

An Amino Acid Substitution Inhibits Specialist Herbivore Production of an Antagonist Effector and Recovers Insect-Induced Plant Defenses^{1[W][OA]}

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Plants respond to insect herbivory through the production of biochemicals that function as either direct defenses or indirect defenses via the attraction of natural enemies. While attack by closely related insect pests can result in distinctive levels of induced plant defenses, precise biochemical mechanisms responsible for differing responses remain largely unknown. Cowpea (*Vigna unguiculata*) responds to Fall armyworm (*Spodoptera frugiperda*) herbivory through the detection of fragments of chloroplastic ATP synthase γ -subunit proteins, termed inceptin-related peptides, present in larval oral secretions (OS). In contrast to generalists like Fall armyworm, OS of the legume-specializing velvetbean caterpillar (VBC; *Anticarsia gemmatilis*) do not elicit ethylene production and demonstrate significantly lower induced volatile emission in direct herbivory comparisons. Unlike all other Lepidoptera OS examined, which preferentially contain inceptin ($Vu\text{-In}$; $^+\text{ICDINGVCVDA}^-$), VBC OS contain predominantly a C-terminal truncated peptide, $Vu\text{-In}^{-A}$ ($^+\text{ICDINGVCVD}^-$). $Vu\text{-In}^{-A}$ is both inactive and functions as a potent naturally occurring antagonist of $Vu\text{-In}$ -induced responses. To block antagonist production, amino acid substitutions at the C terminus were screened for differences in VBC gut proteolysis. A valine-substituted peptide ($Vu\text{-In}^{\Delta V}$; $^+\text{ICDINGVCVDV}^-$) retaining full elicitor activity was found to accumulate in VBC OS. Compared with the native polypeptide, VBC that previously ingested 500 pmol of the valine-modified chloroplastic ATP synthase γ -subunit precursor elicited significantly stronger plant responses in herbivory assays. We demonstrate that a specialist herbivore minimizes the activation of defenses by converting an elicitor into an antagonist effector and identify an amino acid substitution that recovers these induced plant defenses to a level observed with generalist herbivores.

Excluding microorganisms, plants and insects constitute over 75% of the total species on earth (Hawksworth and Kalin-Arroyo, 1995). At a numerical species level, insect herbivores significantly outweigh plants and together display exquisitely complex ecological interactions (Price, 2002). Herbivorous insects are often broadly categorized as either generalists or specialists to denote feeding preferences on either a wide variety or a select subset of plant species, respectively. It is widely envisioned that host plant defenses have less

impact on specialists as compared with generalist herbivores. Comparatively, specialists often manipulate plants to their benefit and demonstrate improved tolerance of host-specific biochemicals. For example, sawfly larvae in the genus *Pontania* induce protective closed galls in numerous willow species (*Salix* spp.) that result in dramatically reduced interior tissue defenses (Nyman and Julkunen-Tiitto, 2000). Swallowtail caterpillars, such as *Papilio polyxenes*, utilize plants in the family Apiaceae through efficient cytochrome P450 monooxygenase enzymes that directly detoxify dietary furanocoumarins (Cohen et al., 1992; Berenbaum, 2002). Cabbage white butterfly (*Pieris rapae*) larvae prevent toxic isothiocyanate formation through the action of a gut nitrile-specifier protein that redirects typical hydrolysis of glucosinolates to instead form nitriles that are excreted in the frass (Wittstock et al., 2004). Although specialists often exhibit greater tolerance to specific biochemicals than generalists, in many cases they are still negatively impacted by high levels of these plant defenses (Adler et al., 1995; Zalucki et al., 2001; Berenbaum, 2002; Agrawal and Kurashige, 2003).

A hallmark of herbivore-inducible plant defense is variability. One method to nondestructively measure this variation is through the assessment of indirect defenses such as herbivore-induced volatile emissions

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that attract natural enemies of the offending pest. In some cases, different feeding habits, such as leaf herbivory versus stem boring, readily account for this variation; however, even similar patterns of leaf herbivory can result in different volatile emission patterns and parasitoid attraction (De Moraes et al., 1998; Turlings et al., 1998). Oral secretions (OS)-derived fatty acid amino acid conjugate (FAC) elicitors, such as *N*-(17-hydroxylinolenoyl)-L-Gln and related analogs, promote plant recognition of Lepidopteran herbivores and trigger induced volatile emission in diverse plant species, including maize (*Zea mays*), eggplant (*Solanum melongena*), and soybean (*Glycine max*; Alborn et al., 1997; Schmelz et al., 2009). A combination of mechanical damage and FAC elicitors can constitute the initial stimuli leading to production of the defensive phytohormones jasmonic acid (JA) and ethylene (ET); however, elicitation can be phylogenetically idiosyncratic and even absent from closely related plants (Schmelz et al., 2009). In receptive plants, a potential for specificity comes from differences in the biochemical content of herbivore OS. For example, in *Nicotiana attenuata*, herbivory and OS of the specialist *Manduca sexta* elicit greater production of JA and ET than comparable treatments with the generalist beet armyworm (*Spodoptera exigua*; Diezel et al., 2009). These phytohormone differences are coincident with markedly different levels of FAC elicitors and also salivary-derived Glc oxidase (GOX) in the herbivore species.

As a salivary secretion, GOX is one of the few herbivore-derived effectors implicated in the suppression of inducible defenses, namely reduced nicotine accumulation in tobacco (*Nicotiana tabacum*) and improved growth of corn earworm (*Helicoverpa zea*; Musser et al., 2002). The suppressive activity of GOX in *Nicotiana* spp. is in part through the formation of hydrogen peroxide, elicitation of induced salicylic acid (SA) production, and associated negative cross talk interactions that attenuate induced JA and ET signaling (Diezel et al., 2009). Transcriptional evidence for similar SA-mediated suppression of JA signaling also exists in different feeding guilds. For example, in *Arabidopsis* (*Arabidopsis thaliana*), whitefly (*Bemisia tabaci*) feeding appears to repress the accumulation JA-regulated defense transcripts such as PDF1.2 (Zarate et al., 2007). Although the effector remains unknown, *B. tabaci* suppression of JA-regulated resistance is associated with strong up-regulation of the SA signaling pathway and associated gene transcript levels (Zarate et al., 2007). Regardless of diet breadth, herbivores cause responses in their hosts that are variable, and more recently, the paradigm of generalist and specialist herbivores inducing predictable plant responses has been challenged (Ali and Agrawal, 2012).

Improved utilization of host plants by any biotic attacker often involves a combination of detoxification and prevention of defense activation. The vast majority of mechanistic advances demonstrating the suppression of inducible defenses have come from plant-pathogen research (Jones and Dangl, 2006; Zhou and

Chai, 2008; Hogenhout et al., 2009). A well-characterized component of plant basal immunity to pathogenic bacteria is recognition of microbe-associated molecular patterns (MAMPs) such as the flagellin peptide fragment, termed flg22. The FLS2 receptor binds flg22 to initiate defense; however, successful pathogens such as *Agrobacterium tumefaciens* and pathovar variants of *Xanthomonas campestris* pv *campestris* exhibit altered sequences of flg22 that neither associate with FLS2 nor activate defense (Felix et al., 1999; Zipfel et al., 2004; Sun et al., 2006). To counter elicitation, pathogenic bacteria, such as *Pseudomonas syringae* pv *tomato* DC3000, inject into plant cells a broad array of multifunctional effector proteins via the type III secretion system that suppress the activation of innate immunity (Jones and Dangl, 2006; Zhou and Chai, 2008). Moreover, *P. syringae* pv *tomato* DC3000 also secretes the jasmonoyl-Ile phytohormone mimic coronatine as a small-molecule effector that co-opts signaling by the E3 ubiquitin ligase Coronatine-Insensitive1 (COI1) and the transcription factor Jasmonate-Insensitive1 (JIN1)/MYC2 to suppress MAMP-mediated resistance responses (Millet et al., 2010). In a conceptually useful scheme detailing the probable evolutionary interplay of resistance and disease susceptibility, plants are hypothesized to have first evolved MAMP recognition systems to trigger innate immune responses. Subsequently, pathogens overcame recognition through effectors (virulence factors) that were able to suppress those immune responses (Bent and Mackey, 2007). Plant R proteins then evolved to either directly recognize pathogen effectors (avirulence factors) or products of their activities on guarded plant proteins to promote defense activation and resistance. In contrast to pathogens, relatively little is known about the mechanisms that Lepidopteran herbivores use to evade plant recognition and activation of defense responses (Bonaventure et al., 2011).

Cowpea (*Vigna unguiculata*) and common bean (*Phaseolus vulgaris*) recognize insect herbivory through the detection of trace amounts of cyclic disulfide-bridged inceptin-related peptides present in caterpillar OS, which are derived from proteolytic fragments of chloroplastic ATP synthase γ -subunit (cATPC) proteins (Schmelz et al., 2006, 2007). Inceptin-related peptides have thus far been described in the OS of Fall armyworm (FAW; *Spodoptera frugiperda*), which prefers grasses but is a confirmed generalist known to feed on over 60 different diverse plant species, including cowpea (Luginbill, 1928). To better understand the adaptations of a legume specialist, we examined the velvetbean caterpillar (VBC; *Anticarsia gemmatilis*), a devastating defoliator of tropical soybean and other legumes, whose host range includes *Vigna* spp. and *Phaseolus* spp. in the Americas (Buschman et al., 1977; Piubelli et al., 2005). Direct comparative assays of induced responses in cowpea to both natural herbivory and OS of both VBC and FAW revealed that VBC induced significantly lower plant defense responses. Compared with eight different Lepidoptera pest species examined, VBC demonstrates a unique preferential processing of

inceptin-related peptides into a biologically inactive form, namely $Vu\text{-In}^{-A}$ ($^+\text{ICDINGVCVD}^-$). Moreover, $Vu\text{-In}^{-A}$ functions as a naturally occurring antagonist of plant defense responses triggered by inceptin ($Vu\text{-In}$; $^+\text{ICDINGVCVDA}^-$). Sequential screening of $Vu\text{-In}$ peptide libraries with substituted C-terminal amino acids revealed a single conservative amino acid change that reduces VBC proteolysis of active inceptin-related peptides and recovers induced plant defense responses to this pest.

RESULTS

Decreased Defense Activation by a Legume Specialist Herbivore

In cowpea, we previously established that FAW OS contain the elicitor $Vu\text{-In}$ that drives the coordinated production of the defense-related phytohormones including JA, ET, and SA as well as increases in emission and leaf pools of the predominant volatile (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT; Schmelz et al., 2006, 2007; Carroll et al., 2008). Volatile DMNT is detected by numerous parasitoid wasps and attracts natural enemies of herbivorous pests (Gouinguéné et al., 2005; Kappers et al., 2005). Cowpea leaves experiencing a FAW feeding bout from larvae devoid of $Vu\text{-In}$ fail to elicit DMNT production (Schmelz et al., 2006). In a direct comparison with FAW OS, larval secretions from the legume specialist VBC elicited 8.8- and 2.2-fold lower levels of ET and DMNT, respectively, when applied to wounded cowpea leaves (Fig. 1, A and B). DMNT leaf pools present at 4 h following VBC OS treatment were not significantly different from those after mechanical damage + water alone (Fig. 1B). Quantified changes in leaf tissue pools of DMNT are used as an estimate for differences in volatile emission at the local site of treatment and herbivore attack. To examine cowpea defense responses to actual herbivory, we performed both short-term assays, which allowed single feeding bouts, and long-term assays, where plants were subjected to intensive sustained attack by either FAW or VBC. Analyses of cowpea leaf tissue surrounding short-term feeding sites that received equivalent damage (Fig. 1C) and long-term whole-plant volatile emissions (Fig. 1D) both demonstrate that VBC herbivory results in significantly lower levels of DMNT compared with FAW herbivory. Thus, cowpea plants produce quantitatively different defense responses to defoliation by these generalist and specialist Lepidopteran herbivores.

Unlike Other Lepidoptera, VBC Predominantly Contain an Inactive Inceptin-Related Peptide

Large-scale purification of FAW OS previously revealed the presence of four inceptin-related peptides, consisting of predominantly $Vu\text{-In}$ and lesser amounts of $Vu\text{-In}^{\text{E}+}$ ($^+\text{EICDINGVCVDA}^-$), $Vu\text{-In}^{\text{GE}+}$ ($^+\text{GEICDINGVCVDA}^-$), and $Vu\text{-In}^{-A}$ ($^+\text{ICDINGVCVD}^-$). Unlike

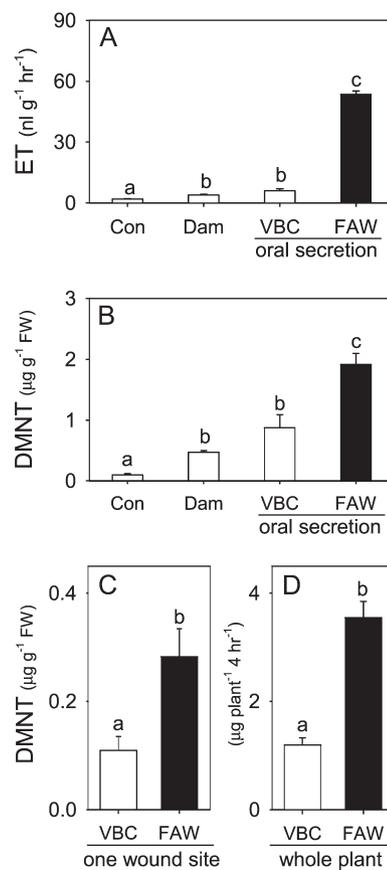


Figure 1. The legume specialist VBC elicits weaker defense responses than the generalist FAW on cowpea leaves. A and B, Average ($n = 4$; \pm SE) ET production (A) at 1 h and DMNT tissue pools (B) at 4 h after cowpea leaves were treated as undamaged controls (Con) or damaged and treated with 5 μL of water (Dam), VBC OS, or FAW OS. C, Average ($n = 7$; \pm SE) cowpea leaf tissue pools of DMNT at 4 h after a single feeding bout by VBC and FAW larvae. D, Average ($n = 4$; \pm SE) whole-plant cowpea volatile emission of DMNT during continuous herbivore feeding damage by eight early sixth instar VBC and FAW larvae. Different letters above the bars (a–c) represent significant differences (all ANOVA P values were $P < 0.007$; Tukey's test corrections for multiple comparisons were at $P < 0.05$). FW, Fresh weight.

the three inceptin-related peptides with additional N-terminal amino acids, the C-terminal truncated $Vu\text{-In}^{-A}$ lacked ET-, SA-, and DMNT-inducing activity (Schmelz et al., 2007). Consistent with our previous findings, FAW accumulates mainly $Vu\text{-In}$ and only low amounts of $Vu\text{-In}^{-A}$ when fed peptide precursors containing the inceptin core sequence (Fig. 2A). In contrast, VBC OS contain largely the inactive $Vu\text{-In}^{-A}$ and only low levels of active $Vu\text{-In}$ (Fig. 2B). To examine if the VBC pattern of inceptin-related peptide processing in OS is unique, we compared VBC, FAW, and seven additional Lepidopteran pests: cabbage looper (*Trichoplusia ni*), tobacco budworm (*Heliothis virescens*), soybean looper (*Pseudoplusia includens*), black cutworm (*Agrotis ipsilon*), *S. exigua*, European corn borer (*Ostrinia nubilalis*), and *H. zea*. After feeding on cATPC precursors containing the

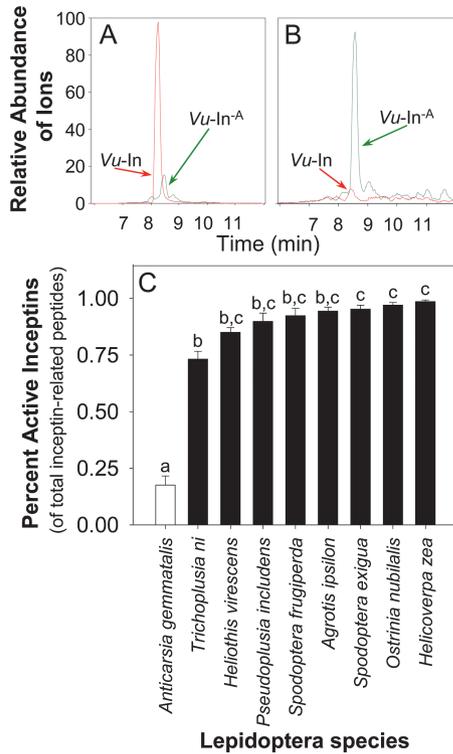


Figure 2. In contrast with other Lepidopteran pests, VBC OS contain predominantly the inactive inceptin $Vu\text{-In}^{-A}$. A and B, HPLC-MS selected $[M+H]^+$ mass-to-charge ratio ion trace of inceptin-related peptides $Vu\text{-In}$ (1,119.5) and $Vu\text{-In}^{-A}$ (1,048.5) in FAW OS (A) and VBC OS (B) following ingestion of the 19-mer- $Vu\text{-In}$ peptide precursor ($^+KGEICDINGVCVDAAEDEF^-$). C, Average ($n = 4$; \pm SE) percentage of active inceptins [(active = $Vu\text{-In}^{GE+}$ + $Vu\text{-In}^{E+}$ + $Vu\text{-In}$)/(total = $Vu\text{-In}^{GE+}$ + $Vu\text{-In}^{E+}$ + $Vu\text{-In}$ + $Vu\text{-In}^{-A}$)] quantified by HPLC-MS in the larval OS of eight different Lepidopteran species following ingestion of 4.5 nmol or less of 19-mer- $Vu\text{-In}$. Different letters above the bars (a–c) represent significant differences (ANOVA $P < 0.0001$; Tukey's test correction for multiple comparisons was at $P < 0.05$).

inceptin core sequence, only VBC exhibited significantly lower percentages (less than 25%) of active OS peptides (sum of $Vu\text{-In}$, $Vu\text{-In}^{E+}$, and $Vu\text{-In}^{GE+}$) compared with the total of all four inceptin-related peptides (Fig. 2C). Compared with the three highest accumulators of active inceptin-related peptides (*S. exigua*, *O. nubilalis*, and *H. zea*), *T. ni* larvae also exhibited a modest ability to accumulate $Vu\text{-In}^{-A}$ (Fig. 2C). While not a preferred host plant, in laboratory settings, *T. ni* is able to complete multiple instars on plants responsive to inceptin, such as common bean (Soo Hoo et al., 1984). Nonetheless, unlike the other eight Lepidopteran pests tested, VBC exhibits preferential processing of inceptin into the predominant inactive OS peptide $Vu\text{-In}^{-A}$.

$Vu\text{-In}^{-A}$ Is a Naturally Occurring Antagonist of Inceptin-Induced Responses

In plants, C-terminal amino acid deletions in synthetic peptide signals can result in the creation of

competitive antagonists of binding and inhibitors of elicitor-induced plant responses (Pearce et al., 1993; Meindl et al., 1998, 2000). To examine if $Vu\text{-In}^{-A}$ functions as a naturally occurring antagonist in cowpea, leaves were wounded and first treated with either water or $Vu\text{-In}^{-A}$ and then subsequently retreated with $Vu\text{-In}$ within 60 s. Prior treatment with 450 fmol leaf $^{-1}$ $Vu\text{-In}^{-A}$ completely suppressed the plant response to subsequent elicitation with an equal level of $Vu\text{-In}$ (Fig. 3A). At 4.5 pmol leaf $^{-1}$, consecutive treatments with $Vu\text{-In}^{-A}$ and $Vu\text{-In}$ resulted in ET responses identical to those of water followed by 45 fmol leaf $^{-1}$ $Vu\text{-In}$. Thus, in the presence of $Vu\text{-In}^{-A}$, a 100-fold greater level of $Vu\text{-In}$ was required to produce an equivalent plant response. This general pattern of antagonism occurred over a wide range of concentrations (Fig. 3A). To function as an antagonist of plant defense responses during herbivory, $Vu\text{-In}^{-A}$ must also exhibit this activity in a mixture of compounds applied simultaneously. In a second experiment, $Vu\text{-In}$ treatments were held constant (1 pmol leaf $^{-1}$) and mixed with increasing amounts of $Vu\text{-In}^{-A}$. At 33% $Vu\text{-In}$ (of the total $Vu\text{-In}$ + $Vu\text{-In}^{-A}$), significant inhibition of elicitor-induced ET responses occurred, with continually increasing inhibition as the proportion of $Vu\text{-In}^{-A}$ increased (Fig. 3B). To examine the linkage between $Vu\text{-In}^{-A}$ -mediated antagonism of $Vu\text{-In}$ -induced ET responses and subsequent metabolic leaf pools, a similar experiment was repeated to include sampling at 4 h. When leaves were treated with a mixture of 1 pmol of $Vu\text{-In}$ and 4 pmol of $Vu\text{-In}^{-A}$, significant ET inhibition at 1 h was again observed compared with an equivalent dose of $Vu\text{-In}$ alone (Supplemental Fig. S1A). Significantly decreased pools of SA and DMNT at 4 h tracked this reduced ET production (Supplemental Fig. S1, C and E). While averages trended lower, significant $Vu\text{-In}^{-A}$ antagonism of $Vu\text{-In}$ -induced JA and cinnamic acid (CA)

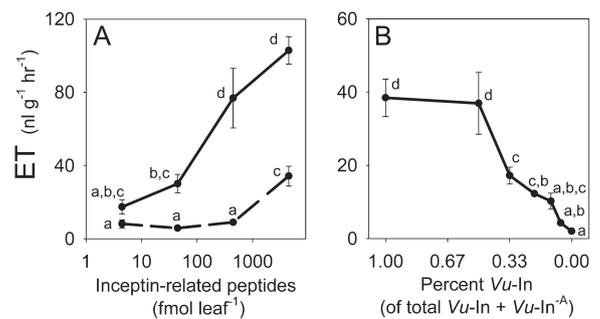


Figure 3. $Vu\text{-In}^{-A}$ is a natural antagonist of inceptin elicitation. A, Average ($n = 4$; \pm SE) ET production in damaged cowpea leaves treated first with either water (solid line) or $Vu\text{-In}^{-A}$ (dashed line) followed by a subsequent treatment 1 min later with $Vu\text{-In}$. Treatments involving both $Vu\text{-In}^{-A}$ and $Vu\text{-In}$ utilized equivalent paired doses separated by time. B, Average ($n = 4$; \pm SE) ET production in damaged cowpea leaves treated simultaneously with a fixed amount of $Vu\text{-In}$ (1 pmol leaf $^{-1}$) and increasing levels of $Vu\text{-In}^{-A}$ in the mixture. $Vu\text{-In}$ ranged from 100% to 5.8% and included a water control (0%). Different letters above the bars (a–d) represent significant differences (all ANOVA P values were $P < 0.0001$; Tukey's test corrections for multiple comparisons were at $P < 0.05$).

pools was not detectable at 4 h (Supplemental Fig. S1, B and D). As expected, individual $Vu\text{-In}^{-A}$ treatments did not result in plant responses different from damage + water alone in any metabolite measured (Supplemental Fig. S1, A–E). Collectively, these results demonstrate that elevated ratios of $Vu\text{-In}^{-A}$ in larval OS can mediate antagonized defense elicitation triggered by active inceptin-related peptides.

Biochemical Screen of Substituted Peptide Precursors Recovers Active Elicitors in VBC OS

Given that amino acid sequences influence protease susceptibility (Poreba and Drag, 2010), one strategy to minimize the production of antagonists and restore plant defense elicitation following VBC attack is to modify the inceptin C terminal. Subtle sequence changes have the potential reduce VBC production of $Vu\text{-In}^{-A}$ relative to active elicitors. Toward this goal, we examined if the C-terminal Ala of $Vu\text{-In}$ can withstand amino acid changes and still retain elicitor activity. As a first step, a $Vu\text{-In}^{\Delta X}$ substitution library was synthesized and assayed for induced ET production in cowpea leaves. With the exception of the basic amino acids, His, Lys, and Arg, as well as Pro and Trp, all other $Vu\text{-In}$ C-terminal substitutions maintained significant elicitor activity (Fig. 4A). To estimate elicitor stability following larval ingestion, all active 11-mer- $Vu\text{-In}^{\Delta X}$ C-terminal substituted peptides Asp, Asn, Gln, Ser, Thr, Val, and Tyr were then fed to VBC larvae and recollected from the OS. The $Vu\text{-In}^{\Delta X}$ C-terminal substituted peptides were recoverable from VBC OS at equal or greater levels relative to $Vu\text{-In}^{-A}$ and thus predictably sufficient to maintain elicitation (Figs. 3B and 4B). Lepidopteran larvae generate inceptin-related peptides through both N- and C-terminal proteolysis of larger cATPC precursors. Given this complexity, we examined the larval production of six substituted peptide candidates from their respective model 19-mer- $Vu\text{-In}^{\Delta X}$ precursors requiring amide bond cleavage for activation. Following ingestion by VBC and subsequent OS collection, the precursor 19-mer- $Vu\text{-In}^{\Delta V}$ (+KGEICDINGVCVDVAEDEF) displayed the highest recovery of the active peptide $Vu\text{-In}^{\Delta V}$ (+ICDINGVCVDV+) and a favorably low percentage of the $Vu\text{-In}^{-A}$ antagonist (Fig. 4C). Induced ET bioassays in cowpea leaves confirmed that both the native 19-mer- $Vu\text{-In}$ and substituted 19-mer- $Vu\text{-In}^{\Delta V}$ precursors are inactive and thus require insect activation (Fig. 4D). Using a combination of plant and insect assays, serial analyses of peptide substitution libraries revealed a conservative amino acid change that retains elicitor activity and enables the preferential accumulation in VBC OS.

A Single Amino Acid Substitution Recovers Plant Elicitation and Defense after Attack by a Legume Specialist Herbivore

Ideally, a modified $Vu\text{-In}$ analog would have either equivalent or greater activity than the native peptide

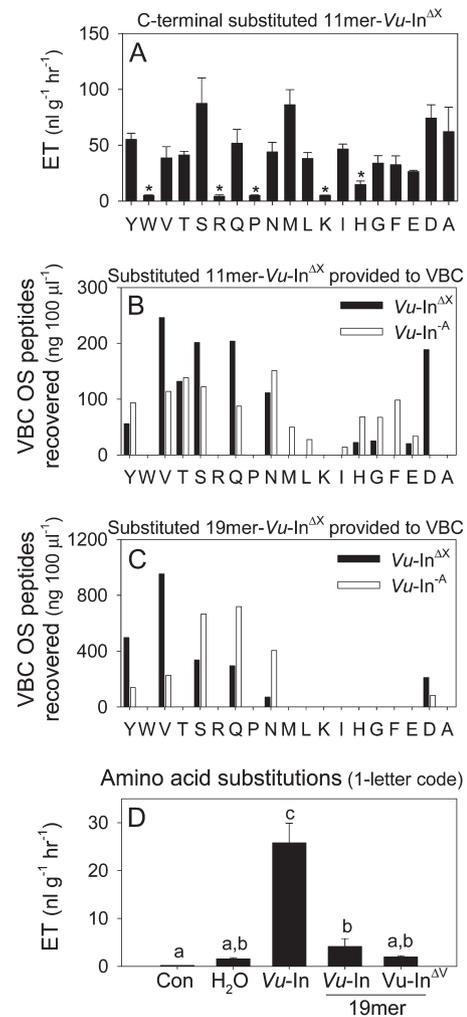


Figure 4. cATPC amino acid substitutions alter inceptin activity and processing in VBC OS. A, Average ($n = 3$; \pm SE) ET production in damaged cowpea leaves treated with 5 μL of water containing 10 pmol of C-terminally substituted peptides $Vu\text{-In}^{\Delta X}$ (+ICDINGVCVD Δ X). Capital letters (D through Y) denote the amino acid analogs of $Vu\text{-In}$. B and C, The 11-mer- $Vu\text{-In}^{\Delta X}$ peptides recovered from VBC OS (ng 100 μL^{-1}) after ingestion of active C-terminally substituted 11-mer- $Vu\text{-In}^{\Delta X}$ (B) and 19-mer- $Vu\text{-In}^{\Delta X}$ (C) analogs (+KGEICDINGVCVD Δ AEDEF). Black and white bars represent C-terminally substituted 11-mer- $Vu\text{-In}^{\Delta X}$ analogs and $Vu\text{-In}^{-A}$, respectively. D, Average ($n = 4$; \pm SE) ET production in undamaged cowpea leaves (Con) or those damaged and treated with 5 μL of water, $Vu\text{-In}$, 19-mer- $Vu\text{-In}$, or 19-mer- $Vu\text{-In}^{\Delta V}$, all at 10 pmol leaf⁻¹. Asterisks represent peptides with ET production significantly lower than $Vu\text{-In}$ (i.e. C terminal in A). Different letters above the bars (a–c) represent significant differences (all ANOVA P values were $P < 0.000$; Tukey's test correction for multiple comparisons was at $P < 0.05$).

signal. A detailed comparison of $Vu\text{-In}$ and $Vu\text{-In}^{\Delta V}$ revealed identical ET-inducing activities in cowpea leaves over a range of concentrations (Fig. 5A). Further analysis of plant metabolite levels 4 h later confirmed the equivalent activity of $Vu\text{-In}^{\Delta V}$ and $Vu\text{-In}$ (Supplemental Fig. S1, F–J). To quantitatively examine VBC peptide-processing patterns, larvae were allowed to feed upon

leaf discs containing 4.9 nmol of either 19-mer-*Vu*-In or 19-mer-*Vu*-In^{ΔV}. Subsequent collections of OS revealed 24 ± 7 and 146 ± 13 pmol of total inceptin-related peptides, respectively. Thus, in addition to 6-fold greater concentrations of inceptin-related peptides, VBC that consumed the 19-mer-*Vu*-In^{ΔV} contained a significantly higher percentage of active elicitors, whereas the antagonist predominated in larvae that consumed the native 19-mer-*Vu*-In (Fig. 5B). Importantly, as demonstrated previously (Fig. 3B), this low proportion (27%) of active inceptin peptides is sufficient to enable *Vu*-In^{-A} antagonism of *Vu*-In elicitation. To examine how trace amounts of substituted inceptin precursors affect plant recognition during herbivory, we fed individual VBC larvae cowpea leaf discs containing water, 0.5 nmol of the native 19-mer-*Vu*-In, or 0.5 nmol of 19-mer-*Vu*-In^{ΔV}. VBC larvae were then placed on intact cowpea plants and allowed to feed for 20 min. Individual herbivore damage sites 4 h later revealed significant increases in JA, SA, CA, and DMNT in plants attacked by VBC that had consumed the modified 19-mer-*Vu*-In^{ΔV} (Fig. 6, A–D). Plants attacked by VBC larvae that consumed the additional native 19-mer-*Vu*-In displayed defense-related metabolite levels statistically identical to those damaged by VBC from the water leaf disc treatment (Fig. 6, A–D).

To examine both the reproducibility and importance of dose, this experiment was repeated by feeding VBC larvae 5-fold greater levels of the 19-mer peptide precursors. At 2.5 nmol of peptide, herbivory triggered 2.1-, 4.1-, 2.6-, and 3.5-fold greater levels of JA, SA, CA, and DMNT, respectively, when VBC larvae previously ingested the modified 19-mer-*Vu*-In^{ΔV} compared with the native 19-mer-*Vu*-In (Fig. 6, E–H). Plant responses to larvae that previously consumed cowpea leaves plus water or 19-mer-*Vu*-In were not statistically different (Fig. 6, E–H). On average, the 2.5-nmol dose of 19-mer-*Vu*-In^{ΔV} resulted in larvae that elicited slightly higher plant responses than those previously ingesting 0.5 nmol (Fig. 6, A–H). Collectively, these results

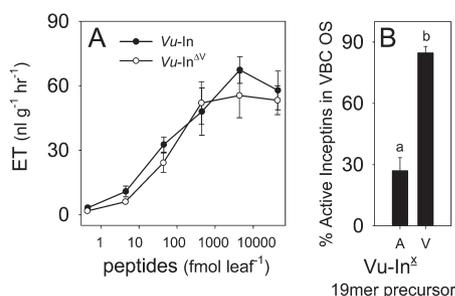


Figure 5. An inceptin Ala-to-Val C-terminal substitution maintains activity and enables the predominant accumulation of active elicitors in VBC OS. A, Dose response of average ($n = 4$; \pm SE) ET production in damaged cowpea leaves treated with either *Vu*-In or *Vu*-In^{ΔV}. B, Average ($n = 4$; \pm SE) percentage of active inceptins recovered from VBC OS after larvae were fed 4.9 nmol of either 19-mer-*Vu*-In or 19-mer-*Vu*-In^{ΔV}. Different letters above the bars (a and b) represent significant differences (Student's *t* test, $P < 0.001$).

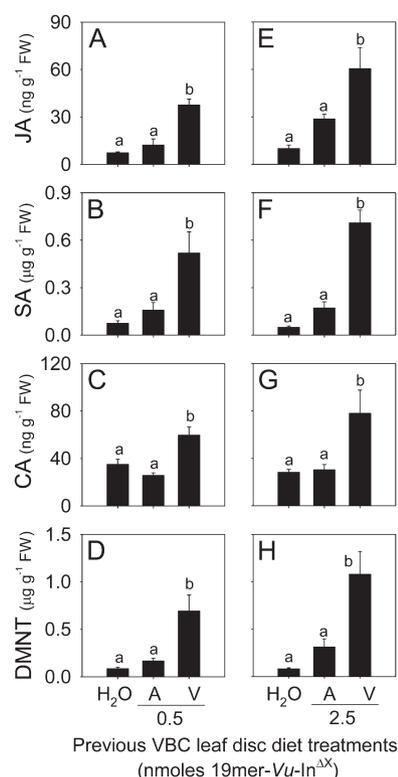


Figure 6. An Ala-to-Val substitution in cATPC polypeptides recovers induced plant defenses during VBC herbivory. A to D, Average ($n = 6$; \pm SE) concentrations of JA (A), SA (B), CA (C), and DMNT (D) 4 h after cowpea leaves experienced a single feeding bout from VBC larvae that had previously ingested cowpea leaf discs containing water, 0.5 nmol of 19-mer-*Vu*-In (A) or 0.5 nmol of 19-mer-*Vu*-In^{ΔV} (V). E to H, Average ($n = 6$; \pm SE) concentrations of JA (E), SA (F), CA (G), and DMNT (H) in a repeated experiment using 2.5 nmol of 19-mer-*Vu*-In (A) or 19-mer-*Vu*-In^{ΔV} (V). Within plots, different letters above the bars (a and b) represent significant differences (all ANOVA P values were $P < 0.015$; Tukey's test corrections for multiple comparisons were at $P < 0.05$). FW, Fresh weight.

demonstrate that an Ala-to-Val substitution in the cATPC polypeptide sequence is able to recover the elicitation of cowpea defense responses following VBC herbivory.

DISCUSSION

The identity and activity of herbivore-associated elicitors of plant defenses have been intensively studied over the past decade (Howe and Jander, 2008). In some cases, insects may avoid the production of active elicitors by feeding on tissues lacking essential precursors, while in other situations, individual herbivore elicitors within a class can vary greatly between conspecific larvae without significantly influencing defense activation (De Moraes and Mescher, 2004; Roda et al., 2004). While the outcome of pathogen and insect attack relies significantly upon their capacity for host plant utilization, the documentation of herbivore

effectors that suppress elicitation is quite limited. A few examples come from aphids that consume phloem for many hours once a sieve element feeding site has been established. In broad bean (*Vicia faba*), secreted salivary proteins from the aphid *Megoura viciae* suppress sieve element occlusion, which typically occurs through the formation of dispersed forisomes (i.e. expanded proteinaceous inclusions), triggered in response to damage and increased local Ca^{2+} levels (Will et al., 2007). These salivary-derived Ca^{2+} -binding proteins also promote dispersed forisomes to convert back into their original compact structure, thereby recovering the flow of phloem sap and enabling successful feeding. Similarly, a specific salivary protein of unknown function, designated as C002, has been demonstrated to be absolutely required for pea aphid (*Acyrtosiphon pisum*) feeding and survival (Mutti et al., 2008). Biochemical mechanisms that Lepidopteran larvae utilize to suppress plant responses during herbivory are less clear but can also involve salivary proteins. *H. zea* herbivory on tobacco results in secretion of the enzyme GOX onto the leaf surface and suppresses induced nicotine accumulation in tobacco (Musser et al., 2002). In cowpea, we here demonstrate a new mechanism of suppression, where legume specialist larvae exhibit the targeted removal of functional OS elicitors in part by converting the active signal into an antagonist of defense elicitation (Fig. 7).

It is unclear if generalist and specialist herbivores consistently differ in their OS elicitors. The transcriptional response of *N. attenuata* to attack from two generalist herbivores, *H. virescens* and *S. exigua*, was more similar to each other than that of the specialist *M. sexta*. This difference was linked to OS-derived FACs (Voelckel and Baldwin, 2004). OS contents of the two generalists are virtually identical, whereas *M. sexta* lacks volicitin and predominantly harbors fatty acid-Glu conjugates (Pohnert et al., 1999; Alborn et al., 2003). FACs from *M. sexta* also have a role in suppressing induced nicotine accumulation in tobacco, yet they remain elicitors for indirect defense responses (Kahl et al., 2000). Unlike a number of solanaceous plants, defense responses in cowpea are not elicited by FACs (Schmelz et al., 2009). Instead, variation within inceptin-related peptides from herbivore OS mediates the activation or suppression of elicitor-induced defenses. The evidence here for specialist antagonism of elicitor-induced plant defenses is further supported by the lack of preferential $Vu\text{-In}^{-A}$ processing in all generalist species examined (Fig. 2C). A future survey of legume specialist Lepidoptera that include *Vigna* spp. and *Phaseolus* spp. as frequent host plants would reveal the breadth and scope of this mechanism in nature.

The theoretical existence of a naturally occurring peptide signal that functions as an antagonist to elicitor-induced plant responses dates back nearly two decades. In 1991, discovery of the 18-amino acid endogenous peptide from tomato (*Solanum lycopersicum*) foliage, termed systemin, marked the first bioactive peptide signal found in plants (Pearce et al., 1991). Shortly

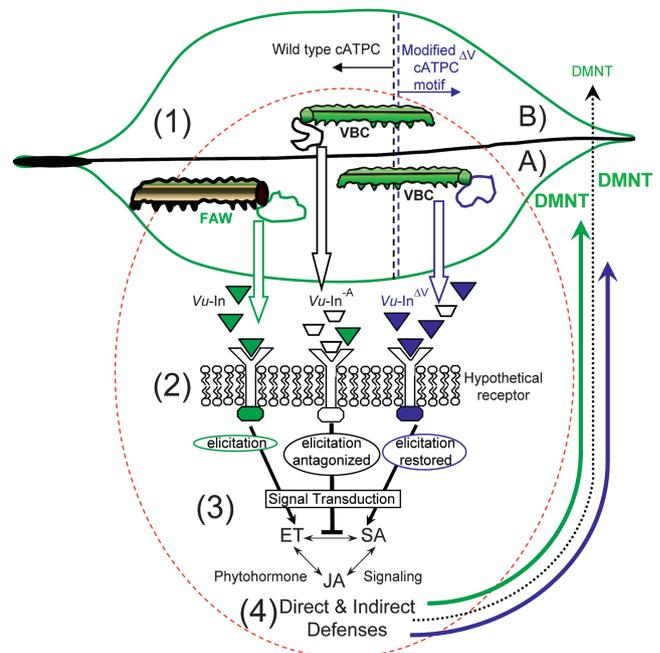


Figure 7. Simplified proposed model for generalist activation, specialist suppression, and engineered recovery of inceptin-elicited plant defenses in cowpea. 1A, FAW and VBC larvae consume cowpea leaves and produce the predominant cATPC digestive fragments $Vu\text{-In}$ and $Vu\text{-In}^{-A}$, respectively. 1B, VBC larvae that consume precursor proteins containing an altered $\text{cATPC}^{\Delta V}$ motif produce the active modified inceptin $Vu\text{-In}^{\Delta V}$. 2, Plants indirectly perceive larval attack when $Vu\text{-In}$ and $Vu\text{-In}^{\Delta V}$ peptides recontact the wounded leaf surface and bind a putative receptor. VBC that contain $Vu\text{-In}^{-A}$ at levels of 66% or greater of the total inceptin-related peptides mediate the antagonism of $Vu\text{-In}$ activity. As a preliminary hypothesis, $Vu\text{-In}^{-A}$ antagonism potentially occurs at the receptor-ligand level through competition. 3, Dependent upon the proportions of inceptin-related peptides present, multiple signaling pathways can be elicited, including the phytohormones JA, ET, and SA, or antagonized (ET and SA). 4, Insect OS elicitors drive quantitatively different levels of induced biochemical defenses such as DMNT that can provide reliable cues to facilitate the attraction of natural enemies.

thereafter, structure-function studies with truncated peptides revealed that the successive removal of C-terminal amino acids inactivated the signal and resulted in peptides that blocked the subsequent binding and activity of native full-length systemin (Pearce et al., 1993; Meindl et al., 1998). Related studies with the bacterial peptide elicitor flg22 also found that C-terminal truncations resulted in competitive antagonist peptides and led to the “address-message” concept that peptide signal N- and C-terminal ends are important for receptor binding and activation, respectively (Meindl et al., 2000). With both systemin and flg22, numerous C-terminal amino acid deletions in synthetic peptides are required to achieve strong competitive antagonism. In this study, a single C-terminal amino acid loss resulting in $Vu\text{-In}^{-A}$ promotes the antagonism of elicitation when present at 66% or more of the total inceptin-related peptides. While comparative phylogenetic

studies are consistent with the existence of specific plant receptors for insect-derived elicitors, the precise mechanism of *Vu*-In-mediated defense activation remains unknown (Schmelz et al., 2009). We identify *Vu*-In^{-A} as a truncated elicitor derivative that antagonizes inceptin-induced responses, including ET, SA, and DMNT; however, the action of *Vu*-In^{-A} as a competitive antagonist at the receptor level remains hypothetical and subject to future empirical examination (Fig. 7).

In contrast with plant-pathogen interactions, receptor-ligand pairs have yet to be identified to regulate plant resistance to insect attack. For aphid resistance, receptors including the *Mi-1.2* gene in tomato and *Vat* genes in melon (*Cucumis melo*) protect against specific biotypes of *Macrosiphum euphorbiae* and *Aphis gossypii*, respectively (Rossi et al., 1998; Boissot et al., 2010). Currently, aphid-associated ligands that bind these receptors are unknown. A common long-term threat in crop breeding is the eventual shift in predominant insect biotypes that favor those able to overcome plant resistance genes. For example, in 1997, a virulent isolate *M. euphorbiae* that performs well on tomato plants harboring the *Mi-1.2* gene was identified in California and currently appears to be predominant in the United States (Goggin et al., 2001). Of relevance to insect biotypes that overcome plant resistance, we demonstrate that VBC possesses a biochemical adaptation that minimizes the activation of plant defenses while feeding. The infrequency of predominant *Vu*-In C-terminal processing in other Lepidoptera species examined is likely due to the high level of protease resistance associated with cyclized peptides (Horton et al., 2002). While the precise mode of cleavage has not been described, VBC larvae may produce a specific gut carboxypeptidase that is capable of cleaving the C-terminal Ala from the largely cyclic *Vu*-In. Thus far, trypsin-like proteases have been predominantly described; however, the gut tissues of *A. gemmatalis* larvae also harbor bacteria potentially capable of further proteolysis (Oliveira et al., 2005; Visóttó et al., 2009).

In pathology terms, the presence of *Vu*-In in FAW OS is analogous to an "avirulence factor," given its ability to trigger rapid plant defense responses in cowpea. Conversely, *Vu*-In^{-A} in VBC OS functions as a "virulence factor" by suppressing elicitation and subsequent induced defenses triggered by trace amounts of *Vu*-In. Unfortunately, these designations are accurate only in the context of each individual system examined, given that virulence and avirulence factors often possess opposite activities in related plant species. Divergent activities of previously designated virulence and avirulence factors result in both confusion and conceptually restrictive usage. The term "effector" is now favored and broadly encompasses biochemicals that either positively or negatively modify plant defense signaling and expression (Hogenhout et al., 2009). The discovery of the elicitor antagonist activity of *Vu*-In^{-A} compels us to view inceptin-related peptides not simply as a class of elicitors but more widely as herbivore-associated effectors capable of multiple activities.

In cowpea, we demonstrate that a conservative amino acid change in the cATPC precursor for *Vu*-In can block the production of an elicitor antagonist and recover elicitor-induced responses following VBC attack (Fig. 7). Curiously, potential mutations that convert precursors of *Vu*-In to *Vu*-In^{AV} may be subject to additional constraints. In an earlier analysis of the coding region of chloroplastic *atpC* spanning *Vu*-In, we observed perfect conservation of the C-terminal Ala in every plant species investigated (Schmelz et al., 2006). This might be due to interactions with the inhibitory ϵ -subunit that in part mediates chloroplastic ATP synthase activity (Hightower and McCarty, 1996). Thus, in legumes that respond to inceptin-related peptides, the transgenic manipulation of plants to improve defense responses to VBC attack may require the embedding of this modified sequence into proteins other than cATPC. An ultimate goal is to impart inceptin-mediated responses to crops that do not currently recognize the elicitor. While this approach has numerous daunting requirements, including the discovery of any putative inceptin receptor(s), successful examples of interfamily resistance transfer exist for bacterially mediated MAMP-triggered immunity (Lacombe et al., 2010). Encouragingly, the majority of Lepidoptera species examined accumulate inceptin-related peptides (Fig. 2C). Thus, while the utility of this perception system hinges partly on elicitor production, active signals already exist in a diversity of pests. Select legume specialists largely avoid inceptin-mediated defenses in cowpea; yet, an amino acid substitution, representing the addition of two methyl groups, can recover elicitor-induced responses. Clearly, little things mean a lot. Detailed biochemical knowledge of both herbivore-associated effectors and the respective plant receptors will remain essential for the directed improvement of crop resistance.

MATERIALS AND METHODS

Plant and Insect Materials

FAW (*Spodoptera frugiperda*) larvae were obtained from Dr. R. Meagher (Center of Medical, Agricultural, and Veterinary Entomology, U.S. Department of Agriculture, Agricultural Research Service, Gainesville, FL) and reared on a pinto bean (*Phaseolus vulgaris*)-based diet (Schmelz et al., 2006). Preliminary trials with VBC larvae utilized wild-caught individuals from outbreak areas on the surrounding margins of peanut (*Arachis hypogaea*) fields (Williston, FL), and these results were readily replicated using commercially obtained insects. All experiments utilized VBC (*Anticarsia gemmatalis*), *Trichoplusia ni*, *Heliothis virescens*, *Pseudoplusia includens*, *Agrotis ipsilon*, *Spodoptera exigua*, *Ostrinia nubilalis*, and *Helicoverpa zea* obtained on from Benzon Research. Cowpea (*Vigna unguiculata* var California Blackeye no. 5; The Wax Company) was germinated in MetroMix 200 (Sun Gro Horticulture) supplemented with 14-14-14 Osmocote (Scotts Miracle-Gro). All plants were maintained in a greenhouse with a 12-h photoperiod, a minimum of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation supplied by supplemental lighting, 70% relative humidity, and a temperature cycle of 24°C/28°C (night/day).

Synthetic Peptides

Inceptin-related sequences *Vu*-GE+In, *Vu*-E+In, *Vu*-In, and *Vu*-In^{-A} were synthesized and purified (more than 95%) at the Protein Core Chemistry Facility of the University of Florida, Gainesville, as described previously (Schmelz et al.,

2006, 2007). C-terminally substituted *Vu*-In 11-mer analogs were synthesized using the PEPscreen Peptides (Sigma-Genosys) service with an 85% average purity. *Vu*-In^{ΔV} and specific substituted 19-mers ("KGEICDINGVCVDXAEDF") utilized in this work were synthesized and received at more than 95% purity (Sigma-Genosys).

Cowpea Leaf Bioassays and Plant Metabolite Analysis

All experiments used 14- to 18-d-old plants containing two fully expanded pairs of trifoliolate leaves. For ET 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 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 water were immediately applied and dispersed over the damage sites. For ET quantification, leaves remained on the intact plants for 1 h, were excised, sealed in 13-mL tubes, and analyzed via head space sampling and gas chromatography as described previously (Schmelz et al., 2006). To quantify leaf tissue pools of DMNT, JA SA, and CA, a 4-cm² section of leaf surrounding the treated site was weighed (50–100 mg), frozen in liquid N₂, processed, and analyzed by gas chromatography isobutane-chemical ionization mass spectrometry as described elsewhere (Schmelz et al., 2004, 2006). The collection and quantification of leaf volatile emission from intact plants followed established protocols (Carroll et al., 2008).

Individual Insect Feeding Studies

Sixth instar VBC and FAW larvae were allowed to feed for 12 h on cowpea leaves and then individually isolated on polystyrene 12-well Cell Culture Plates (BD Falcon) for 1 h in the absence of plant tissue. The larvae were carefully transferred onto the leaf canopies of tightly spaced cowpea plants and allowed to feed for 20 min. At this time, all larvae were removed from plants, and only insect-damaged sites with comparable leaf area consumed (typically 50 mm²) were selected for analysis (*n* = 6). To ensure maximal uniformity of feeding time and surplus of larval wound sites with equivalent leaf area removed, each of these trials utilized groups of 48 insects on 16 plants for each insect species or treatment comparison. To control the elicitor content of larval OS, VBC on 12-well plates were allowed to consume a 65-mm² cowpea leaf disc containing the native and substituted 19-mer cATPC peptides. Within 30 min of consumption, larvae were placed on leaves as described above.

Quantification of Inceptin-Related Peptides in Insect OS

Chemical verification and quantification of inceptin-related peptides from insect OS utilized HPLC-mass spectrometry (MS) as described previously (Schmelz et al., 2006). An isotopically labeled ([¹³C]Val and [¹⁵N]Val; +ICD-ING-V*-CVDA*) *Vu*-In analog was used as an internal standard for quantification. Aliquots of crude OS, typically 50 to 100 μL derived from a pool of 12 larvae, were sequentially spiked with 100 ng of the internal standard peptide and 5 μL of HCl, vortexed, and centrifuged at 12,000g for 5 min. The aqueous phase was mixed with an equal volume of ethanol, stored at -70°C for 30 min, and centrifuged at 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 CH₃CN:water. Samples were then concentrated to dryness under vacuum and brought up in 50 μL of 5:95 CH₃CN:water containing 10 mM NH₄COOH. Using an HPLC system composed of a P4000 pump, an AS3000 autosampler, and a UV6000LP detector (Thermo Separation Products), 10-μL OS samples were injected onto a YMC ODS-AQ RP-C18 (250 × 4.6 mm, 5–5 μm, 20 nm; Waters) analytical column heated to 60°C, using a flow rate of 1 mL min⁻¹, with mobile phases A and B containing 95:5 water:CH₃CN and 9:1 CH₃CN:water, respectively, as well as 10 mM NH₄COOH buffer. The post-column eluent was split, allowing 0.1 mL min⁻¹ to enter the ion source for positive-ion MS using a LCQ Deca XP MAX ion trap (Thermo Electron) as described previously (Schmelz et al., 2006). Chemical verification was based on comparing retention times and MS² daughter ion mass spectra of synthetic standards against natural products. Quantification was based on extracting [M+H]⁺ ions from full-scan spectra using the following common ion-analyte pairs: 1,125.5 (internal standard), 1,048.5 (*Vu*-In^{-A}), 1,119.5 (*Vu*-In), 1,248.5 (*Vu*-E⁺In), 1,305.5 (*Vu*-GE⁺In), 1,147.5 (*Vu*-In^{ΔV}), 1,276.5 (*Vu*-E⁺In^{ΔV}), and 1,333.5 (*Vu*-GE⁺In^{ΔV}). A complete list of all amino acid-substituted peptides used in this study and analyzed for in insect OS via HPLC-MS is given in Supplemental Table S1.

Lepidoptera Species Comparison

The OS were collected and pooled from groups of six larvae (*n* = 4) for inceptin quantification. Early sixth instar larvae (*n* ≥ 48) were removed from diet and isolated for 1 h prior to feeding on leaf discs (65 mm²) containing 4.5 nmol of 19-mer-*Vu*-In. OS were collected and frozen immediately after the larvae consumed at least 50% of the treated leaf disc, which required between 30 and 120 min depending on the species. With the exception of *A. ipsilon*, *O. nubilalis*, and *T. ni*, which utilized either maize (*Zea mays*) or bok choy (*Brassica rapa*) leaves, all other species were fed cowpea leaves. Resulting levels of the four inceptin-related peptides *Vu*-In^{-A}, *Vu*-In, *Vu*-E⁺In, and *Vu*-GE⁺In were then quantified by LC-MS as described.

Determination of *Vu*-In^{-A} Inceptin Antagonist Activity

Four paired groups of cowpea leaves (*n* = 4; 32 total) were wounded and treated with either 5 μL of water or *Vu*-In^{-A} at 0.0045, 0.045, 0.45, and 4.50 pmol leaf⁻¹. One minute later, both leaves in each paired group were treated with a secondary application of *Vu*-In at 0.0045, 0.045, 0.45, and 4.50 pmol leaf⁻¹. To assess the simultaneous application of *Vu*-In^{-A} and *Vu*-In, all peptide-treated leaves (*n* = 4) received 10 μL of water containing 1 pmol of *Vu*-In in the presence of 0, 1, 2, 4, 8, or 16 pmol of *Vu*-In^{-A}. A damage + water-only control group was included. All leaves were excised after 1 h and analyzed for ET production. To examine *Vu*-In^{-A} antagonism of *Vu*-In activity in the context of leaf metabolites at 4 h, a subset of these treatments was reexamined. Cowpea leaves (*n* = 4; 48 total) were wounded and treated with 5 μL of water, 1 pmol of *Vu*-In, *Vu*-In + *Vu*-In^{-A} (1:1; 1 pmol leaf⁻¹ for each peptide), or *Vu*-In + *Vu*-In^{-A} (1:4; 1 and 4 pmol, respectively), or 1 pmol of *Vu*-In^{-A} alone. To address the similarity of *Vu*-In^{ΔV} activity to *Vu*-In, leaves were also treated with 1 pmol of *Vu*-In^{ΔV}. For each of the six treatments, one set of leaves (24) was analyzed at 1 h for ET confirmation and the other set (24) was analyzed at 4 h for JA, SA, CA, and DMNT pools.

Screening Activity and Stability of Substituted Inceptins in VBC OS

With the exception of Cys, which would have complicated peptide cyclization, a C-terminal substituted peptide of *Vu*-In library was first analyzed by LC-MS for purity, normalized for concentration, and tested at 10 pmol leaf⁻¹ for E-inducing activity in cowpea. *Vu*-In C-terminal substituted peptides with activity similar to *Vu*-In were then fed to 14 groups of 12 VBC larvae (20 μg leaf disc⁻¹), and OS were collected 6 h later and pooled for HPLC-MS analysis. To examine VBC proteolytic processing of precursors into inceptin-related peptides, six of the more promising *Vu*-In^{ΔX} C-terminal substituted peptides (where X = D, N, Q, S, V, Y) were synthesized as 19-mers ("KGEICDINGVCVDXAEDF"). Similarly, the substituted 19-mers were fed to six groups of 12 VBC larvae (20 μg leaf disc⁻¹), and within 1 h of consumption, the OS were collected and pooled for HPLC-MS analysis.

Data Analysis

ANOVAs were performed on quantified inceptin-related peptides, phytohormones, leaf tissue metabolites, and volatile emissions of DMNT. Significant treatment effects were investigated when the main effects of the ANOVAs were significant (*P* < 0.05). Where appropriate, Tukey's tests were used to correct for multiple comparisons between control and treatment groups. Before statistical analysis, all nonpercentage data were subjected to square root transformation to compensate for elevated variation associated with larger mean values. The analysis was accomplished with JMP 4.0 statistical discovery software (SAS Institute).

Supplemental Data

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

Supplemental Figure S1. *Vu*-In^{-A} antagonism of inceptin-induced ET at 1 h parallels subsequently observed patterns for SA and DMNT at 4 h.

Supplemental Table S1. Native and modified inceptin-related peptides used for ethylene bioassays, larval feeding studies, and HPLC-MS-based quantification.

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