATGGCTGATGATGTTTCTCAC and GCGGCCGCA-TAGATCTATCCCTCTCCACAGTCAAC, which contain *XhoI* and *NotI* sites, respectively, were used to amplify cDNA from soybean leaves with the resulting 253-bp PCR product cloned into a TOPO pCRII vector (Invitrogen). The soybean chloroplast *atpC* partial sequence generated was deposited in GenBank (EF185283). This fragment contains a *BamHI* site 95 bp downstream of the *XhoI* site and a *BglII* site 34 bp upstream of the *NotI* site. The 230-bp *XhoI*-*BglII* fragment and the 145-bp *BamHI*-*NotI* fragment were ligated together with pBluescript SK+ cut with *XhoI* and *NotI* to create a construct with two inceptin fragments. This SK+ *GmIn2* construct was cut with *BglII* + *NotI* and *BamHI* + *NotI*, and then the gel-purified 3.2-kb and 145-bp fragments were ligated to create SK+ *GmIn4*. This process was repeated once more to generate the SK+ *GmIn8* construct. To introduce a polyhistidine tag, primers *Bgl2HIS-F* (GATCACCACCACCACCACCAC CACCAC) and *Bgl2HIS-R* (GATCGTGGTGGTGGTGGTGGTGGT) were phosphorylated with T4 polynucleotide kinase (Invitrogen), annealed with one another to create *BglII*-compatible overhangs, and ligated with *BglII*-cut SK+ *GmIn8*. The 1.2-kb *XhoI*-*NotI* *GmIn8H* fragment was then ligated to *Sall*-*NotI* cut pET41b(+) (Novagen). This construct was transformed into BL21DE3pLysS *Escherichia coli* (Novagen) and was used to express GST-*GmIn8H* upon the addition of 1 mM isopropylthio- $\beta$ -galactoside to the culture medium. After induction, cultures were grown overnight at room temperature on a rotary shaker. Cells were ultrasonically lysed and pelleted by centrifugation. Total insoluble inclusion body proteins were enriched by resolubilization of the pellet in 1:1 ethanol:water, precipitation at  $-70^{\circ}\text{C}$ , and centrifugation at 12,000g for 10 min. This process was repeated three times to ensure removal of soluble peptides. The protein pellet was suspended in 20% aqueous solution of Suc to aid in larval feeding (Chapman, 2003) and divided into multiple aliquots for storage at  $-70^{\circ}\text{C}$ .

To estimate the amount of GST-*GmIn8H* fed to armyworm larvae, a dilution series containing the equivalent of 0.1, 0.3, 1.0, and 3.0  $\mu\text{L}$  of protein was subject to SDS-PAGE next to known concentrations of pure His-tagged GST. Duplicate gels were either stained with Coomassie Blue or transferred to polyvinylidene difluoride membrane for western blot with mouse monoclonal anti-6 $\times$  His antibody (Sigma) followed by peroxidase conjugated antimouse (Sigma). Reactive proteins were visualized by chemiluminescent staining followed by exposure to x-ray film. Both films and stained gels were photographed with the Bio-Rad Gel-Doc system and quantified with Quantity One software (Bio-Rad). The slope of the line for the GST standard was used to calculate the concentration of GST-*GmIn8H* in each lane and average molecules/microliter was determined based on the molecular mass of GST and GST-*GmIn8H*.

### Larvae Feeding Studies with GST-*GmIn8H* and Other Plants

To examine inceptin-related peptide production in armyworm OS, we allowed 24 individual larva to consume 10-mm<sup>2</sup> pieces of cellulose filter paper saturated with 20  $\mu\text{L}$  of 20% Suc solution containing 9.6  $\mu\text{g}$  GST-*GmIn8H* for either 2 or 6 h. Amounts of filter paper consumed varied greatly, and larval OS were pooled into single 280- $\mu\text{L}$  samples at each time point. The 2- and 6-h samples were purified using a combination of RP-C18/strong anion exchange SPE and RP-C18 HPLC with all active fractions combined for the final NP-HPLC separation. To aid in reproducibility of diet consumption by larvae and quantitatively explore the dynamics of inceptin production, isolated sixth instar armyworm were deprived of artificial diet for 12 h, then allowed to consume a 5-mm<sup>2</sup> piece of cellulose filter paper saturated with 10  $\mu\text{L}$  of 20% Suc solution containing 4.8  $\mu\text{g}$  GST-*GmIn8H*. All larvae completed consumption of the protein between 45 and 60 min after initial access to the filter paper diet. Within 15 min of completed diet consumption ( $\leq 1$  h from the start of feeding) larval OS were collected from the first group, defined as time zero, and pooled from three individual larva ( $n = 4$ ). Similar larvae were sampled 2, 4, 6, and 8 h later. The peptides *Gm*-<sup>GE+</sup>In, *Gm*-<sup>E+</sup>In, *Gm*-In, and *Gm*-In<sup>-A</sup> were quantified in these samples using RP-C18 LC/MS analysis. To examine OS activity from larvae on different host plants, sixth instar armyworm were

allowed to feed on either cowpea or spinach for 48 h prior to removal. A total of 96 larvae were used to generate 32 independent samples, with the OS of three larvae pooled ( $n = 4$ ) at 0, 1, 2, and 4 h after isolation from host plants and used for induced E bioassays in cowpea leaves.

### Inceptin Quantification

Quantification of inceptin-related peptides was achieved using ubiquitously labeled <sup>13</sup>C and <sup>15</sup>N Val-N-FMOC (V\*; Cambridge Isotope Laboratories) incorporated into the synthetic peptide [Vu-I; <sup>13</sup>ICDING-V\*-CVDA<sup>15</sup>]. Aliquots of crude OS (typically 50–100  $\mu\text{L}$ ) were sequentially spiked with 50 ng of the internal standard peptide, 5  $\mu\text{L}$  HCOOH, vortexed, and centrifuged at 12,000g for 5 min. The aqueous phase was mixed with an equal volume of ethanol, stored at  $-70^{\circ}\text{C}$  for 30 min, and centrifuged 12,000g for 2 min. Samples were diluted to 5% ethanol, loaded on 100-mg RP-C18 SPE columns, washed with 2 mL of water, and eluted with 9:1 CH<sub>3</sub>CN:water. Samples were then concentrated to dryness under vacuum, brought up in 50  $\mu\text{L}$  5:95 CH<sub>3</sub>CN:water containing 10 mM HCO<sub>2</sub>NH<sub>4</sub> and 10  $\mu\text{L}$  analyzed by LC-MS as described. Quantification was based on peak retention times (9.7–10.2 min) and monitoring of [M + H]<sup>+</sup> ions with a *m/z* of 1,125.5 (ISTD), 1,034.5 (*Gm*-In<sup>-A</sup>), 1,105.5 (*Gm*-In), 1,234.5 (*Gm*-<sup>E+</sup>In), 1,291.5 (*Gm*-<sup>GE+</sup>In), 1,048.5 (*Vu*-In<sup>-A</sup>), 1,119.5 (*Vu*-In), 1,248.5 (*Vu*-<sup>E+</sup>In), and 1,305.5 (*Vu*-<sup>GE+</sup>In). The identity of each peptide was confirmed with MS<sup>2</sup> daughter ion spectra.

### Phytohormone and Biochemical Analyses

Gas chromatography-based quantification of elicitor-induced E production followed from Schmelz et al. (2006). In standard E bioassays, experimental leaves were treated, excised 1 h later, and sealed in tubes for an additional 1 h prior to headspace sampling. To estimate the time course of OS-induced phytohormone and DMNT pools, leaves were either left as undamaged controls or damaged and treated with 5  $\mu\text{L}$  of an aqueous solution containing either 1  $\mu\text{L}$  cowpea root-derived OS or shoot-derived OS. For E production, leaf headspace ( $n = 5$ ) was collected for 15 min starting at 0, 30, 60, 120, and 240 min. For phytohormones and DMNT, leaf tissues ( $n = 5$ ) were harvested in liquid N<sub>2</sub> at 0, 30, 60, 90, 120, and 240 min after treatments and analyzed by isobutane chemical-ionization gas chromatography-MS as described (Schmelz et al., 2004). Leaf pools of DMNT were quantified based on an external standard curve of synthetic DMNT and monitoring of the [M + H]<sup>+</sup> *m/z* ion 151 at retention time 7.75 min.

### Activity of Ala-Substituted Inceptin

To consider amino acid residues important for inceptin signaling, peptides with additional and deleted amino acids at both the N and C termini were examined along with an Ala substitution series of *Vu*-In. These peptides were synthesized by Genosys (Sigma-Aldrich) and subsequently HPLC purified using RP-C18 to >90%. Significant solubility differences in these peptides necessitated estimation of actual aqueous concentrations using LC/MS and selected [M + H]<sup>+</sup> *m/z* ion monitoring to determine peak areas. Analysis of dilutions resulted in a final concentration of 4 pmol leaf<sup>-1</sup> for all peptides assayed. Based on the timing of the dynamics of induced phytohormone changes (Fig. 7, A–D), we focused on E production between 60 and 120 min and SA/DMNT pools at 240 min. In this experiment, the cowpea leaf tip ( $n = 4$ ) was excised at 1 h for E analysis while the treated basal leaf section remained attached and was harvested at 4 h for metabolite analysis.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EF185283.

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** LC-MS confirmation of natural and synthetic inceptin-related peptides.

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