Title: A receptor for herbivore-associated molecular patterns mediates plant immunity

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Short title: An anti-herbivore receptor-ligand pair

One Sentence Summary: A plant cell surface receptor directly perceives peptides associated with caterpillar herbivory.

Abstract:

Plant-herbivore interactions are ubiquitous across nature and drive major agricultural losses. Inducible defense responses triggered through immune recognition aid in host plant protection; however, specific ligand-receptor pairs mediating the initial perception of herbivory remain unknown. Plants in the subtribe Phaseolinae detect herbivore-associated peptides in caterpillar oral secretions and the defined ligands are proteolytic fragments of chloroplastic ATP synthase termed inceptins. Using forward genetic mapping of inceptin-induced responses, we identify a cowpea (*Vigna unguiculata*) leucine-rich repeat receptor-like protein as an inceptin receptor (INR) sufficient for elicitor-induced responses and enhanced defense against armyworms (*Spodoptera exigua*). INR defines a receptor by which plants perceive herbivore-associated molecular patterns (HAMPs) and expands the paradigm of surface immune recognition to attack with mandibles.

Main text:

Herbivores defoliate and often devastate plants, making effective inducible defense responses critical for plant resilience (*1*, *2*). To combat insect herbivores, plants respond to specific molecular patterns present in oral secretions (OS) and frass by amplifying wound-induced

50   defenses and activating resistance (*3*, *4*). Analogous immune responses to microbes and other pests are mediated by ligand-receptor interactions mediated by receptor kinases (RKs) or receptor-like proteins (RLPs) (*5*). While signaling and defensive outputs after herbivory are increasingly understood (*6*), receptors directly recognizing biochemically-defined herbivore-associated molecular patterns (HAMPs) have remained elusive (*7*).

55   Among defense-eliciting HAMPs, inceptins are a potent bioactive series of proteolytic fragments derived from the chloroplastic ATP synthase γ-subunit (cATPC) found in Lepidopteran larvae OS (*4*, *8*). An abundant inceptin present during caterpillar herbivory on cowpea (*Vigna unguiculata*) is an 11 amino acid (AA) peptide, termed *Vu*-In ($^{+}$ICDINGVCVDA^{-}). The epitope is highly conserved among plant cATPC sequences; however,

60   only species within the Phaseolinae subtribe respond to inceptins *(9)*.

To identify inceptin receptor (INR) candidates, we examined plant response variation to both *Vu*-In and C-terminal truncated inceptin, termed *Vu*-In^{-A} ($^{+}$ICDINGVCVD^{-}), a less bioactive variant that uniquely accumulates in the OS of *Anticarsia gemmatalis,* a legume specialist herbivore (*10*). Anticipating an arms race pattern of evasion and re-establishment consistent with

65   other elicitors (*11*, *12*), we screened for cowpea germplasm with positive *Vu*-In^{-A}-induced 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 hypothesized that the existence of qualitative response variation to the weak elicitor variant

70   could be mediated by INR genetic variation.

To map *INR,* we used a biparental population of 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 worldwide accessions belonging to the UC Riverside Minicore collection for a genome-wide association study (GWAS). *Vu*-In[-A] elicited

75   variable ethylene production across accessions (Fig. S1A-B, Table 1). Using QTL mapping and GWAS, we observed that *Vu*-In[-A] responses strongly associated with a single genetic locus (Fig. 1B, 1C, Fig S2). The most highly-associated markers in both studies were SNPs 2_22560, 2_22561, and 2_09070 (*13*), spanning a 22-kb region on chromosome Vu07 (Fig. 1D, S1C). Both markers 2_22560 and 2_22561 fell within the receptor-encoding gene *Vigun07g219600*

80   suggesting that this locus is associated with inceptin responsiveness in cowpea.

To examine function of the INR candidate, we transiently expressed *Vigun07g219600* from the reference accession IT97K-499-35 (*14*) 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

85   expression of the *EF*-Tu receptor (EFR) (*15*) in *N. benthamiana* conferred responses to elf18 peptide (Fig. 2A). Expression of *Vigun07g219600* conferred *Vu*-In-inducible responses*,* in the form of ROS (Fig. 2B, 2C) and inducible ethylene production (Fig. 2D), indicating that *Vigun07g219600* encodes a functional INR.

To understand the basis of cowpea phenotypic variation for inceptin responsiveness, we

90   cloned and expressed INR alleles from 6 cowpea accessions with differential *Vu*-In[-A] responses. Only alleles from highly *Vu*-In[-A] responsive accessions conferred significant *Vu*-In-induced ROS

and ethylene production in *N. benthamiana*, thus mirroring *Vu*-In^{-A} response variation originally observed in cowpea (Fig. 2E, Fig S3). Interestingly, none of the tested alleles conferred *Vu*-In^{-A} responses in *N. benthamiana* (Fig. S3, S4). Given that all 364 cowpea accessions could respond

95 to *Vu*-In (Table S1), natural variation in INR may thus specify an activation threshold for (1) the weak elicitor, *Vu*-In^{-A}, in cowpea and (2) the robust elicitor, *Vu*-In, when expressed heterologously in *N. benthamiana.*

INR is a leucine-rich repeat (LRR)-RLP, a receptor class distinguished from LRR-RKs by lack of an intracellular kinase domain (*16*). INR contains 30 semi-regular LRRs preceding a

100 transmembrane and short cytosolic domain (Fig. S5). The *INR* locus in cowpea contains a paralog *Vigun07g219700* (72% AA similarity) that is unable to confer *Vu*-In induced ethylene production when expressed in *N. benthamiana* (Fig. S6A). In contrast, two orthologs with >90% similarity, *Phvul.007G077500* (from *P. vulgaris*) and *Vradi08g18340* (from *V. radiata*), conferred *Vu*-In-induced ethylene production (Fig. S6B). In soybean (*Glycine max)*, the most

105 closely related RLPs are four genes at the same syntenic locus, each with 73-76% similarity to cowpea INR-*Vu* (Fig. S6C). Neither of two tested soybean homologs (*Glyma.10G228000* or *Glyma.10G228100*) enabled *Vu*-In-induced ethylene production (Fig. S6B). Soybean plants are both unresponsive to inceptin and lack representation in a subclade of functional *INR* genes (Fig. S6C) (*9*). We conclude that *Phaseolus* and *Vigna*, both within the subtribe Phaseolinae, encode

110 functional INRs.

RLPs from Arabidopsis and tomato have been demonstrated to bind peptide ligands (*17, 18*). To measure inceptin binding, we generated an N-terminal acridinium-tagged *Vu*-In conjugate. *N. benthamiana* expressing cowpea INR-*Vu* retained acridinium-*Vu*-In luminescent signal, while tissue expressing an unrelated receptor, EFR, did not (Fig. 3A). Furthermore,

115   acridinium-*Vu*-In-derived luminescence was abolished by pre-incubating with excess unlabeled

Vu-In (Fig. 3A). To better understand the receptor-ligand interaction, we constructed a homology

model of INR based on the crystal structure of the LRR ectodomain of FLAGELLIN-SENSING

2 (FLS2) (*19*), and performed *Vu*-In docking simulations. The predicted lowest-energy

conformations were ranked by peptide binding scores. In multiple low-energy conformations of

120   *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. 3B). Consistent with

critical binding activity, a previous Ala substitution study demonstrated that Asp10 was the only

AA essential for *Vu*-In elicited ethylene production (*8*). To test the interaction, a receptor mutant

was generated with Ala substitutions at His495/Arg497, which resulted in loss of both *Vu*-In

125   binding activity and *Vu*-In-induced ROS production in *N. benthamiana* (Fig. 3A, 3B). His495

and Arg497 are highly conserved in *Phaseolus* and *Vigna* but not in soybean RLP homologs

(Fig. S7). Our data supports INR-*Vu* binding to inceptin with key interactions specifically

mediated by His495/Arg497.

       LRR-type surface receptors in plants typically associate with Somatic Embryogenesis

130   Receptor Kinase (SERK) co-receptors for signal transduction (*20*). In addition, characterized

RLPs constitutively associate with the adapter RK Suppressor of BIR1 (SOBIR1) (*21*). We

tested if INR associates with Arabidopsis and cowpea orthologs of SOBIR1 by co-

immunoprecipitation. Association of INR with both AtSOBIR1 and VuSOBIR1

(*Vigun09g096400*) was constitutive (Fig. 3C), while INR associated more strongly with AtSERK

135   co-receptors after peptide treatment (Fig. 3D, 3E). Thus, INR associates with SERK and

SOBIR1 receptor kinases in a manner similar to characterized LRR-RLPs.

To test if INR enhances anti-herbivore defenses in plants lacking native inceptin responses, we stably transformed *N. benthamiana* and *N. tabacum* with either 35S:INR-*Vu* or INR-*Pv* transgenes. Multiple independent transgenic lines expressing 35S:INR responded to *Vu*-In as measured by ethylene and a direct defense output, induced peroxidase activity (Fig. 4A,B, Fig. S9). We then challenged INR-expressing lines with second instar larvae of the generalist Lepidopteran herbivore, beet armyworm (*Spodoptera exigua*). Caterpillars consistently displayed 29-38% lower growth rates on transgenic lines than on wild-type (WT) when caged on single young seedlings of either *Nicotiana* species for 4 days (Fig. 4C, 4D). A similar magnitude of caterpillar growth reduction was previously observed after pretreatment of cowpea with *Vu*-In (*4*). Thus expression of INR-*Vu* or INR-*Pv* is sufficient to confer enhanced anti-herbivore defense responses.

Dynamic plant defense responses to herbivory have been examined for nearly 50 years (*22–24*) and are induced by numerous insect associated elicitors or HAMPs (*3, 4*). In parallel with pathogen- and damage-associated molecular patterns (PAMPs and DAMPs), inceptin is detected by INR, an LRR-type surface receptor that establishes a HAMP-receptor pair. INR shares features of pattern recognition receptors, such as recent emergence in plant evolution and association with conserved signaling machinery (*25*). Despite similarities in mode of recognition, plant immunity to pathogens and herbivores is often characterized by downstream antagonistic interactions between defense hormones (*26*). INR and other diverse surface immune receptors will facilitate the comparison of signaling tradeoffs against a wide breadth of pest classes. A defined ligand-receptor pair for plant-herbivore interactions represents a step forward in both understanding and controlling plant immunity to diverse biotic pressures.

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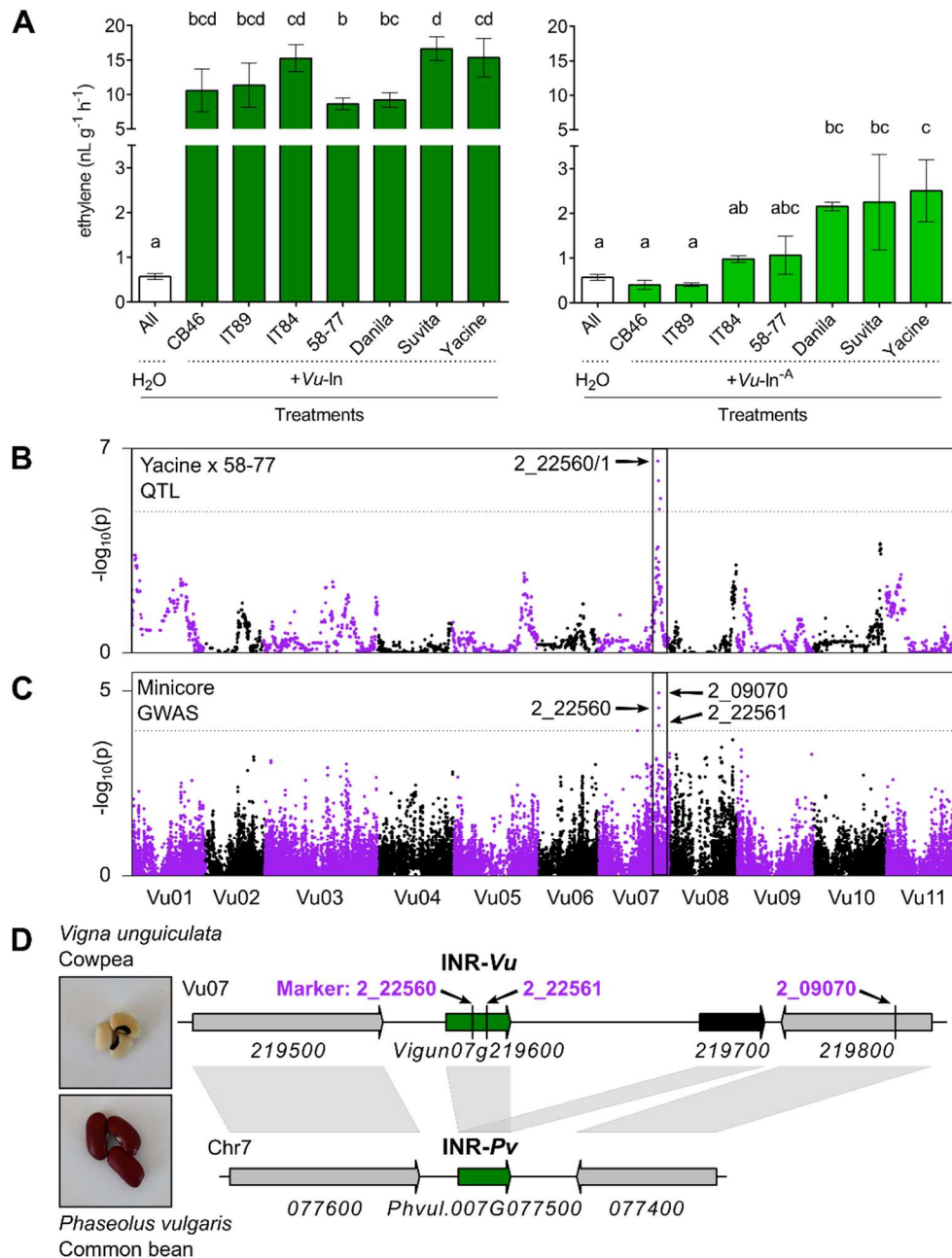
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Supplementary Materials:

Materials and Methods

205   Figures S1-S8

Tables S1-S2

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Fig.1, Cowpea sensitivity to Vu-In^{-A} associates with a single genetic locus. (A) Ethylene production of cowpea accessions after treatment with H_2O or 1 μM inceptin peptides. Bars show means +/- SEM of n=3 replicate leaflets or all controls (n=21). Left, F=63.8, right, F=9.69, letters indicate significantly different means (Tukey HSD, α=0.05). (B) QTL statistics for ethylene production ratio, Vu-In^{-A}/H_2O, in the Yacine x 58-77 RIL population. Dotted line indicates significance cutoff using modified Bonferroni correction at α=0.05, $-\log_{10}(p) > 4.84$. (C) Manhattan plot of GWAS results for ethylene production ratio, Vu-In^{-A}/H_2O, in the panel of Minicore accessions. Dotted line indicates false discovery rate (FDR) significance cutoff at α =0.05, $-\log_{10}(p) > 3.93$. $-\log_{10}$ of the probability (p-values) is shown for 17638 (B) or 42686 (C) SNPs at respective physical coordinates in the cowpea genome (Lonardi et al. 2019). (D) Genomic region of Vu07 (positions 34,220,090-34,258,839) containing highly associated marker SNPs, and syntenic genes on common bean Chr7. Green and black filled arrows indicate RLP genes.
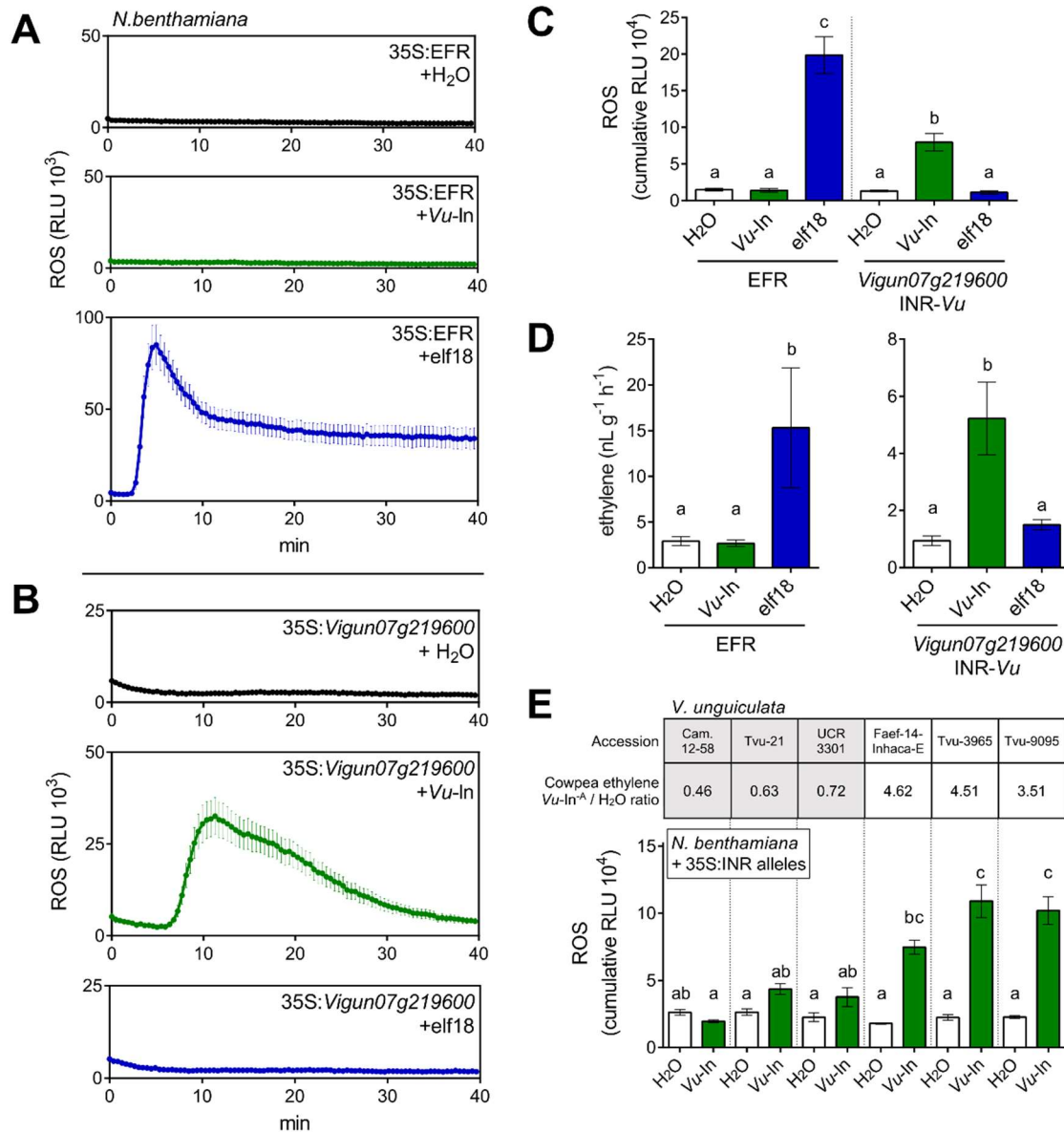
Fig. 2, *Vigun07g219600,* **Inceptin Receptor (INR), confers inceptin sensitivity in** *N. benthamiana*
(A-C) Receptor-dependent reactive oxygen species (ROS) production in *N. benthamiana*. RLU, relative
luminescence units, min, minutes after treatment with 1 µM peptide. Average is shown for n=8 leaf discs
+/- SEM. (A) elf-18 receptor (EFR) expressed, (B) *Vigun07g219600* expressed. (C) Sum of RLU, 0-20
min. (D) Receptor-dependent ethylene production in *N. benthamiana* after treatment with water or 1 µM
peptide. Bars show average of n=6 leaf discs +/- SEM. (E) Allelic variation in INR-dependent ROS in *N.
benthamiana*. Bars show mean +/- SEM of n=8 leaf discs expressing alleles from indicated lines. Table
indicates source accession for INR alleles. Letters indicate significantly different means (Tukey HSD,
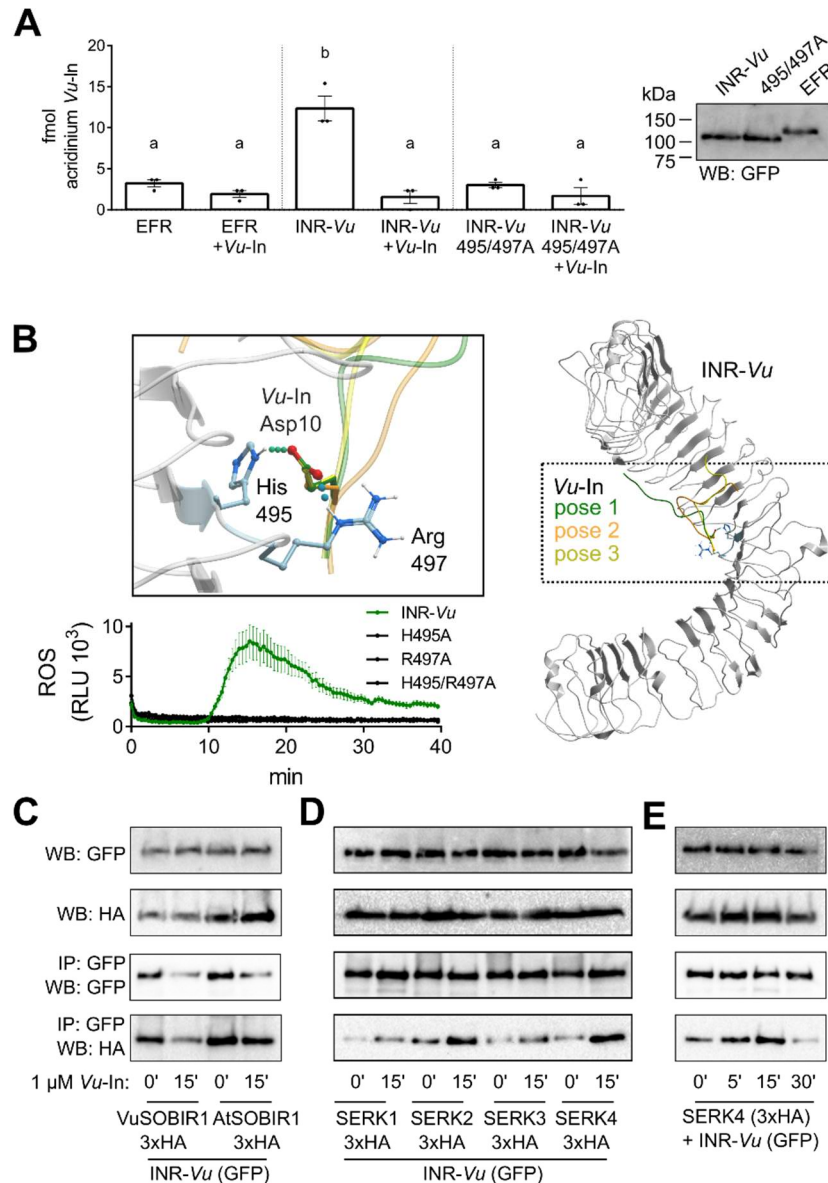α=0.05).

Fig. 3, INR binds *Vu*-In peptide and associates with co-receptor RLKs. (A) INR-*Vu* but not EFR or mutant INR H495/R497A binds acridinium-*Vu*-In. Bars indicate mean +/- SEM (n=3 incubations) retained
235   acridinium signal. Letters indicate significantly different means (Tukey HSD, α=0.05) (B) INR-*Vu* model in complex with predicted binding pose of the *Vu*-In peptide. Right, extracellular domain of INR-*Vu* was modeled by homology with the FLS2 ectodomain template (PDB code: 4mn8). The predicted top three *Vu*-In poses are shown. Left, close-up with *Vu*-In Asp10 residue sidechain shown interacting to INR-*Vu* His495 and Arg497 sidechains. Bottom, ROS burst (1 μM *Vu*-In) is shown for *N. benthamiana* leaf punches (n=8)
240   expressing INR-*Vu* (green) or mutant variants (black). (C-F) INR-*Vu* co-immunoprecipitates (IP) Arabidopsis (At) and cowpea (Vu) homologs of SOBIR1 and SERK RLKs. C, 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.
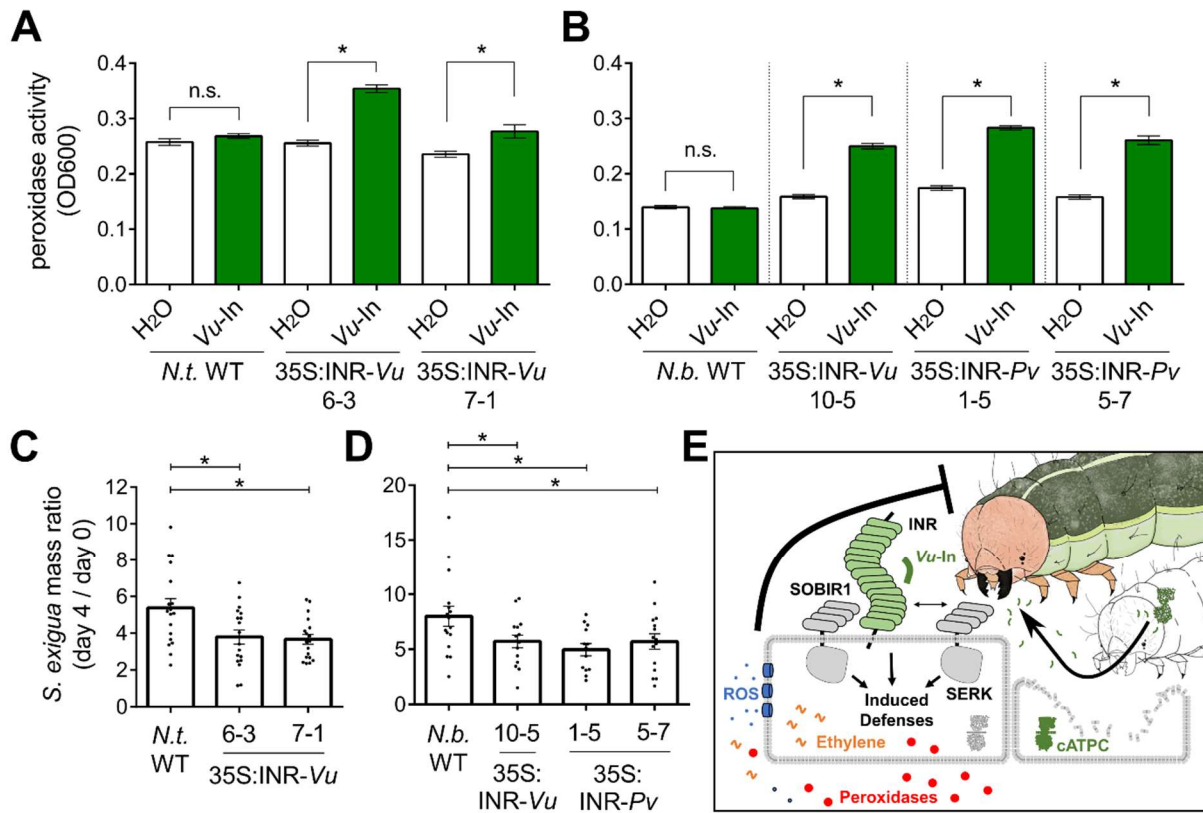245   Representative results are shown from 2-3 independent experiments.

Fig. 4, INR confers receptor activity and herbivore resistance to stable *Nicotiana* lines. (A-B) *Vu*-In induces peroxidase activity in stable INR transgenic lines of (A) *N. benthamiana (Nb)* and (B) *N. tabacum*. Bars show average +/- SEM of n=16-24 leaf discs after incubation with 1 µM inceptin. *, p<0.05 Student's t-test. (C-D) Relative growth rates of beet armyworm (*Spodoptera exigua*) larvae reared on transgenic or WT lines of *N. benthamiana* (C) and *N. tabacum* (D). Bars show average +/- SEM of n=13-16 *N. benthamiana* plants or n=18 *N. tabacum* plants. For *N. benthamiana,* F=3.43, *N. tabacum*, F=5.93. * p<0.05, Fisher's LSD. (E) Model for INR perception of *Vu*-In. INR associates with adapter and co-receptor kinases leading to burst of reactive oxygen species (ROS), ethylene, and peroxidases. Combined defense outputs negatively affect herbivore growth rate.