**Main Manuscript for**

A Receptor-Like Protein Mediates Plant Immune Responses to Herbivore-Associated Molecular Patterns

**Keywords:** Herbivory, LRR-RLP, PRR, HAMP, receptor

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Main Text

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**Abstract:**

Herbivory is fundamental to the regulation of both global food webs and the extent of agricultural crop losses. Induced plant responses to herbivores promote resistance and often involve the perception of specific herbivore-associated molecular patterns (HAMPs); however, precisely defined receptors and elicitors associated with herbivore recognition remain elusive. Here, we show that a receptor confers signaling and defense outputs in response to a defined HAMP common in caterpillar oral secretions (OS). Staple food crops, including cowpea (*Vigna unguiculata*) and common bean (*Phaseolus vulgaris*), specifically respond to OS via recognition of proteolytic fragments of chloroplastic ATP synthase, termed inceptins. Using forward-genetic mapping of inceptin-induced plant responses, we identified a corresponding leucine-rich repeat receptor, termed INR, specific to select legume species and sufficient to confer inceptin-induced responses and enhanced defense against armyworms (*Spodoptera exigua*) in tobacco. Our results support the role of plant immune receptors in the perception of chewing herbivores and defense.

**Significance statement:**

Plants respond to biotic attack using an immune system of receptors to recognize molecules associated with danger. We identified an immune receptor, termed Inceptin Receptor (INR), able to confer responses to defined inceptin peptide fragments present in caterpillar oral secretions. Like many plant immune receptors, INR is encoded only by certain plant species, but can be transferred across families to confer new signaling and defense functions. While INR is only found in the genomes of cowpea, common bean and related legumes, it confers defined elicitor responses to transgenic tobacco and suppresses the growth of attacking beet armyworm larvae. INR expands the breadth of plant pattern recognition receptors to detection of chewing insect herbivores.

**Introduction:**

The global balance between autotroph and heterotroph biomass is dictated by a nearly immeasurable number of plant-herbivore interactions. Similarly, historic pest challenges and modern herbivore invasions still threaten global food security [(1–3)](https://paperpile.com/c/gQ0VBZ/Epy4R%2BX19lW%2BNqBpv). Plant resilience in both natural and agricultural settings is mediated by inducible biochemical defenses against herbivores. Importantly, the nature and magnitude of plant responses is often amplified by specific biochemical elicitors, termed herbivore-associated molecular patterns (HAMPs), associated with attackers [(4, 5)](https://paperpile.com/c/gQ0VBZ/vW7uh%2BmWUf3). Despite the critical need for a mechanistic understanding of induced defense responses, specific receptors perceiving HAMPs have remained elusive [(6)](https://paperpile.com/c/gQ0VBZ/Qxp9k).

 Plants can specifically perceive Lepidopteran herbivores by sensing HAMPs in oral secretions (OS) and frass [(7, 8)](https://paperpile.com/c/gQ0VBZ/teX19%2BynrU6). Among defense-eliciting molecular patterns, inceptins are a potent bioactive series of proteolytic fragments derived from host chloroplastic ATP synthase γ-subunit (cATPC) and found in the OS of all examined Lepidopteran species [(8–10)](https://paperpile.com/c/gQ0VBZ/ynrU6%2BITjW4%2BCqG4W). The dominant inceptin present during caterpillar herbivory on cowpea (*Vigna unguiculata*) is an 11-amino acid (AA) peptide, termed *Vu-*In (+ICDINGVCVDA-). The *Vu-*In epitope is highly conserved among plant cATPC sequences; however, only species within the legume subtribe Phaseolinae respond to inceptins [(11)](https://paperpile.com/c/gQ0VBZ/g9879).

In plants, both non-self and modified-self extracellular peptide signals can be recognized by specific pattern recognition receptors (PRRs) [(12, 13)](https://paperpile.com/c/gQ0VBZ/5rZRz%2BQjP3Z). We hypothesized that legumes encode an inceptin receptor (INR) enabling *Vu-*In recognition, analogous to perception of pathogen-associated molecular patterns (PAMPs) by receptor kinases (RKs) and receptor-like proteins (RLPs) [(12, 14)](https://paperpile.com/c/gQ0VBZ/fjLSl%2B5rZRz). Here we identify and characterize closely related RLP-encoding genes from legumes in subtribe Phaseolinae that confer signaling and defense outputs in response to a specific HAMP elicitor in caterpillars.

**Results:**

To identify INR candidates, we examined plant response variation to both *Vu*-In and a less bioactive C-terminal truncated inceptin, termed *Vu*-In-A (+ICDINGVCVD-), found in the OS of a legume specialist herbivore (*Anticarsia gemmatalis*) [(10)](https://paperpile.com/c/gQ0VBZ/CqG4W). Anticipating an arms race pattern of evasion and re-establishment consistent with other elicitors [(15, 16)](https://paperpile.com/c/gQ0VBZ/c7qq7%2BLONsj), we screened cowpea germplasm for positive *Vu*-In-A elicited responses. Accessions Danila, Suvita, and Yacine displayed induced ethylene production after applying *Vu*-In-A to wounded leaves, while other accessions failed to respond (Fig. 1a). Although responses to *Vu*-In-A were quantitatively low compared to fully active *Vu*-In (Fig. 1a), we reasoned that the existence of qualitative response variation to the weaker elicitor variant could be mediated by *INR* genetic variation.

To map *INR,* we used a biparental population of 85 recombinant inbred lines (RILs) derived from a cross between accessions Yacine(*Vu*-In-A responsive) and 58-77 (*Vu*-In-A unresponsive) (Fig. 1a) for QTL mapping, as well as a panel of 364 cowpea accessions belonging to the UC Riverside Minicore collection for a genome-wide association study (GWAS) [(17)](https://paperpile.com/c/gQ0VBZ/5TpuH). *Vu*-In-A elicited variable ethylene production across accessions (Fig. S1, Table S1, Table S2). Using QTL mapping and GWAS, we observed that *Vu*-In-A responses strongly associated with a single genetic locus in both populations (Fig. 1b, 1c, Fig. S2). In contrast to qualitative variation in plant responses to *Vu*-In-A, quantitative variation in response to *Vu*-In resulted in different candidate loci that did not meet statistical thresholds for co-association between QTL and GWAS efforts (Fig. S2), were less well supported and thus not pursued further. The most highly-associated markers with *Vu*-In-A response in both QTL mapping and GWAS were SNPs 2\_22560, 2\_22561, and 2\_09070 [(18)](https://paperpile.com/c/gQ0VBZ/Dj6Mt), spanning a 22-kb region on chromosome 7 (Fig. 1d, Fig. S1). Both markers 2\_22560 and 2\_22561 fell within a RLP-encoding gene *Vigun07g219600,* consistent with a potential role in receptor-mediated inceptin responses.

To examine function of the INR candidate, we transiently expressed *Vigun07g219600* from the reference accession IT97K-499-35 [(19)](https://paperpile.com/c/gQ0VBZ/a84EL) in *Nicotiana benthamiana*,and tested responsiveness to *Vu*-In. Hallmarks of receptor-mediated defense responses include a burst of reactive oxygen species (ROS) and ethylene production. As a positive control, transient expression of the *EF*-Tu receptor (EFR) [(20)](https://paperpile.com/c/gQ0VBZ/lBOQL) in *N. benthamiana* conferred responses to elf18 peptide, but not *Vu-*In (Fig. 2a). Expression of *Vigun07g219600* selectively conferred *Vu-*In induced ROS (Fig. 2b,c) and ethylene production (Fig. 2d) to *Vu*-In but not elf18, supporting the hypothesis that *Vigun07g219600* encodes a functional INR.

To understand the basis of phenotypic variation in cowpea, we cloned and expressed *INR* alleles from 6 accessions with differential *Vu*-In-A responses (Table S2). *Vu*-In-A response variation originally observed in cowpea corresponded with *INR* allelic strength, as only *INR* alleles from *Vu*-In-A responsive cowpea accessions conferred significant *Vu*-In-induced ROS and ethylene production in *N. benthamiana* (Fig. 2e, Figs. S3, S4). Both active and inactive RLP variants tagged with GFP co-localized with the plasma membrane marker PIP2A-mCherry (Fig. S5). Interestingly, none of the tested alleles conferred *Vu*-In-A  responses in *N. benthamiana* (Fig. S4). Given that all 364 tested cowpea accessions respond to *Vu*-In (Table S1), our data support a model where cowpea natural variation in INR specifies an activation threshold for the weak elicitor, *Vu*-In-A. When expressed heterologously in *N. benthamiana*, the same allelic series cannot confer responses to *Vu*-In-A but is instead differentially activated by the stronger *Vu*-In elicitor(Fig. S6)*.*

INR is a leucine-rich repeat (LRR)-RLP, a receptor class distinguished from LRR-RKs by lack of an intracellular kinase domain [(13)](https://paperpile.com/c/gQ0VBZ/QjP3Z). It contains 29 semi-regular LRRs with intervening motif, preceding a transmembrane domain and short cytosolic segment (Fig. S7). The *INR* locus in cowpea contains a paralog *Vigun07g219700* (72% AA similarity) that is unable toconfer *Vu*-In induced ethyleneproduction when expressedin *N. benthamiana* (Fig. S8). In contrast, orthologs with >90% AA similarity, *Phvul.007G077500* (from *P. vulgaris*)and *Vradi08g18340* (from *Vigna radiata*), conferred *Vu-*In-inducedethylene production (Fig. S8). Notably, genome-sequenced *P. vulgaris* accession G19833 contains a single RLP receptor at the *INR* locus (Fig. 1d) and responds robustly to inceptin (Fig. S9), excluding a strict requirement for duplicated receptor genes in responsive varieties. In more distantly related soybean (*Glycine max)*, four syntenic homologs share 73-76% similarity to cowpea INR-*Vu* (Fig. S7). Neither of two tested soybean homologs (*Glyma.10G228000* or *Glyma.10G228100*) enabled *Vu-*In*-*induced ethylene production (Fig. S8). Soybean plants are both unresponsive to inceptin [(11)](https://paperpile.com/c/gQ0VBZ/g9879) and phylogenetic analysis of its four syntenic homologs showed that they fall outside the subclade of functional *INR* genes(Fig. S8). We conclude that a subtribe of legume species including *Phaseolus* and *Vigna* uniquely encode functional INRs sufficient to confer HAMP-induced responses in tobacco.

In plants, LRR-RLPs can either directly bind ligands or can instead modulate the binding activities of other LRR-RKs [(21, 22)](https://paperpile.com/c/gQ0VBZ/fNed1%2BAluAk). Unlike modulating RLPs, which display clear orthologs across plant families and contain comparatively short ectodomains [(23)](https://paperpile.com/c/gQ0VBZ/XomLd), INR shares structural and phylogenetic features common in Arabidopsis and tomato ligand-binding receptors [(24–26)](https://paperpile.com/c/gQ0VBZ/Q3sqc%2B8jMCW%2B7d4LY) including a large ectodomain, an intervening motif [(27)](https://paperpile.com/c/gQ0VBZ/qbOmH), and membership in a large clade-specific gene family (Fig. S10). Our data can not exclude the presence of a latent inceptin receptor endogenous to tobacco, which, upon activation by heterologous expression of *Vigun07g219600* or its homologs, allows *Vu-*In-induced responses. However, the most parsimonious model is that legume-specific perception of inceptin peptides is mediated by INR as a lineage-specific RLP.

To examine inceptin binding *in vitro*, INR was expressed heterologously in insect cells and purified but interpretation was confounded by predominant aggregation of the protein product (Fig. S11). As an alternative method to obtain support for *in planta* peptide binding activity to INR, we generated an N-terminal acridinium-tagged *Vu-*In conjugate and measured retention of luminescence signal by immunoprecipitated, semi-purified INR-GFP expressed in *N. benthamiana* leaves. Acri-*Vu*-In was similarly bioactive in eliciting ethylene release in cowpea (Fig. S12). Immunoprecipitated INR-*Vu* retained acridinium-*Vu-*In luminescent signal, while an unrelated RLP control, Phvul.007g087600 showed no retained signal (Fig. 3a). Pre-incubation with excess unlabeled *Vu-*In, but not flg22, competed acridinium-derived signal, supportive of specific binding (Fig. 3a-c). Both *Vu*-In and *Vu*-In-A compete for acridinium-*Vu*-In retention at concentrations from 50-500 nM (Fig. 3b).

To better understand potential sites that could mediate direct physical interactions of INR with inceptin, we constructed a homology model of INR based on the crystal structure of the LRR ectodomain of FLAGELLIN-SENSING 2 (FLS2) [(28)](https://paperpile.com/c/gQ0VBZ/mwuHi), and performed *Vu*-In docking simulations. The predicted lowest-energy conformations were ranked by peptide binding scores. In multiple low-energy conformations of *Vu*-In, the ligand acidic residue Asp10 showed a conserved binding position and conformation by interacting with both basic INR-*Vu* residues His495 and Arg497 (Fig. 3d, Fig. S12). Consistent with a predicted role in binding, a previous Ala substitution study demonstrated that Asp10 was the only AA essential for *Vu*-In elicited ethylene production [(9)](https://paperpile.com/c/gQ0VBZ/ITjW4). To examine the predicted interaction, we substituted INR-*Vu* at His495/Arg497 to Ala495/Ala497, which resulted in loss of both acridinium-*Vu-*In retention and *Vu*-In-induced ROS production in *N. benthamiana* (Fig. 3d, Fig. S12)*.* Substituted INR co-localized with plasma membrane marker PIP2A-mCherry (Fig. S5) and was present in biochemical plasma membrane fraction (Fig. S12). We further investigated the role of His495 and Arg497 through charge swap substitutions to Asp and Asp/Glu respectively; however, these combinations were not sufficient to confer responsiveness to a respectively charge substituted *Vu-*In peptide (Asp10 → Lys/Arg) (Fig. S13). His495 and Arg497 are conserved in several *Vigna* and *Phaseolus* INR homologs but not in non-functional soybean RLP homologs (Fig. S14), consistent with a role in inceptin recognition with other conserved features of INR controlling signaling outputs. Our data show that INR-*Vu* is sufficient to confer acridinium-*Vu*-In retention in tobacco, and that this activity is in part mediated by His495/Arg497.

Plant LRR-type surface receptors typically associate with Somatic Embryogenesis Receptor Kinase (SERK) co-receptors for signal transduction [(29)](https://paperpile.com/c/gQ0VBZ/ZMlDu). In addition, characterized RLPs constitutively associate with the adapter RK Suppressor of BIR1 (SOBIR1) [(30)](https://paperpile.com/c/gQ0VBZ/lo41R). We tested if INR associates with Arabidopsis and cowpea orthologs of SOBIR1 by co-immunoprecipitation (co-IP). Association of INRwith both AtSOBIR1 and VuSOBIR1 (*Vigun09g096400*) was constitutive (Fig. 3e), while INR associated more strongly with AtSERK co-receptors after peptide treatment (Fig. 3f, 3g). Likewise the co-IP of SERKs requires INR (Fig. S15). Thus, INR associates with co-receptor and adapter RKs in a manner similar to characterized LRR-RLPs.

To test if INR is sufficient to enhance anti-herbivore defenses in plants lacking native inceptin responses, we stably transformed *N. benthamiana* and *N. tabacum* with either 35S:INR-*Vu* or 35S:INR-*Pv* transgenes. Multiple independent transgenic lines expressing 35S:INR responded to *Vu*-In as measured by induced ethylene and induced peroxidase activity as a direct defense output (Fig. 4a, 4b, Fig. S16). Transcriptomic characterization of *Vu-*In induced responses in *N. benthamiana* INR-*Pv* line 1-5 showed upregulation of characteristic anti-herbivore defense genes (Fig. S17). We confirmed that two classical defense markers, a Kunitz trypsin inhibitor (KTI) [(31)](https://paperpile.com/c/gQ0VBZ/9xkKT) and ascorbate oxidase (AscOx) [(32)](https://paperpile.com/c/gQ0VBZ/Hb3wL), were upregulated in the presence of both INR and *Vu*-In application (Fig. 4c, Fig. S18).

We challenged INR-expressing tobacco lines with second instar larvae of the generalist Lepidopteran herbivore, beet armyworm (*Spodoptera exigua*). Both *N. benthamiana* and *N. tabacum* harbor a substitution in the inceptin precursor cATPC (V256N), creating a *Nb/Nt-*In peptide upon proteolytic processing. We confirmed both the presence of *Nb/Nt-*In in *Spodoptera* OS after consumption of tobacco at similar levels to *Vu-*In, and the bioactivity of *Nb*/*Nt-*In on plants expressing INR-*Vu* (Fig. S19). Consistent with INR enabling recognition of inceptin peptides, caterpillars displayed 32-37% lower Relative Growth Rates on transgenic lines than on wild-type (WT) plants when caged on either *Nicotiana* species for 4 days (Fig. 4d, 4e). A similar magnitude of caterpillar growth reduction was previously observed after pretreatment of cowpea with *Vu-*In [(8)](https://paperpile.com/c/gQ0VBZ/ynrU6). Our data supports that the heterologous expression of either INR-*Vu* or INR-*Pv* can confer enhanced anti-herbivore defense responses.

**Discussion:**

Plant recognition of modified-self and non-self patterns enables increased resistance to diseases and pests [(12)](https://paperpile.com/c/gQ0VBZ/5rZRz). Immune recognition of specific PAMPs is often mediated by pattern recognition receptors (PRRs), but defined HAMP receptors have remained largely unknown. Here we describe INR, a LRR-RLP family protein sufficient to confer signaling and defense responses to precisely defined HAMPs. Our work builds on previous findings implicating cell surface signaling elicited by HAMPs. Plant responses to specific HAMPs can be reduced when candidate receptors or downstream pathway components are silenced [(33–36)](https://paperpile.com/c/gQ0VBZ/9jC71%2BU5kYW%2BjoJFl%2BkoqW6). Similarly, a fatty acid amide HAMP elicitor was shown to bind maize plasma membrane preparations [(37)](https://paperpile.com/c/gQ0VBZ/diG0g). We demonstrate that INR confers retention of acridinium-*Vu*-In signal when expressed in tobacco (Fig. 3a-c) and identify AA residues that mediate both signaling and acridinium-*Vu*-In retention (Fig. 3d, Fig. S12). Collectively, our data is consistent with a role for INR as a genuine inceptin receptor. Further technical advances in LRR *in vitro* biochemistry will be needed to generate additional support and evidence for direct INR-inceptin binding interactions.

INR is only found in certain non-model legume species and is consistent with lineage-specific HAMP perception in plant families [(11)](https://paperpile.com/c/gQ0VBZ/g9879). Our findings are consistent with the majority of demonstrated PRRs in the RLP gene family, which often belong to large lineage-specific clades [(24)](https://paperpile.com/c/gQ0VBZ/Q3sqc). Consistent with a similar PRR function in legumes, we observed natural variation in strength of INR alleles (Fig. 2e, Fig. S4), the restricted presence of functional INR homologs in select, *Vu*-In responsive legume genomes (Fig. 2a, Fig. S8), and shared structural and phylogenetic features of INR with demonstrated lineage-specific receptors (Fig. S10). While a modeled binding site can be found in other RLP homologs at the locus, other conserved features are shared in the INR clade, for example a truncated intracellular sequence (Fig. S14) consistent with sensitive roles for this sub-domain in effective RLP signaling [(38)](https://paperpile.com/c/gQ0VBZ/JsqW). Demonstrating a precise requirement of *INR* for *Vu*-In response in legumes will require reliable reverse genetic tools in cowpea, currently limited by poor receptivity to transformation.

Inceptin peptides have been identified in the OS of all examined Lepidopteran species and are produced after feeding on a variety of host plant species [(8, 10)](https://paperpile.com/c/gQ0VBZ/CqG4W%2BynrU6) (Fig. S19). The receptor activity of INR is thus consistent with other plant PRRs recognizing conserved patterns associated with danger or attack [(12)](https://paperpile.com/c/gQ0VBZ/5rZRz). Despite similarity in peptide recognition as an immune strategy, responses to distinct peptide PAMPs can vary in genetic requirements [(39)](https://paperpile.com/c/gQ0VBZ/pW639), and defense outputs to herbivores differ from pathogen-induced responses [(40, 41)](https://paperpile.com/c/gQ0VBZ/ROTTv%2B4BWid). INR now provides a new genetic tool to define attacker-specific signaling pathways in plants.

Dynamic plant defense responses to herbivory have been examined for nearly 50 years [(5, 31, 42–45)](https://paperpile.com/c/gQ0VBZ/9xkKT%2By58us%2BqxxL8%2BmWUf3%2BIkJTu%2BtD5XF). Our data provides support for a current working hypothesis and model where INR directly recognizes inceptin peptides via a plant immune network of adapters and co-receptors, mediating defense outputs (Fig. 4f). INR mediates plant defense in response to a common oral secretion pattern in Lepidoptera [(10)](https://paperpile.com/c/gQ0VBZ/CqG4W), and represents a functional immune surveillance module that can be imparted to non-legume plant families. With pest invasions routinely threatening food security, a greater understanding of mechanisms underpinning plant-herbivore recognition is critical [(44)](https://paperpile.com/c/gQ0VBZ/IkJTu). More broadly, defined receptor-ligand pairs for plant-herbivore interactions are needed to provide essential mechanistic tools to understand and regulate interactions between autotrophs and animals.

**Methods:**

**Plant materials and SNP genotyping**

*P. vulgaris* accession G19833 was kindly provided by Dr. Phil Miklas (USDA), accession Red Hawk was kindly provided by Dr. Jim Kelly (Michigan State University). *V. radiata* accession VC1973A (“Tex-Sprout”) was kindly provided by Dr. Creighton Miller (Texas A&M University). All other soybean and common bean germplasm was made available through USDA Germplasm Information Network, specifically with invaluable assistance from Western Regional Plant Introduction Station (Pullman, WA) and Soybean Germplasm Collection (Urbana, IL).

Two populations were used for mapping of *Vu*-In-A response: 85 lines from a bi-parental recombinant inbred line (RIL) population derived from a cross between Yacine and 58-77 that was developed previously [(46)](https://paperpile.com/c/gQ0VBZ/LKw1X), and a set of 364 cowpea accessions representing worldwide diversity of cultivated cowpea [(17)](https://paperpile.com/c/gQ0VBZ/5TpuH). Both populations were genotyped with the Cowpea iSelect Consortium Array containing 51,128 SNPs [(18)](https://paperpile.com/c/gQ0VBZ/Dj6Mt) at the University of Southern California Molecular Genomics Core facility (Los Angeles, CA, USA). SNPs were called using GenomeStudio software (Illumina, Inc., San Diego, CA, USA) with the custom file from Muñoz‐Amatriaín *et al* [*(18)*](https://paperpile.com/c/gQ0VBZ/Dj6Mt). Data curation was performed by removing SNPs with more than 20% missing or heterozygous calls.

**Linkage and QTL mapping**

For linkage map construction, RILs with high heterozygosity and those carrying non-parental alleles were eliminated prior to mapping. Of the remaining 100 RILs, 17,638 SNPs that were polymorphic in both the parents and the RIL population, and that had minor allele frequencies (MAFs) >0.20 were used. MSTmap [(47)](https://paperpile.com/c/gQ0VBZ/YWkwR) (http://www.mstmap.org/) was used for genetic map construction, with the following parameters: grouping LOD criteria = 10; population type = DH (doubled haploid); no mapping size threshold = 2; no mapping distance threshold: 10 cM; try to detect genotyping errors = no; and genetic mapping function = kosambi. The linkage groups were numbered and oriented according to cowpea pseudomolecules [(19)](https://paperpile.com/c/gQ0VBZ/a84EL). Since the DH function inflated the cM distance for a RIL population, cM distances were divided by two to correct for the extra round of effective recombination occurring in a RIL population compared to a DH population.

QTL analysis was performed using a linear mixed model described by Xu [(48)](https://paperpile.com/c/gQ0VBZ/BfCgH) and implemented in R following Lo et al. [(49)](https://paperpile.com/c/gQ0VBZ/bx0Wz). A modified Bonferroni correction (α =0.05) that uses the effective number of markers or effective degrees of freedom instead the total number of SNPs as a denominator was used to set the genome-wide critical value, as in Lo et al. [(49)](https://paperpile.com/c/gQ0VBZ/bx0Wz). This set the significance cut-off to a -log10(p-value) of 4.84 for mapping *Vu*-In-A response.

**Genome-wide association studies (GWAS)**

The GWAS was performed in a panel of UCR Minicore accessions (Extended Data Table 1) using the mixed linear model (MLM) function [(50)](https://paperpile.com/c/gQ0VBZ/5Jf1F) implemented in TASSEL v5 (www.maizegenetics.net/tassel), with a principal component analysis (5 principal components) accounting for population structure in the dataset and a kinship matrix correcting for genetic relatedness between accessions. A total of 42,686 SNPs with MAF>0.05 were used for GWAS. SNPs were ordered based on their physical position in the cowpea reference genome [(19)](https://paperpile.com/c/gQ0VBZ/a84EL). A false discovery rate (α =0.05) was used for multiple testing correction of the GWAS results, which set the significance threshold to a -log10(p-value) of 3.93 for mapping *Vu*-In-A response.

**Peptide-induced ethylene production**

Inceptin peptides based on *Vigna unguiculata* cATPC sequence, *Vu-*In (ICDINGVCVDA) and *Vu*-In-A (ICDINGVCVD), were synthesized (Genscript, New Jersey, USA) and reconstituted in H2O. Induced ethylene accumulation in cowpea or common bean was measured in first fully-extended trifoliate leaves of 3-week old greenhouse-grown seedlings, grown from March to November in San Diego, CA in 3.5 inch pots using commercial potting soil (Berger Mix 2) supplemented with 5 mL Florikan 18-5-12. Leaflets were lightly scratch-wounded in each corner with a fresh razor blade to remove cuticle over an area of 1 cm2, and 10 μL of H2O with or without peptide was equally spread over the 4 wounds with pipette tip. After 1 hour, leaflets were excised and placed in sealed tubes for 1 hour before headspace sampling. Ethylene was measured by gas chromatography using a short 1 m column (Supelco 13018-U, 80/100 μm Hayesep Q) with flame ionization detection and quantified using a standard curve following Schmelz *et al.* 2003 [(51)](https://paperpile.com/c/gQ0VBZ/6litE). The experimental design for forward genetic studies using ethylene as a response output was as follows: 2 plants of each of 85 recombinant inbred lines (RILs) were treated with H2O or *Vu*-In-A as described above, on paired leaflets of either trifoliate or primary leaves. The ratio of ethylene production per gram of tissue (*Vu*-In-A:H2O) was calculated for each individual pair of leaflets, and the log-corrected average of the 4 pairs was used for QTL mapping. For GWAS, experimental design was similar except 1 trifoliate leaf of a single seedling of each of 364 lines were treated.

For ethylene assays in *N. benthamiana*, plants were grown in Berger Mix 2 with weekly supplemental fertilizer in a growth room (12 h light, 150 μmol m-2s–1) at 22°C. A recent fully expanded leaf of 4-week old plants was infiltrated with a blunt syringe, patted dry with paper towel, and 4 leaf discs within infiltrated area were immediately excised with a #5 cork borer and sealed in tubes. Headspace ethylene was measured after 3 hours of accumulation.

**Molecular cloning, transient and stable expression**

Full length cDNA sequences of all described receptors and co-receptors were PCR amplified using primers (Table S2) from 5’ SMARTer RACE cDNA libraries (Takara Biosciences). Unless otherwise noted, INR-*Vu* and INR-*Pv* indicate the genes or protein products of *Vigun07g219600.1* and *Phvul.007g077500.1*, obtained from reference sequenced accessions IT97K-499-35 and G19833 respectively. All other genes were cloned from reference accessions Tex-Sprout (*V. radiata*) and Williams 82 (*G. max*). For the highly similar soybean genes *Glyma.10G228000* and *Glyma.10G228100*, a larger fragment was subcloned from genomic DNA using primers with local homology for flanking sequences on chromosome 10, then a single primer pair was used to amplify either coding sequence. Amplicons were inserted using Gateway technology (Invitrogen) into plant expression vectors pEarleyGate103 [(52)](https://paperpile.com/c/gQ0VBZ/pvNOV) for C-terminal GFP or pGWB414 [(53)](https://paperpile.com/c/gQ0VBZ/7izQF) for C-terminal 3xHA tag. Constructs were electroporated into *Agrobacterium tumefaciens* strain GV3101 (pMP90) [(54)](https://paperpile.com/c/gQ0VBZ/9V1ax). Agrobacterium strains for expression of individual constructs were induced with 150 μM acetosyringone in 10 mM MES pH 5.6, 10 mM MgCl2 and infiltrated into *N. benthamiana* leaves at OD600 of 0.45. Western blotting was performed with α-GFP polyclonal (Thermo A-6455) or α-HA monoclonal (clone HA-7, Sigma H3663) primary antibodies at 1:1000 dilution, and α-rabbit (Sigma A6154) or α-mouse (Sigma A4416) secondary antibodies at 1:10,000 dilution. Transgenic lines of *N. benthamiana* and *N. tabacum* var. SR1 were obtained from the UC Davis Plant Transformation Facility using GV3101 strains (pEG103) with INR-*Vu* or INR-*Pv* (C-terminal GFP) inserts. T1 lines with segregation of glufosinate resistance consistent with single transgene insertions were selfed and homozygous T2 lines were selected.

**Ectodomain expression and purification**

INR-*Vu* (residues 23 – 845) coding sequence was sub-cloned in a modified pFastBac vector (Geneva Biotech) containing the *Drosophila* BiP signal peptide, a C-terminal TEV cleavable StrepII – 10x His tag and non-cleavable Avi-tag[(55, 56)](https://paperpile.com/c/gQ0VBZ/SGcHw%2BOihvx). *Trichoplusia ni* (strain Tnao38) [(57)](https://paperpile.com/c/gQ0VBZ/w3BSC) cells were infected with a multiplicity of infection (MOI) of 5 at a density of 2 x 106 cells ml-1 and incubated for 26 h at 28 °C and for additional 46 h 22 °C. The secreted protein was purified from supernatant by Ni 2+ (HisTrap Excel; GE healthcare; equilibrated in 50 mM KP i pH 7.6, 250 mM NaCl, 1 mM 2- Mercaptoethanol) and StrepII (Strep-Tactin XT Superflow high affinity chromatography: IBA; equilibrated in 20 mM Tris pH 8.0, 250 mM NaCl, 1 mM EDTA) affinity chromatography. Proteins were then dialyzed in 20 mM sodium citrate pH 5.0, 250 mM NaCl for 3 h and further purified by size-exclusion chromatography on a HiLoad 26/600 Superdex 200 pg column (GE Healthcare), equilibrated in 20 mM sodium citrate pH 5.0, 250 mM NaCl. Monomeric peak fractions were dialyzed in 50 mM Tris pH 8.0, 250 mM NaCl, 1 mM 2- Mercaptoethanol, and the tag was cleaved with TEV protease at 4 °C overnight and removed by size-exclusion chromatography on a Superdex 200 increase 10/300 GL column (GE Healthcare), equilibrated in 20 mM sodium citrate pH 5.0, 250 mM NaCl.

**Biotinylation and grating-coupled interferometry**

INR-*Vu* ectodomain was biotinylated with biotin ligase BirA (2 μM) [(56)](https://paperpile.com/c/gQ0VBZ/Oihvx) for 1 h at 25°C, in a volume of 200 μl; 25 mM Tris pH 8, 150 mM NaCl, 5 mM MgCl2, 2 mM 2-mercaptoethanol, 0.15 mM biotin, 2 mM ATP, followed by size-exclusion chromatography to purify the biotinylated protein.Binding kinetic measurements were performed with Creoptix WAVE system (Creoptix AG, Switzerland) using 4PCP chips (thin quasiplanar polycarboxylate surface; Creoptix, Switzerland ). Chips were conditioned with borate buffer (100 mM sodium borate pH 9.0, 1 M NaCl; Xantec, Germany) and streptavidin (20 µg ml-1; Sigma, Germany) was immobilized on the chip surface using amine-coupling; activation (1:1 mix of 400 mM *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride and 100 mM *N*-hydroxysuccinimide (Xantec, Germany) for 7 min, followed by injection of streptavidin in 10 mM sodium acetate pH 5.0 (Sigma, Germany) until the desired density was reached, passivation of the surface (0.5 % BSA (Roche, Switzerland) in 10 mM sodium acetate pH 5.0) for 10 min and final quenching with 1M ethanolamine pH 8.0 (Xantec, Germany) for 7 min. Biotinylated INR-*Vu* ectodomain (10 µg ml-1) was captured on the streptavidin-coupled chip surface until the desired density was reached. Kinetic analyses were performed at 25°C with a 1:2 dilution series from 10 µM in 20 mM citrate pH 5.0, 250 mM NaCl, 0.01 % Tween 20. Blank injections were used for double referencing and a dimethylsulfoxide (DMSO) calibration curve for bulk correction. Analysis and correction of the obtained data was performed using the Creoptix WAVE control software (correction applied: X and Y offset; DMSO calibration; double referencing).

**Homology modeling of INR-*Vu* and docking of *Vu*-In**

The crystal structure of LRR ectodomain of FLS2 (PDB ID: 4MN8) was used as the template. Sequence of INR-*Vu* was aligned with sequence of the template through the zero end-gap global alignment (ZEGA) method with the Gonnet comparison matrix [(58, 59)](https://paperpile.com/c/gQ0VBZ/enmAH%2BVcykr). The penalty of gap opening and extension were set as 2.4 and 0.15, respectively. Based on the alignment and template structure, a homology model of INR-*Vu* was built with the homology modeling tool and default parameters in ICM-Pro [(60)](https://paperpile.com/c/gQ0VBZ/YpkjD). All side chains and insertions/deletions were refined via a biased probability Monte Carlo (BPMC) sampling [(61)](https://paperpile.com/c/gQ0VBZ/K0fQd)

The co-crystallized 22-amino-acid peptide in the crystal structure template (PDB ID: 4MN8) was used to define the docking region. A set of potential maps were generated for the docking region on a 0.5 Å 3D grid, containing: (i) van der Waals interaction; (ii) electrostatic interaction; (iii) hydrogen bond; and (iv) hydrophobic potential grids. With potential maps, docking and scoring of *Vu*-In was performed using a stochastic global energy optimization procedure in internal coordinates implemented in the ICM-Pro v3.8-6a [(62)](https://paperpile.com/c/gQ0VBZ/13xGD), described as the following steps. 1) The *Vu*-In peptide was sampled with the implicit solvation model to generate a series of starting conformations via BPMC method, and each starting conformation was placed into the docking region with four principal orientations. 2) *Vu*-In was sampled in the pre-calculated potential maps through BPMC sampling to optimize its positional and internal variables. 3) After sampling, 10 top ranking conformations were re-scored with ICM full atom scoring function and conformations were re-sorted by the docking score.

**Acridinium-labeled peptide**

Acridinium-labeled *Vu-*In (acri-In) was synthesized using the N-hydroxy-succinimidyl (NHS) acridinium ester (Cayman Chemical, Ann Arbor USA) quenched with Tris pH 8 and purified by reverse phase high performance liquid chromatography (HPLC; Agilent Poroshell 120 EC-C18) by tracking absorbance at 372 nm. Final labeled peptide concentration was determined using a standard curve of absorbance of NHS-acridinium standard.

Acridinium retention assays were performed according to Butenko *et al.* [*(63)*](https://paperpile.com/c/gQ0VBZ/nbHHU) and Wang *et al.* [*(64)*](https://paperpile.com/c/gQ0VBZ/ru5Ur) with modification. Protein was extracted from 1 g *N.benthamiana* tissue expressing RLP genes (48 hpi Agrobacterium), using a solution of 50 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM PMSF, 1x Roche Protease Inhibitor Cocktail. Homogenized extracts were cleared for 30’ at 20,000 rcf, and supernatant was then incubated with end-over-end mixing with 10 uL GFP-Trap MA resin (Chromotek, Germany) for 3 h. Immunoprecipitated receptor was washed twice in extraction buffer (1 mL) and twice in binding buffer (1 mL per wash, 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM PMSF). Immunoprecipitates were aliquoted using 80 μL per replicate tube. Four microliters of either H2O or excess competitor peptide was added, to a final concentration of 4 nm - 40 μM, and the preparation was pre-incubated on ice for 2 h. Acri-In in binding buffer was added to a final concentration of 200 nM with or without additional competitor peptide (final concentration 80 μM in 100 μL volume). After 20 min incubation on ice with occasional mixing, pellets were washed twice with 1 mL binding buffer and resuspended in 100 μL 5 mM citric acid. Pellet-retained luminescence was measured using a Biotek Synergy H2 Multimode plate reader by injecting 100 μL trigger buffer (0.2 N NaOH, 0.1% H2O2) and reading 10s luminescence, and a standard curve of acri-In was used to determine retained peptide concentration.

**Co-immunoprecipitation of co-receptor and adaptor kinases**

Following *Agrobacterium* infiltration (48 h), *N. benthamiana* leaves expressing both INR or EFR (C-terminal GFP) and SOBIR1 or SERKs (C-terminal 3xHA) were infiltrated with peptide solutions and harvested on liquid N2 at specified timepoints. Tissue was ground on N2-chilled mortar and pestle and homogenized in 2 mL g-1 extraction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM DTT, 1x Roche Protease Inhibitor) then cleared by centrifugation (30m, 20,000 rcf). Supernatant was incubated with 10 μL GFP-Trap A beads (Chromotek, Germany) by end-over-end mixing at 4°C and eluted in 30 uL of Laemli buffer (95°C, 5 min).

**Plasma membrane purification**

*N. benthamiana* tissue expressing INR-*Vu* or mutant receptor was homogenized in lysis buffer (0.33 M sucrose, 50 mM Tris pH 7.5, 5 mM EDTA, 1x Roche Protease Inhibitor), cleared by centrifugation (10’, 6000 rcf), and supernatant was filtered through Miracloth. Membranes were pelleted by ultracentrifugation (30’, 100,000 rcf). Membranes were resuspended in resuspension buffer (0.33 M sucrose, 5 mM KPO4 pH 7.8, 3 mM KCl, 1x Roche Protease Inhibitor) and separated in two-phase solution of 6.2% Dextran T500 and PEG 3350. Upper and lower phases were pelleted by ultracentrifugation to yield plasma membrane and microsomal fractions respectively [(65)](https://paperpile.com/c/gQ0VBZ/J93rw). Total protein was quantified by BCA assay (Thermo) and equal protein was loaded for Western blotting.

**Reactive Oxygen Species (ROS) production and peroxidase activity assays**

Following *Agrobacterium* infiltration for receptor expression (24 h), leaf punches were taken with a 4 mm biopsy punch and floated in 50 μL H2O using individual cells of a white 96-well plate. After overnight incubation, ROS production was measured using luminol-horseradish peroxidase (HRP) over 40 min as described [(66)](https://paperpile.com/c/gQ0VBZ/ijFnC) using a Biotek Synergy H2 Multimode plate reader. Peroxidase activity was measured as described by Mott *et al.* [*(67)*](https://paperpile.com/c/gQ0VBZ/H969p) with the following modifications. Leaf discs were taken from fully extended leaves of 4-week old *Nicotiana* seedlings, washed for 1 h in ½ Murashige Skoog salt (4.4 g L-1), and incubated overnight in ½ MS with 1 μM peptide prior to reaction with 5-aminosalicylic acid.

**Transcriptomic and qPCR Analysis**

*N. benthamiana* stable transgenic line expressing *P. vulgaris* INR (INR-Pv 1-5) was syringe infiltrated with H2O or 1 µM *Vu*-In, and total leaf tissue was harvested 6 h later. Total RNA was extracted using Nucleospin Plant RNA kit (Macherey-Nagel). RNA was used to generate Lexogen Quantseq 3’ RNA seq libraries at Cornell University Institute of Biotechnology Genomics Facility. 3’ reads were mapped to *N. benthamiana* genome v1.0.1 (Sol Genomics Network) using HISAT2 [(68)](https://paperpile.com/c/gQ0VBZ/OlqfF), counts by gene were analyzed using HTSeq-Count [(69)](https://paperpile.com/c/gQ0VBZ/GyZm2), and differential expression was analyzed by DESeq2 [(70)](https://paperpile.com/c/gQ0VBZ/XFCi0).

Highly *Vu-*In upregulated genes relative to H2O-infiltrated tissue were prioritized for qPCR analysis. In replicated experiments, plant material was treated as before in the transcriptome analysis and cDNA libraries were generated using SuperScript III (Life Technologies). qPCR was performed using primers in Table S2.

**Measurement of inceptin peptides in oral secretions**

OS was collected from 4th instar *S. frugiperda* caterpillars that had fed for 48 h on leaves of defined host plants (*V. unguiculata*, *N. tabacum*, and *N. benthamiana*). Stable isotope internal standard-based quantification of OS inceptin levels, following Schmelz *et al.* 2006 [(8)](https://paperpile.com/c/gQ0VBZ/ynrU6), were obtained using ultra-high performance liquid chromatography - tandem mass spectrometry (UHPLC-MS/MS). Aliquots of 25 µL of spit were extracted on Oasis HLB cartridges (30 mg Oasis HLB, Waters, Milford, MA), evaporated and reconstituted in 200 µL of 35% methanol for injection. The peptide separation was achieved on a Cortecs C18 column (100 mm length x 2.1mm i.d., 1.6 µm, Waters) using formic acid 0.05% in H2O and acetonitrile as mobile phases. Detection was performed in electrospray positive ionization using the doubly charged ions [M+2H]2+ as precursor ions for the MRM transitions, using selected MRM transitions 567.9 > 205.2, 492.2, 501.0 for 11-mer tobacco inceptin (*Nb/Nt*-In), 560.3 > 205.2, 493.1 for 11-mer cowpea inceptin (*Vu*-In), and 563.4 > 205.2, 304.2, 495.9 for the labelled internal standard). Data are from 3 independent replicates.

**Herbivory Assays**

Beet armyworm (*Spodoptera exigua*)larvae were obtained from Benzon (Carlisle, PA) as neonates, incubated at 28°C for 48 h, and newly molted second instars were selected, pre-weighed and placed on individual 3-week old plants. Each plant was contained with individual larvae using PVC cages secured at the base with surrounding potting soil. After 4 d, larvae were re-weighed and Relative Growth Rate was calculated [(71)](https://paperpile.com/c/gQ0VBZ/3uFfv).

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**Figure 1, Cowpea responses to an inceptin peptide associate with a single genetic locus**. **a,** Ethylene production in cowpea accessions after treatment with H2O or 1 μM inceptin peptides. Bars show means +/- SEM of replicate leaflets for individual inceptin treatments (n=3) and all combined respective H2O controls with label “All” (n=21). Different letters represent significant differences (All ANOVAs p<0.05; Tukey HSD, α=0.05). **b,** QTL statistics for ethylene production ratio, *Vu*-In-A/H2O, in the Yacine x 58-77 RIL population. Dotted line indicates false discovery rate (FDR) significance cutoff (17638 SNPs; modified Bonferroni correction at α=0.05) **c,** Manhattan plot of GWAS results for ethylene production ratio, *Vu*-In-A/H2O, in 364 cowpea Minicore accessions. Dotted line indicates FDR cutoff at α=0.05 for 42686 SNPs assigned respective physical coordinates in the cowpea genome [(19)](https://paperpile.com/c/gQ0VBZ/a84EL). **d,** Genomic region of chromosome 7 (Vu07; positions 34,220,090-34,258,839) containing highly associated marker SNPs (2\_22560/1) and syntenic genes on common bean chromosome 7. Green and black filled arrows indicate leucine rich repeat (LRR)-RLP encoding genes.

**Figure 2, Inceptin Receptor (INR) confers novel inceptin signaling responses in heterologous model *N. benthamiana,* corresponding with cowpea response variation.** **a-c,** Ligand-dependent reactive oxygen species (ROS) production following the heterologous expression of receptors in *N. benthamiana*. RLU, relative luminescence units, minutes (min) after treatment with H2O or 1 μM the peptides elf-18 or *Vu*-In. **a,** Peptides applied to plants expressing elf-18 receptor (elongation factor-Tu; EFR), **b,** Peptides applied to plants expressing Inceptin Receptor (INR-*Vu*; *Vigun07g219600*), **c,** Cumulative RLU counts from a,b after indicated peptide treatments. Bars show average of n=8 leaf discs +/- SEM. **d,** Receptor-dependent ethylene production in *N. benthamiana* after treatment with H2O or 1 μM peptides. Bars show average of n=6 leaf discs +/- SEM. **e,** *Vu-*In-A / H2O induced ethylene response ratio in select cowpea germplasm (top), with corresponding ROS responses when INR alleles are expressed in *N. benthamiana* (bottom). Gray boxes indicate accessions with GG allele at mapped marker 2\_22561, white boxes indicate AA allele. See Table S2 for full list of UCR Minicore responses. Bars show average of n=8 leaf discs +/- SEM. Within panels **c, d** and **e**, different letters indicate significant differences (All ANOVAs P < 0.05; Tukey HSD, α=0.05)

**Fig. 3, INR confers acridinium-*Vu*-In retention and displays both constitutive and inducible co-receptor/adapter RLK associations. a,** Quantified acridinium-In retention to immunoprecipitated, GFP-tagged LRR-RLPs in the presence or absence of competitor peptide. **b**, Competition curve of free *Vu*-In or *Vu-*In-A peptide against 0.3 μM acri-In bound to immunoprecipitated INR-*Vu.* **c**, *Vu-*In but not flg22 competes for peptide retention to immunoprecipitated INR-*Vu.*  In **a-c**, points indicate replicate luminescence measurements. **d**, Predicted binding site for *Vu-*In with His495 and Arg497 of INR-*Vu.* ROS production after 1 μM *Vu*-In treatment is shown for *N. benthamiana* leaf punches (n=8) expressing INR-*Vu* (green) or mutant variants (black). **e-g,** INR-*Vu* co-immunoprecipitates (IP) cowpea (*Vu*) and Arabidopsis (*At*) homologs of SOBIR1 and AtSERK RLKs(1-4). VuSOBIR1 (*Vigun09g096400*) or AtSOBIR1 (*AT2G31880*) were co-expressed with INR-*Vu*. D-E, AtSERK1-4 were co-expressed with INR-*Vu.* All receptor (C-terminal GFP) and coreceptor (C-terminal 3xHA) combinations were expressed in *N. benthamiana.* Western blots (WB) were probed with either GFP or HA antibody. Representative results are shown from 3 independent experiments.

**Figure 4, Heterologous INR expression regulates inducible plant defenses and herbivore resistance in tobacco species. a-b,** *Vu*-In induces peroxidase activity in stable INR transgenic lines of *N. tabacum (N.t.)* and *N. benthamiana* (*N.b.*). Bars show average +/- SEM of n=16-24 leaf discs after incubation with 1 μM *Vu*-In. Asterisk (\*) denote statistical significance (*P* < 0.05 Student’s t-test). **c,** qPCR quantification of *Vu*-In induced defense transcripts for a *Kunitz Trypsin Inhibitor* (*KTI*) and an *Ascorbate Oxidase* (*AscOx*) in 35S:INR-*Pv* transgenic *N. benthamiana* line 1-5. Relative expression of defense markers in n=6-7 replicate plants are shown (see Fig. S15 for additional details). **d,e,** Relative Growth Rates (RGR) of beet armyworm (*Spodoptera exigua*)larvae reared on transgenic or wildtype (WT) lines of *N. tabacum* (d) and *N. benthamiana* (e). Bars show average of n>22 *N. tabacum* plants from two independent experiments or n>33 *N. benthamiana* plants from four independent experiments +/- SEM, with individual dots showing individual larvae. Different letters indicate significantly different means (Tukey HSD, α=0.05). **f,** Conceptual model of chewing herbivore recognition and induced defense elicitation in legumes via INR. Foliar attack by caterpillars on cowpea (*Vigna unguiculata*, Vu) results in gut proteolysis of chloroplastic ATP synthase -subunits (ATPC) and the production of inceptin peptides, such as Vu-In, in oral secretions (OS). Subsequent bouts of herbivory stimulate recognition of Vu-In by INR, a legume-specific LRR-RLP. INR constitutively and dynamically associates with SOBIR1 and SERK (LRR-RLK) co-receptors, respectively. The collective outcome is the enhanced production of subsequent induced defense signals such as reactive oxygen species (ROS) and ethylene which in part contribute to the up-regulation of additional proteinaceous defenses. Inceptin elicited production of direct defenses such as peroxidases and trypsin inhibitors are part of a complex array of biochemical changes that collectively suppress insect growth rates.