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Characterization of *Arabidopsis thaliana* INHIBITOR OF PEP ACTION (IPA), a truncated PEPR homolog

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by

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The Thesis of Carleen M Villarreal is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Characterization of *Arabidopsis thaliana* INHIBITOR OF PEP ACTION (IPA), a truncated PEPR homolog

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Professor Alisa Huffaker, Chair

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Plants rely on innate immunity to perceive pathogens and attackers at a cellular level. Once recognized, defense signaling and amplification can be accomplished though production of plant elicitor peptides (Peps) and their interaction with their receptors (PEPRs) to initiate a number of defense responses. Here, we characterize INHIBITOR OF PEP ACTION (IPA) as a negative regulator of the Pep signaling pathway in Arabidopsis. This protein was identified as apoplastic and soluble with 77% identity to PEPR1's extracellular domain, resembling a truncated version of the Pep receptors and similar in structure to a mammalian decoy receptor. Plants overexpressing IPA showed reduced production of reactive oxygen species (ROS),

while IPA-deficient plants exhibited more robust immune responses. *IPA* expression was also shown to be induced by AtPep1 in root tissue, but was not elicited by other microbe-associated molecular patterns (MAMPs). Suspension cells pretreated with high concentrations of AtPep1 transcribed *IPA* at higher levels and were desensitized to subsequent AtPep1 treatment. Competition assays utilizing transgenic *Nicotiana benthamiana* demonstrated that IPA was able to compete with PEPR2 for binding of AtPep1, reducing signal transduction. Taken together, this data implicates IPA as a potentially novel decoy receptor responsible for regulating innate immunity in plants.

1. Introduction

1.1 The role of plant immunity in agriculture

Sustaining the world's growing population presents a number of challenges that must be overcome in the near future. Among these is the need to produce enough food for such vast numbers, an issue already acutely felt in some places. However, a limiting amount of viable land on which to grow these crops necessitates finding more efficient ways of utilizing already available land. One major approach has focused on reducing crop losses to pests and disease, a strategy that could greatly increase land use efficiency and crop yield. A better understanding of how plants defend themselves from attackers, as well as how they perceive such attacks in the first place, will help facilitate the development of such strategies.

1.2 The role of PAMPs in pathogen recognition and response

Although plants lack the specialized, differentiated immune cells of mammals, both kingdoms are able to recognize foreign invaders through microbe-associated molecular patterns (MAMPs), or conserved molecular signatures that are often common to a variety of microbes or fungi (Boller *et al*., 2009; Nürnberger *et al*., 2004). These MAMPs are recognized by specific pattern recognition receptors (PRRs), which activate a series of immune responses known as patern-triggered immunity (PTI) (Zipfel 2014). For example, recognition of bacterial flagellin or elongation factor Tu by the plant PRRs FLS2 or EFR, respectively, result in a rapid production of reactive oxygen species (ROS), as well as the alkalinization of the cell wall and an inhibition of pathogenic growth (Gómez-Gómez *et al*., 1999; Zipfel *et al*., 2006).

1.3 The role of Peps in the activation of plant immunity

Plant elicitor peptides (Peps) are endogenous peptide signals that amplify defense responses and signal the threat of infection to other cells within the plant (Huffaker *et al*., 2006). These signals, produced as a result of PTI or treatment with plant defense hormones such as jasmonic acid or ethylene (Bartels *et al*., 2013; Huffaker and Ryan *et al*., 2007), were the first discovered endogenous peptide regulators of plant defense against microbes. Originally isolated from *Arabidopsis thaliana*, orthologs of Pep exist in all higher plants (Huffaker *et al*., 2013, Trivilin *et al*., 2014). Peps are recognized through Pep receptors (PEPRs), a class of leucine rich repeat receptor-like kinases (LRR-RLKs) common to diverse kingdoms of organisms for their role in initiating innate immune responses (Bell *et al*., 2003). In Arabidopsis, two of these Pep receptors have been identified – AtPEPR1 and AtPEPR2 (Yamaguchi *et al*., 2006; Yamaguchi *et al*., 2010). In order to initiate signaling in Arabidopsis, AtPep1 must first be produced from cleavage of a 92 amino acid precursor peptide, AtPROPEP1 by the cysteine protease METACASPASE4 (MC4) (Hander *et al*., 2019). After processing, the 23 amino acid AtPep1 is able to move to the apoplast, where it can bind PEPR1 or PEPR2 and induce heterodimerization with an LRR-RLK brassinosteroid insensitive 1-associated kinase (BAK1) coreceptor (Postel *et al*., 2010; Yamaguchi *et al*., 2010, Tang *et al*., 2015). BAK1 is then able to phosphorylate *Botrytis*-induced kinase 1 (BIK1), which further transduces this signal via activation of respiratory burst oxidase homolog D (RbohD) to produce ROS (Kadota *et al*., 2014, Li *et al*., 2014). Additionally, activation of the PEPRs allows for an influx of cytosolic Ca^{2+} through production of cGMP and activation of cyclic

nucleotide gated cation channels (CGNCs) such as CGNC2 (Qi *et al*., 2010, Ma *et al*., 2012). This calcium allows for the activation of a number of calcium-dependent protein kinases (CDPKs) which ultimately induce transcriptional changes within the cell and upregulate defense related genes such as *PDF1.2, WRKY33,* and *PROPEP1* (Huffaker *et al*., 2006, Ma *et al*., 2013). PEPR activation also induces a variety of other changes, such as alkalinization of the apoplast and production of the defense signal nitric oxide, as well as production of secondary metabolites (Huffaker *et al*., 2006; Huffaker *et al*., 2015). As an output, these effects culminate in a decrease in susceptibility to pathogens such as *Pseudomonas syringae, Pythium irregulare* and *Cochliobolus heterostrophus* in various plants (Huffaker *et al*., 2006, Yamaguchi *et al*., 2010; Huffaker *et al*., 2011). Pep signaling has also been implicated as a mediator of antiherbivore defenses in maize, as it stimulates production of defense chemicals that were demonstrated to limit larval growth and attract beneficial parasitoids (Huffaker *et al*., 2013, Huffaker *et al*., 2015). However, many components of the Pep signaling pathway have yet to be identified and characterized, meaning that there are still many avenues to explore and better understand this pathway in plant immunity.

1.4 Decoy receptors and a possible role for INHIBITOR OF PEP ACTION

We recently identified a gene locus (At1g73066) with a high degree of similarity to *PEPR1* and *PEPR2*. However, this gene appeared to lack the transmembrane and kinase domains of the aforementioned receptors. Additionally, this gene was tandemly arrayed with *PEPR1* in the genome. This fact, along with altered *IPA* gene expression patterns upon treatment with elicitors or inoculation with pathogens (http://bar.utoronto.ca/eplant/), suggests that the gene may play a role in

AtPep signaling. The presence of an LRR domain and possible ligand binding site with a lack of any other obvious functional domains is suggestive of a decoy receptor regulatory mechanism well-characterized in mammals, but to not widely observed in plants.

Decoy receptors generally act as negative regulators by competing for a specific ligand, thus sequestering it away from activating the cognate receptor (Jenkins *et al*., 2000, Felix *et al*., 2017). Decoy receptors tend to exist in two forms – membrane-bound or soluble. Membrane-bound decoy receptors tend to mimic the structure of a ligand's true receptor but lack an intracellular catalytic domain and thus are unable to transduce a signal upon ligand binding (Jenkins *et al*., 2000). These decoy receptors are most frequently produced by alternative splicing of a functional receptor gene (Vorlová *et al*., 2011). Soluble decoy receptors are not bound to the membrane and usually consist solely of a ligand binding domain. While soluble receptors can also be generated by alternative splicing of a receptor gene, they are more commonly produced by ectodomain shedding, or cleavage of a pre-existing receptor protein to release the ligand binding domain (Peschon *et al*., 1998, Reddy *et al*., 2000). Possibly the most well characterized example of this can be found in the signaling pathway of Interleukin-1 (IL-1), a pro-inflammatory signal in mammals. Activation of this pathway is achieved through the binding of IL-1 to its receptor, IL-1 receptor 1 (IL-1R1) (Wang *et al*., 2010). However, this activation is modulated by the presence of IL-1 receptor 2 (IL-1R2), a separately encoded protein that resembles IL-1R1 but lacks an integral intracellular signaling domain, as well as a soluble form of

the decoy receptor (sIL-1R2) produced via ectodomain cleavage (Wang *et al*., 2010, Re *et al*., 1994).

This evidence suggests that the gene identified in Arabidopsis, referred to as INHIBITOR OF PEP ACTION (IPA), appears to be a separately encoded soluble decoy receptor for PEPR1 and PEPR2. In order to evaluate the accuracy of this hypothesis, IPA was characterized with regards to its localization and effects on AtPep1 signaling in Arabidopsis. IPA is shown to localize in the apoplast, where AtPep1 is secreted in order to activate signaling, as well as to negatively regulate AtPep1 signaling. Finally, the ability of IPA to compete with PEPR2 for AtPep1 binding is shown in order to demonstrate a mode of action for regulation of the Pep signaling pathway. These results suggest that IPA may serve as a novel decoy receptor in the Pep signaling pathway of Arabidopsis.

2. Materials and Methods

2.1 Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used as a wild-type reference plant. The *pepr1/pepr2* double mutant has been previously described (Yamaguchi *et al.*, 2010) and was used as a knockout mutant for all relevant bioassays. Arabidopsis seeds were sterilized in a sealed chamber containing chloric gas (50 mL 100% bleach, 1.5 mL HCl 36.5%) for 2-4 hours. Seeds were plated aseptically on half-strength Murashige and Skoog (MS) media containing 0.8% phytoagar and stratified for 2-5 days at 4°C. Seeds were germinated in a light- and humidity- controlled growth chamber (22°C, 12h light/12h dark) and transplanted to soil after 2 weeks. *N. benthamiana* seeds were sowed in soil and grown at 22°C under

16h light / 8h dark conditions. 5- to 6- week old plants were utilized for all *N. benthamiana* assays.

2.2 Sequence homology analysis

Full-length amino acid sequences for *IPA* (At1g73066), *PEPR1* (At1g73080), and *PEPR2* (At1g17750) were obtained from the Arabidopsis Information Resource (TAIR, https://www.arabidopsis.org/) and aligned using Clustal Omega version 1.2.4. Percent identity between proteins was determined using BlastP (Stephen *et al*., 1997) version 2.9.0. The shaded diagram of amino acid homology was constructed using Boxshade (https://embnet.vital-it.ch/software/BOX_form.html). Domain annotations were based on previously published work (Yamaguchi *et al.*, 2010).

2.3 Construction of plasmid constructs and transgenic plant materials

The *IPA* and *PEPR2* coding sequences were amplified from Arabidopsis cDNA using gene-specific primers and standard PCR. The primers used are listed in Table 1. The PCR fragments were initially cloned into the pENTR D-TOPO vector and later recombined into expression vectors pGWB441 and pGWB414. The amiRNA IPA construct was generated using the genomic-scale artificial microRNA library, as described (Hauser *et al*., 2013, Hauser *et al*., 2016). All constructs were sequenced in order to verify a lack of mutations prior to transformation into *Agrobacterium tumefaciens* strain GV3101. The constructs were subsequently expressed in plants either through transient transformation of *N. benthamiana* or floral dip transformation of Arabidopsis.

2.4 Arabidopsis floral dip transformation

Wild-type or *pepr1/pepr2* double knockout plants were transformed using the floral dip method (Clough and Bent 1998) with modifications. Briefly, *A. tumefaciens* strain GV3101 containing the plasmid construct to be transformed was grown in 50 mL of Luria-Bertaini medium (LB) containing selective antibiotics to a final OD_{600} of 0.5-0.8. The bacterial culture was added to a 120 mL solution of 5% sucrose and 0.03% Silwet L-77. Arabidopsis flowers and bolts were submerged in the bacterial solution and gently agitated for approximately 10 seconds. After dipping, flowers were covered and stored in low-light conditions for 16-24 hours before being returned to normal growth conditions.

2.5 *Agrobacterium tumefaciens* growth and preparation

Agrobacterium strain GV3101 containing the plasmid construct to be expressed was streaked on LB medium containing 1.5% agar with selective antibiotics and grown at 28℃ for 2 days. A singular colony from the streaked plate was used to inoculate a liquid culture of LB containing appropriate antibiotics. The OD_{600} of the culture was measured until it fell within the range of 0.6-1.25 (20-24 hours after inoculation). Cells were harvested via centrifugation (5000xg, 5 minutes) and subsequently used for agroinfiltration or floral dip.

2.6 Agroinfiltration and transient transformation of *Nicotiana benthamiana*

Harvested *Agrobacterium tumefaciens* cells were resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 200 μM acetosyringone) to a final OD_{600} of 0.45 for single-construct infiltrations. For co-infiltrations, the cells were diluted to an OD_{600} of 0.9 for each individual construct and combined to achieve a final OD_{600} of

0.45 for each construct. The bacteria was allowed to equilibrate in buffer for 1 hour at 22°C prior to infiltration. The third and fourth leaves of 5- to 6- week old *Nicotiana benthamiana* plants were infiltrated using a blunt end syringe. The leaves were sampled for relevant bioassays 24 hours after infiltration unless otherwise noted.

2.7 Subcellular localization of IPA and plasmolysis

N. benthamiana leaves transiently transformed with YFP-tagged constructs of genes under the control of the 35S promoter were sampled for microscopy and visualized using a confocal microscope (Nikon Eclipse TE2000-U) at 20x magnification. The wavelengths for YFP excitation and emission were 514 nm and 527 nm, respectively. Plasmolysis was performed by incubating leaf tissue in 0.75 M mannitol solution for 15 minutes. All image processing and colorization was completed using ImageJ.

2.8 ROS assay (Luminol-based ROS detection and measurement)

Detection of ROS responses in Arabidopsis were performed as previously described (Smith and Heese 2014), with modifications. 1 day prior to the assay, leaf disks from 4-to 5- week old plants were floated adaxial side up in wells of a 96-well microtiter plate filled with 100 µl ddH2O. In trials involving Arabidopsis, two leaf disks from independent plants were placed in each well without overlap whereas trials involving *N. benthamiana* utilized one leaf disk per well. To reduce wounding response, the plate was then incubated overnight at 22°C under continuous light. Prior to elicitation, a 2x elicitation solution containing 20 µg/mL HRP, 34 µg/mL Luminol, as well as 20 nM AtPep1, 100 nM Flg22, or 2 µM flg22,was prepared. 100 µl of solution was added to each well using a multichannel pipette and the plate was placed

without delay into a BioTek Synergy H1 microplate reader (BioTek) to measure ROS production over the course of 30 minutes.

2.9 Gene expression analysis of Arabidopsis seedlings

Col-0 Arabidopsis seedlings were grown in half-strength MS medium for 7 days. Seedlings were then treated with 1 μM AtPep1 and harvested after the indicated amount of time. For assays testing root gene expression, root tissue was excised and harvested after the given amount of time. RNA was extracted from samples to analyze gene expression of *PEPR1* and *IPA*.

2.10 Protein extraction

Plant tissue was snap-frozen in liquid nitrogen and ground into powder. 3x Lamellae buffer (0.24 M Tris-Hcl pH 6.8, 6% SDS, 30% glycerol, 16% Beta-mercaptoethanol, 0.006% bromophenol blue, 10M urea) was added to the tissue in a 1:1.5 (m/v) tissue to buffer ratio and boiled at 95℃ for 5 minutes. The samples were then centrifuged (3 minutes, 13,000xg) and the supernatant collected for Western blotting.

2.11 Western blot

Extracted protein samples were separated on an 8% SDS-PAGE gel and transferred to a nitrocellulose membrane using a Trans-Blot Turbo Transfer System (Bio-Rad) according to the manufacturer's instructions. The membrane was blocked using a TBS-Tween (TBST) solution containing 5% non-fat milk prior to incubation with TBST containing 2% BSA and a 1:1000 dilution of primary antibody (α-GFP rabbit or α-HA mouse, Sigma) overnight at 4℃. After at least five washes with TBST for 5 min each, the blot was incubated with TBST containing a 1:1000 dilution of

secondary antibody (α-rabbit-HRP conjugate or α-mouse-HRP conjugate, respectively, Sigma) for at least 2 hours. After at least five more TBST washes, the blot was visualized using the SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific) and a Bio-Rad Molecular Imager Chemidoc™ XRS+ imaging system. The blot was stained with Ponceau in order to visualize protein loading.

2.12 RNA extraction / RT-qPCR

Total RNA was isolated from snap-frozen and powdered tissue samples using Trisure (Bioline) according to manufacturer's instructions. 6 ug of the isolated RNA was treated with DNAseI (Invitrogen) in order to remove genomic DNA contamination and run on an agarose gel to ensure RNA integrity. 2 ug of this RNA was reverse-transcribed in order to synthesize cDNA using M-MLV reverse transcriptase (Invitrogen) according to manufacturer's instructions. cDNA integrity was tested via PCR prior to further use. Quantitative PCR was performed using a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad) in conjunction with SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad). The expression levels of each gene of interest were normalized to those of a reference gene, *ACTIN2* (At3g18780) or *GAPDH* (At1g13440).

2.13 Alkalinization assay

Arabidopsis T87 suspension-cultured cells were pretreated with water or the indicated concentrations of AtPep1 prior to the experiment. 24 hours after pretreatment, the pH of the suspension cells was measured and cells were treated with water or 20 nM AtPep1. The pH of the media was measured 15 minutes post treatment and the suspension cells were harvested for gene expression analysis.

2.14 Statistical analyses

Statistical analyses were conducted using Excel 2016 (Microsoft Inc.) and vassarstats.net. Student's unpaired t-test was utilized to make pairwise comparisons, and unpaired ANOVAs were used for multiple comparisons between groups. The Tukey test was utilized to analyze significance of differences between trials with multiple variables.

3. Results

3.1 The structure of *IPA* resembles that of the *PEPR1* and *PEPR2* LRR domain

The LRR domain of an LRR-RLK canonically is the region of protein responsible for direct interaction with a ligand (Ng and Xavier 2011). Therefore, the structure of this domain largely determines binding affinity and specificity. The At1g73066 gene encodes a predicted 598 amino acid (65.5 kDa) protein with a high degree of similarity to the LRR domains of *PEPR1* and *PEPR2* (Fig. 1). This protein, comprised of a hydrophobic N-terminal secretion signal sequence followed by 21 sequential repeats of a 24-residue LRR motif (residues 75 to 574), maintains a 77% and 65% identity to the LRR domains of *PEPR1* and *PEPR2*, respectively. Additionally, of the 25 residues directly involved in the interaction of PEPR1 with AtPep1, 22 are conserved in IPA (Tang *et al*., 2015). Two of the residues that differ still share similar properties between their PEPR1 and IPA counterparts (D179 to E177, I463 to V461, respectively). Only one interacting residue, E439, has a dissimilar analog in IPA (I437). This similarity to the *PEPR*s served as an indicator of the protein's possible role in the AtPep1 signaling pathway. Moreover, At1g73066 appears to resemble a truncated form of the receptors, lacking the amino acids that

comprise the transmembrane and kinase domains of a canonical LRR-RLK. In addition to the fact that At1g73066 (subsequently referred to as *IPA*) does not seem to contain any other predicted functional domains suggests that the protein may function solely to bind to a ligand. This observation spurred further analysis as to the effects of *IPA* on plant innate immune responses, as well as indicated the protein's possible role as a soluble decoy receptor.

3.2 Induction of *IPA* expression is specific to root cells treated with AtPep1

Activation of the Pep signaling pathway results in an overall transcriptional reprogramming of the cell, resulting in an upregulation of genes such as *PEPR1* and *PEPR2*, as well as the precursor peptide *PROPEP1* (Yamaguchi *et al*., 2010). These effects suggest that other genes involved in the regulation and activation of Pep signaling may show an altered transcriptional profile as a result of AtPep1 treatment. Additionally, transcriptional analysis of wild-type Arabidopsis plants indicates that *IPA* appears to be induced upon treatment with *Pseudomonas syringae* (Waese *et al*., 2017), indicating a possible role for the gene in general PTI responses. In order to determine if these *IPA* transcriptional changes are connected specifically to AtPep1 signaling, Col-0 seedlings were treated with a 1μM concentration of AtPep1 and incubated at different timepoints (0, 0.5, 1, 1.5, 2, 3, 4 and 5 hours). qRT-PCR analysis of these seedlings revealed an expected upregulation of *PEPR1* expression, peaking at an 8-fold increase 1 hour post treatment, but no corresponding induction of *IPA* over the course of 5 hours (Fig. 2A). Despite this apparent lack of change in *IPA* transcription in whole seedlings, a previous observation that expression of the *PROPEP* genes is largely root-specific (Bartels *et al*., 2013) suggested that *IPA*

expression might also mirror this expression pattern, especially given the protein's suspected interaction with AtPep1. A similar analysis of gene expression was conducted again, and this time seedling roots were excised after treatment in order to test whether *IPA* expression was specific to root tissue (Fig. 2B). In this assay, a clear upregulation of *IPA* was readily observed, with transcript levels doubling only 45 minutes post elicitation before reaching a maximum 5-fold increase after 2 hours. Additionally, the magnitude of root *PEPR1* expression resembled that of whole seedlings, with a 10-fold expression increase peak. However, the timeline of this upregulation appeared slightly delayed and extended, with root expression remaining high through 2 hours post-elicitation and peaking at 90 minutes. Peak expression of *IPA* transcripts appeared to be induced later than *PEPR1* by approximately 30 minutes. This indicates that *IPA* may serve a more downstream role in the Pep signaling pathway. To investigate whether this altered *IPA* expression was specific to AtPep1, seedlings were also treated with other elicitors, such as the MAMPs derived from bacterial flagellin (flg22) and elongation factor Tu (elf18) (Fig. 2C). Treatment with these MAMPs produced no obvious fluctuation in transcript levels over the course of 4 hours. These results suggest that rapid *IPA* expression pattern changes are indeed specific to AtPep1. However, these results do not preclude the possibility that *IPA* upregulation may occur at a later time point in response to elicitation with other upstream MAMPs.

3.3 IPA localizes to the apoplast

Similarities between *IPA* and the *PEPR*s, particularly with regard to their secretion signals, suggests that the proteins may share similar subcellular localizations.

In order to better understand IPA localization, a p35s:IPA-YFP construct was transiently expressed in *N. benthamiana* leaves and visualized using confocal microscopy. In order to differentiate between apoplastic and plasma membrane localization, plasmolysis was used in order to expand the apoplastic space and shrink the plasma membrane away from the cell wall (Zavaliev *et al*., 2016). IPA-YFP appeared to localize to the periphery of the cell, similar to other LRR-RLKs under normal conditions (Fig. 3A, B). However, plasmolysis revealed an expansion of IPA-YFP into the apoplastic space, confirming that the protein is soluble rather than anchored to the plasma membrane (Fig. 3C). This localization places IPA in a similar space to that occupied by the LRR domain of the PEPRs and secreted AtPep1 (Yamaguchi and Huffaker 2011). As such, the spatial possibility of IPA competing with the PEPRs for AtPep1 binding appears supported.

3.4 Upregulated *IPA* expression correlates with a decrease in Arabidopsis Pep-induced alkalinization

Decoy receptors tend to act as negative regulators of signaling pathways due to their ability to sequester ligands away from active receptors (Felix *et al*., 2017). The presence of a decoy receptor would thus be expected to downregulate responses associated with ligand-receptor interactions, such as the extracellular alkalinization response observed after AtPep1 treatment in Arabidopsis cells (Huffaker *et al*., 2006). To determine whether *IPA* acts as a negative regulator of Pep signaling, the expression of *IPA* in Arabidopsis suspension-cultured cells, which closely resemble root cells (Axelos *et al*., 1992), was correlated with differing levels of extracellular alkalinization after AtPep1 treatment. Suspension cells were pretreated 24 hours prior

to testing with either low (10 nM) or high (100 nM) concentrations of AtPep1 or a water control. After 24 hours, cells were challenged with water, AtPep1, or Flg22 treatments. Cells pretreated with low concentrations of AtPep1 exhibited much stronger AtPep1-induced alkalinization than water-pretreated cells (Fig. 4A), indicating a potentiation effect. This relates to previous work suggesting that pretreatment of cells with Peps can "prime" them to better respond to subsequent elicitation (Yamaguchi *et al*., 2010). However, cells pretreated with a higher concentration (100 nM) of AtPep1 exhibited no extracellular alkalinization beyond that of water controls upon subsequent Pep treatment. This indicated that treatment with high levels of AtPep1 triggered a desensitization to subsequent elicitation. Responses to flg22 were also somewhat dampened in cells pretreated with high concentrations of AtPep1. Interestingly, the apparent desensitization to AtPep1 correlated with an upregulation of *IPA* expression in cells pretreated with 100 nM AtPep1 (Fig. 4B). This suggests a possible link between IPA and AtPep1 elicitation of the extracellular alkalinization response, and AtPep1 elicitation. Specifically, the data seems to indicate that *IPA* expression is not only induced by high concentrations of AtPep1, but also may negatively regulate PTI-induced responses. This is also supported by the similar levels of both *PEPR1* and *PEPR2* expression maintained among pretreatments, suggesting that the differences in alkalinization response were not due to an increase in receptor expression for AtPep1 (Fig. 4B).

3.5 IPA is capable of competing with PEPR2 for AtPep1 binding

One of the key characteristics of a decoy receptor is the ability to directly interact with and compete for a target ligand as a means of regulation (Felix *et al.*,

2017). This competition serves to make the ligand unavailable for binding to its intended receptor, reducing signal transduction and thereby negatively regulating the signal transduction pathway as a whole. The ability of IPA to compete with PEPRs for AtPep1 ligand binding was examined in order to determine if IPA was able to function as expected in this context. Leaves of wild-type *N. benthamiana* plants were infiltrated with constructs such that they transiently expressed combinations of PEPR2-YFP, IPA-YFP, and EV-YFP (empty vector). It has been shown previously that the heterologous expression of novel PRRs can confer the ability to respond to the corresponding ligands with no additional modifications to the plant (Yamaguchi *et al*., 2006, Lacombe *et al*., 2010). Additionally, the observation that functional PEPR1 has been successfully expressed in *N. benthamiana* suggests that the proteins expressed should remain similarly functional in this system (Yamaguchi *et al*., 2006). Using this framework, the ability of IPA to competitively bind AtPep1 was tested by measuring effects on inducible ROS production. Transformed leaves corresponding to the combinatorial constructs were tested for their ability to produce ROS after AtPep1 elicitation (Fig. 5A). Leaves expressing PEPR2-YFP alone showed a strong response to AtPep1 elicitation with high levels of ROS produced. In contrast, in leaves expressing EV-YFP, AtPep1 did not induce the production of a significant amount of ROS. Interestingly, leaf samples expressing both PEPR2-YFP and IPA-YFP exhibited reduced responsiveness to AtPep1 treatment compared to PEPR2-YFP-expressing leaves. 5 nM of AtPep1 resulted in a ROS burst only slightly higher than that produced by nonresponsive cells, while 10 nM of peptide elicited only half the response achieved in cells expressing PEPR2-YFP alone. These responses suggest a

dose dependent regulatory mechanism for this inhibition. Additionally, cells expressing IPA-YFP did not appear to perceive and respond to AtPep1, indicating that the truncated structure of IPA renders it unable to transduce a signal. These differences in responsiveness appeared to be linked solely to construct expression, as all transformed leaves appeared to respond similarly to elicitation with 100 nM flg22 (Fig. 5A). Transformed leaves were also shown to express comparable levels of protein (Fig. 5B), suggesting that the different responses of PEPR2-YFP leaves compared to IPA-YFP plus PEPR2-YFP expressing leaves were due to the presence of IPA rather than a depletion of PEPR2. Taken together, these results suggest that IPA is capable of competing with PEPR2 for AtPep1 and support the hypothesis that IPA acts as a decoy receptor for Pep signaling.

3.6 Generation of transgenic p35S:IPA and amiRNA IPA lines

In order to better understand the effects of *IPA* levels on inducible immune responses, stable transgenic lines expressing an IPA-YFP or IPA-3xHA construct under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter were created. Additionally, amiRNA lines with depleted levels of *IPA* were generated by transforming Arabidopsis plants with an artificial microRNA (amiRNA) construct designed to specifically bind *IPA* transcripts (Schwab *et al*., 2006; Hauser *et al*., 2013; Hauser and Ceciliato *et al*., 2016). Binding of this amiRNA to the transcripts of the targeted gene lead to the formation of double stranded RNA, which is recognized by the plant as a degradation target for the DICER enzyme (Tijserman and Plasterk 2004). This causes the gene transcript to be degraded and creates a signal for more distal parts of the plant to also degrade the target transcript (Smith *et al*., 2007). To

confirm the altered *IPA* levels of each transgenic line, protein and gene transcript levels were quantified and compared to a wild-type standard. For the overexpressor lines, a Western blot was performed to visualize accumulation of IPA-YFP or IPA-3xHA protein and confirm the expression of the construct in T_3 lines. The amount of IPA-YFP present in each of the tested independent lines appeared roughly equivalent relative to the amount of total protein loaded (Fig. 6A), indicating that these lines expressed relatively similar quantities of increased IPA. The levels of IPA-3xHA, while not directly comparable to those of the other p35s:IPA lines, also indicated successful transformation and overexpression (Fig. 6B). Quantification of *IPA* expression in the amiRNA lines was carried out through gene expression analysis of T_2 plants, revealing a strong reduction in transcript levels (Fig. 6C). Most lines produced less than 20% of the transcripts found in similarly grown wild-type plants, while one line (number 9) only a 50% reduction of gene levels. These transgenic lines were utilized to characterize the effects of altered *IPA* levels on inducible defenses in subsequent bioassays.

3.7 IPA negatively regulates Pep-induced ROS production in Arabidopsis

In order to determine if IPA has a role in regulating the functional Arabidopsis Pep signaling pathway, Pep-induced ROS production was measured in the transgenic lines described previously. Upon elicitation, plants constitutively expressing *IPA* appeared to have reduced ROS accumulation after AtPep1 treatment (Fig. 7A). This phenotype, while somewhat variable between individual plants, remained consistent overall between the p35s:IPA lines with an average 40% reduction of ROS burst intensity. This reduction in ROS production represented a partial inhibition of AtPep1

signaling, as compared to full Pep signaling blockage exhibited by the *pepr1/pepr2* double knockout. AtPep1 treatment of the amiRNA IPA lines resulted in a 20-30% increase of ROS production (Fig. 7B), an effect that supports a role for IPA as a negative regulator. These results suggest that IPA negatively regulates early Pep signaling events in particular, as reactive oxygen species generated through RbohD activation is a rapid response to PEPR activation. Notably, constitutive expression of *IPA* had a very weak effect on ROS production after treatment with flg22 (Fig. 7C). This effect is possibly due to the fact that FLS2 activation by flg22, which can also lead to phosphorylation of RbohD to produce ROS, also leads to PEPR activation for signal amplification (Ross *et al*., 2014). This weak effect on ROS production after flg22 treatment may indicate that while IPA does not affect ROS produced by initial FLS2 activation, it does affect the strength of the downstream PEPR activation and response. This may cause a less severe reduction of ROS burst intensity as observed here, although no such effect in observed in the *pepr1/pepr2* knockout line. No increased flg22 responses in amiRNA IPA lines were reproducibly observed (data not shown).

4. Discussion

4.1 IPA resembles a soluble decoy receptor form of PEPR1 and PEPR2

This study aimed to better characterize and understand the AtPep1 signaling pathway by investigating the potential regulatory role of IPA. Characterization of this protein was an investigation into a regulatory mechanism not widely observed in plants – namely, regulation by decoy receptors. The LRR domain of a canonical PRR directly interacts with a ligand and determines binding specificity. Therefore, the high

degree of similarity between the IPA LRR domains and that of the PEPRs suggested a similarity in binding function (Fig. 1). The lack of other obvious functional domains within the structure of IPA also seems to suggest that the protein serves no other function than binding AtPep1. Together this evidence, along with the observed localization of IPA to the apoplast (Fig. 3), indicated a possible role as a soluble decoy receptor for the PEPRs. Soluble decoy receptors commonly resemble the LRR domains of their corresponding receptors, often because they are formed by cleavage of this domain from the original receptor (Reddy *et al*., 2000). While IPA is encoded on a separate gene than the two PEPRs (At1g73066), the *IPA* gene is tandemly arrayed with PEPR1 in the Arabidopsis genome, suggesting a possible selective pressure for co-inheritance of both genes. This data suggests that IPA may serve an important regulatory role in AtPep1 signaling as a novel decoy receptor.

4.2 *IPA* expression patterns are similar to those of other AtPep1-responsive genes

AtPep1 signaling triggers transcriptional changes for numerous genes which play a role in Pep-induced innate immune responses (Huffaker *et al*., 2006, Yamaguchi *et al*., 2010). Because of this, altered expression of *IPA* after AtPep1 treatment suggested that the gene might play some role in the Pep signaling pathway (Fig. 2C). The fact that this induction was specific to root tissue coincidentally mirrored expression patterns of many of the *PROPEP* genes (Bartels *et al*., 2013). The observed lack of altered *IPA* expression after treatment with MAMPs such as elf18 and flg22 (Fig. 2E) also suggested a role specific to the Pep signaling pathway. There is the possibility that *IPA* expression is affected by MAMP elicitation, but on a slower timescale than that which was measured here. Notably, experiments involving

suspension-cultured cells indicated that *IPA* expression was upregulated after pretreatment with high concentrations of AtPep1 (Fig. 4B). Pretreatment with AtPep1 has been shown to alter the plant's response to subsequent elicitation, with some data indicating increased pathogen resistance and others revealing decreased intensity of defense-related phenotypes (Yamaguchi *et al*., 2010; Flurry *et al*., 2013). Based on this information, it is possible that while pretreatment with high concentrations of AtPep1 induces a transcriptional change, MAMP-induced cleavage of PROPEP1 may not produce enough AtPep1 for drastic changes in *IPA* expression to occur. This would still suggest that *IPA* is produced specifically as a result of high-capacity Pep signaling. *IPA* may reduce Pep signaling in these situations as a negative feedback loop.

4.3 IPA negatively regulates early AtPep1-triggered immune responses

Activation of the AtPep1 signaling pathway and other PRR signal cascades triggers a variety of shared downstream responses. One of the earliest of these responses is the rapid production of ROS, resulting from PEPR and BAK1 heterodimerization, BIK1 activation, and subsequent phosphorylation of RbohD (Lin *et al*., 2014; Kadota *et al*., 2014). This response, which occurs within minutes of elicitation, transpires in parallel with the alkalinization of the surrounding environment due to the inactivation of a membrane proton ATPase (Huffaker *et al*., 2006, Pearce and Ryan 2003). Due to the relatively rapid nature of these responses, alterations of early signaling pathway components should result in changes regarding these phenotypic responses. *IPA* was identified as a regulator of early AtPep1 signaling in this way, as an increase in *IPA* expression of suspension cells was correlated with a

decreased alkalinization response (Fig. 4A,B). This correlation did not directly indicate that IPA caused this change in alkalinization, but the protein's role as a negative regulator was supported by the observation that transgenic p35s:IPA lines exhibited a decreased ROS response, while amiRNA IPA lines produced a more robust response in Arabidopsis plants (Fig. 7A, B). These pieces of evidence, taken together, suggest that IPA acts to inhibit early signaling responses in the AtPep1 pathway. However, whether or not this regulation is specific to AtPep1 signaling needs be explored more deeply, as *IPA* expression also appeared to somewhaty affect ROS production in response to Flg22 elicitation in Arabidopsis (Fig. 7C). This may be due to an effect of IPA on downstream ROS produced from the Pep signaling pathway, rather than a direct effect of IPA on FLS2 activation.

4.4 IPA competitively binds to AtPep1

Decoy receptors act as negative regulators by sequestering ligands away from their receptors, thus preventing the initiation of signal transduction (Felix and Savvides 2017). Therefore, a decoy receptor would be expected to affect even the earliest observable responses of the pathway. IPA fulfilled these expectations, as it was shown to compete with PEPR2 for binding of AtPep1 and reduce the resulting intensity of ROS response in *N. benthamiana* (Fig. 5A). This significant reduction in signaling intensity occurred despite consistent PEPR2 protein levels in leaves expressing and not expressing IPA, as well as a lower concentration of IPA in comparison to PEPR2 (Fig. 5B). This indicates that IPA may exhibit a higher binding affinity for AtPep1 than PEPR2. However, a repetition of this assay in which IPA competes with PEPR1 for AtPep1 binding may provide valuable insight as to whether

this trend holds true for both receptors. While IPA is likely to act as a decoy receptor for both PEPR1 and PEPR2 based on sequence homology (Fig. 1), it is possible that the protein may exhibit different effects on PEPR1. PEPR1 contributes more significantly to the activation of AtPep1 signaling, yet has a lower affinity for AtPep1 than PEPR2 (Yamaguchi *et al*., 2010). Additionally, while these results seem to strongly indicate a competitive binding mechanism for AtPep1 signaling, there is a possibility that IPA may instead serve as a negative regulator due to interaction with the coreceptor BAK1 rather than via direct binding of AtPep1. However, analogs to the PEPR1 LRRs responsible for interacting with BAK1 do not exist in the truncated IPA protein (Tang *et al*., 2015). Additionally, the seemingly dose-dependent nature of IPA regulation appears to suggest otherwise. A more conclusive demonstration of this could be achieved by treating cells with increasing concentrations of AtPep1. This would show whether a complete restoration of ROS production is achieved in tissue expressing both IPA and PEPR2, as sufficiently high concentrations of ligand should be able to saturate both receptor and decoy receptor alike

4.5 Proposed model

Although previously uncharacterized in plants, this work describes the existence of a possible novel decoy receptor as a regulator of the Pep signaling pathway. Upon activation of the Pep signaling pathway, additional PROPEP1 is produced and processed, resulting in the production and release of additional AtPep1 (Yamaguchi *et al*., 2010). This positive feedback loop increases the concentration of extracellular AtPep1 and amplifies AtPep1 signaling. However, at some high concentration of extracellular AtPep1, possibly caused by overstimulation of the

pathway or a buildup of Pep over time, *IPA* transcription is upregulated and the produced protein is secreted into the extracellular space. The presence of IPA in the apoplast allows it to bind to AtPep1 and sequester the ligand away from the PEPRs, negatively regulating the activation of the pathway (Fig. 8). In this way, IPA may act as a mechanism to reduce Pep signaling or avoid possible consequences associated with overstimulation of innate immune responses. Based on the data collected in this work, this model serves to explain both the expression patterns for *IPA* and its larger role within the Pep signaling pathway.

5. Future directions in IPA characterization

While a tentative model for IPA function has been established, additional work will allow for a better understanding of the protein. The data obtained here suggests that IPA is produced in the presence of high levels of AtPep1, directly binding to the peptide and sequestering it away from the PEPRs (Fig. 8). However, the ability of IPA to compete similarly with PEPR1 for ligand binding remains to be determined. Additionally, a direct interaction between IPA and AtPep1, or any other AtPep, has yet to be shown directly. Further characterization of the transgenic Arabidopsis lines with reduced or increased *IPA* expression would also aid in understanding the effects of IPA on Pep signaling and immunity. Further phenotypic examinations of these lines, evaluating defense responses such as marker gene expression, ethylene emission, and especially susceptibility to pathogens, would further outline the function of IPA and may help to elucidate any specificity in regulating the AtPep1 pathway. Additionally, the ability of IPA to repress defense responses may make it a target for upregulation by pathogens attempting to invade the plant. This is suggested by an

apparent upregulation of IPA expression in Arabidopsis leaves treated with *Pseudomonas syringae* pv. *tomato* DC3000, a virulent bacterial pathogen (Waese *et al*., 2017). When compared with the downregulation of IPA that seems to occur upon infection with the avirulent form of the pathogen, the possibility of IPA as a target for manipulation seems likely. Thus far, we have not been able to reliably replicate these results in our lab (data not shown), but it still remains an avenue of exploration. As a final point, *ipa* knockout lines were not used in this work due to what may be a lethal phenotype. Multiple SALK T-DNA lines for *ipa* are available to order – however, attempts to grow these lines resulted in poor germination rates across all 5 lines examined (data not shown). Of the plants that did germinate, all were genotyped as wild-type. For this reason, amiRNA IPA lines displaying a reduced level of *IPA* gene expression were utilized as an alternative to true knockout mutants. This observation, coupled with an observation that *IPA* expression is incredibly high in pollen granules, suggests that IPA may play a crucial role in maintaining pollen viability. This would be consistent with the low expression of *PROPEP1* in pollen (Waese *et al*., 2017). It is possible that IPA serves as an additional regulatory mechanism to prevent perturbations in pollen homeostasis that would result from activation of AtPep1 signaling, as pH changes alone have been shown to negatively affect pollen germination and growth (Fan *et al*., 2001). If this is the case, then generation of a stable genetic knockout may prove futile.

6. Conclusions

The identification of IPA as a negative regulator for the Pep signaling pathway helps provide more insight into potential mechanisms by which Pep signaling is

inactivated once a pathogen or attack has been dealt with. The existence of IPA also paves the way for new and closer examinations into other possible decoy receptors in plant systems, either as regulators of other PRRs or in other contexts. Although IPA appears to be a decoy receptor encoded by a separate gene, it is possible that other such receptors are generated by alternative splicing and proteolytic cleavage, as described previously (Peschon *et al*., 1998, Vorlová *et al*., 2011). Understanding the mechanisms by which *IPA* expression is induced may also provide insight into how pathogens evade or neutralize the plant immune system. Altogether, continued study of IPA opens a new door for many lines of inquiry into plant immune regulation, as well as possible regulation of other pathways as well.

Figures

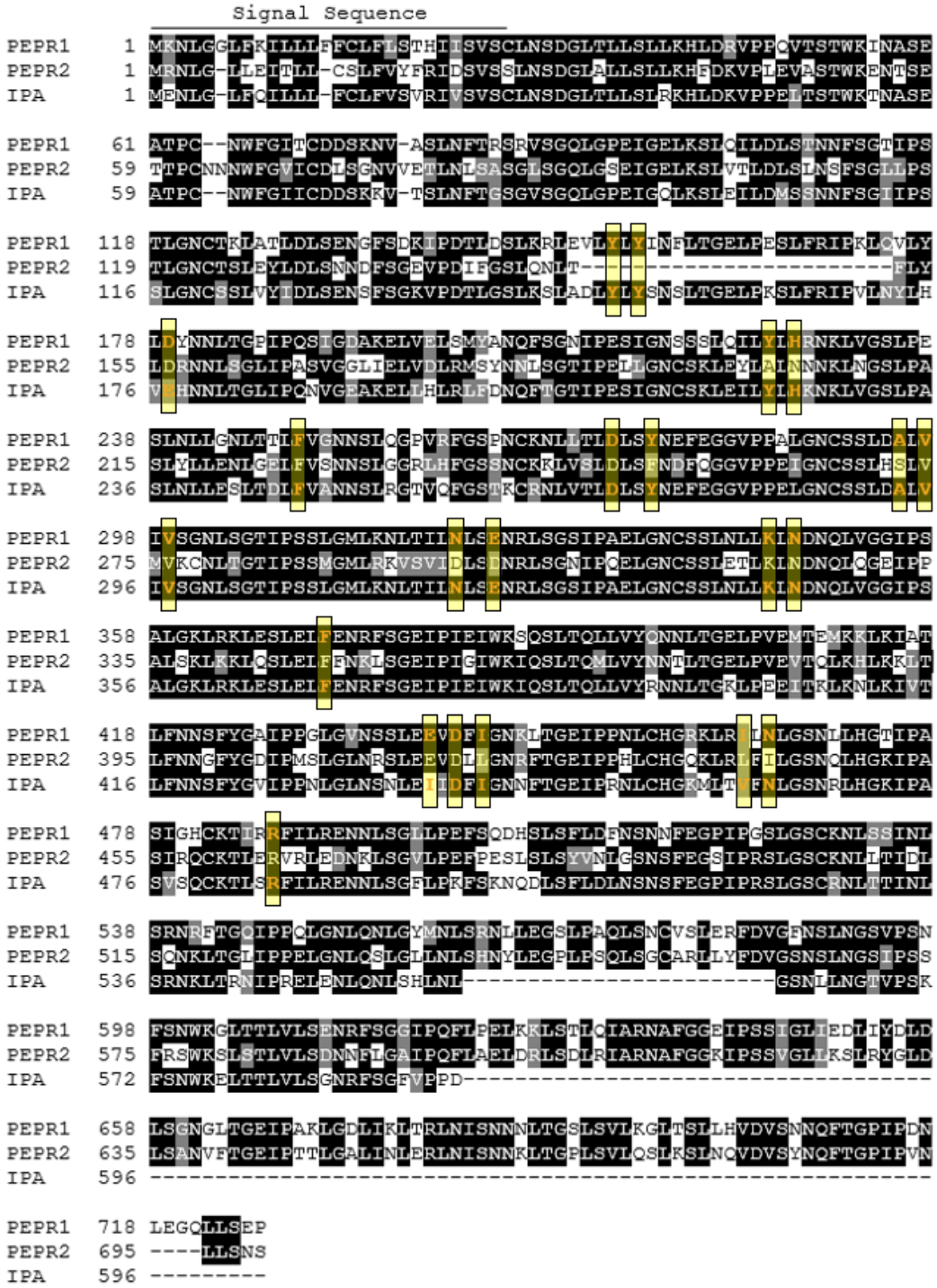

Figure 1 IPA shows a high degree of sequence homology to the LRR domain of PEPR1 and PEPR2 . The amino acid sequences of the LRR domains of PEPR1, PEPR2, and IPA were aligned using the Clustal Omega program. Identical amino acids are highlighted in black, while residues with similar properties are highlighted in gray. PEPR1 residues directly involved in interactions with AtPep1 and the corresponding residues in other proteins are highlighted in yellow boxes (Tang *et al*., 2015). The predicted signal peptide sequence is indicated above the first 28, 26 and 26 amino acids for PEPR1, PEPR2, and IPA, respectively.

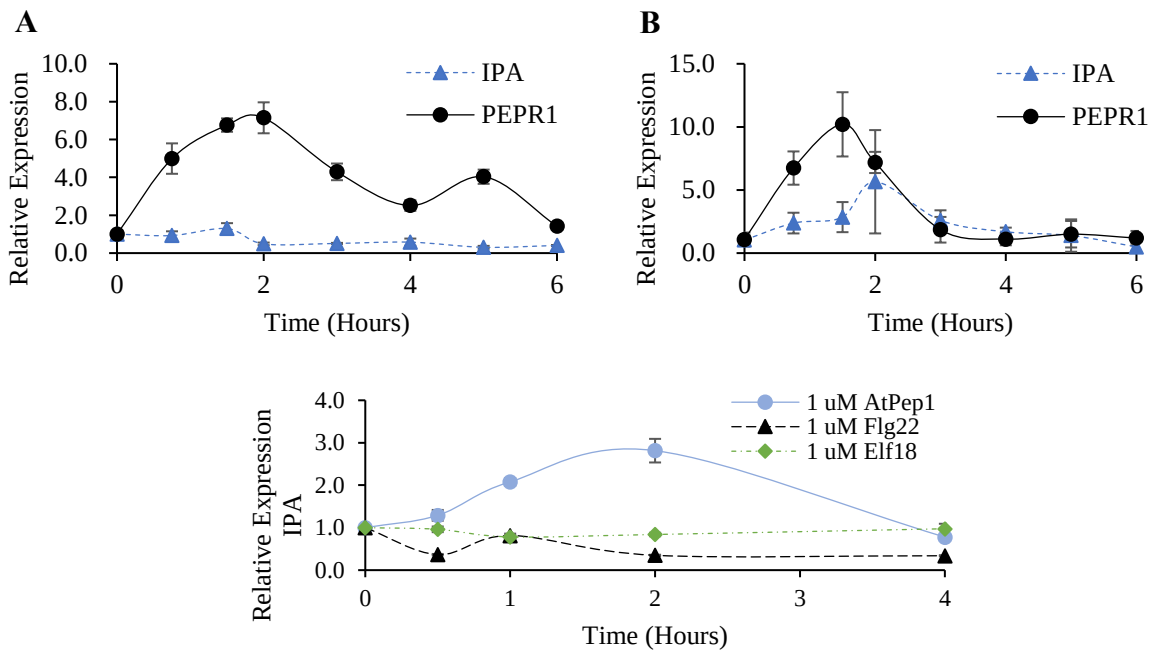

Figure 2. *IPA* expression in root tissue is induced by AtPep1. Wild-type seedlings were grown in 1/2x MS medium for 7 days prior to treatment. At 0 minutes, seedlings were treated with AtPep1, elf18, or flg22 at a concentration of 1μM. At the indicated time points, (A) whole seedlings or (B,C) seedling roots were harvested to analyze expression of the indicated genes. *GAPDH* expression was used as a reference gene. Error bars represent standard deviation between two biological replicates (A,B) or two technical replicates (C).

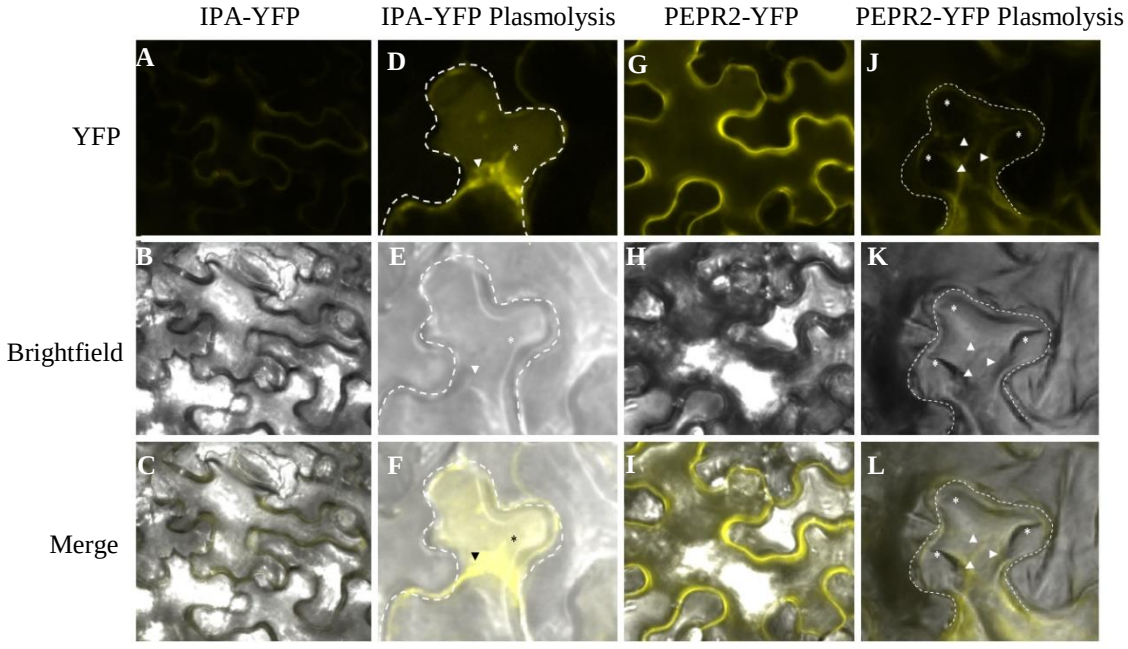

Figure 3. IPA localizes in the apoplast. (A-C, G-I) IPA-YFP and PEPR2-YFP were transiently expressed in *N. benthamiana* cells and visualized using confocal microscopy. (D-F, J-L)) Plasmolysis of cells expressing these proteins. In plasmolyzed cells, asterisks indicate the expanded apoplastic space, dotted lines indicate the cell wall, and arrows indicate the position of the receding plasma membrane.

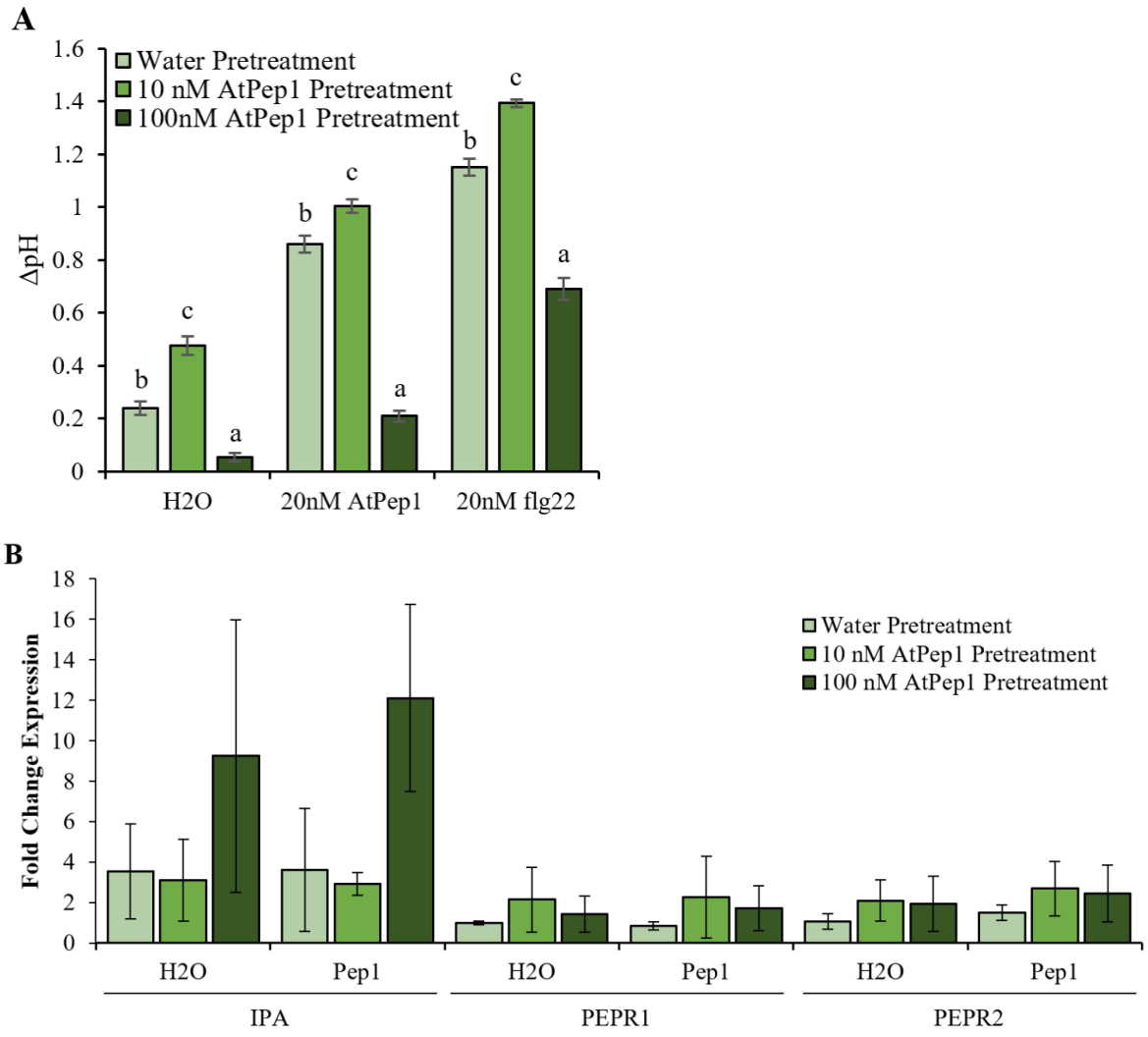

Figure 4. *IPA* expression correlates with a decreased alkalinization response to AtPep1 treatment. T87 Arabidopsis suspension cells were pretreated with water, 10 nM AtPep1, or 100 nM AtPep1 (light green, green, and dark green bars, respectively) for 24 hours, prior to treatment with water, 20 nM AtPep1 or 20 nM flg22. After 15 minutes, the pH of the surrounding media (A) and relative expression of *IPA*, *PEPR1*, and *PEPR2* (B) was measured. In both cases, samples were compared to untreated suspension cells as a control. *GAPDH* expression was utilized as a reference gene (n=3, error bars represent standard deviation).

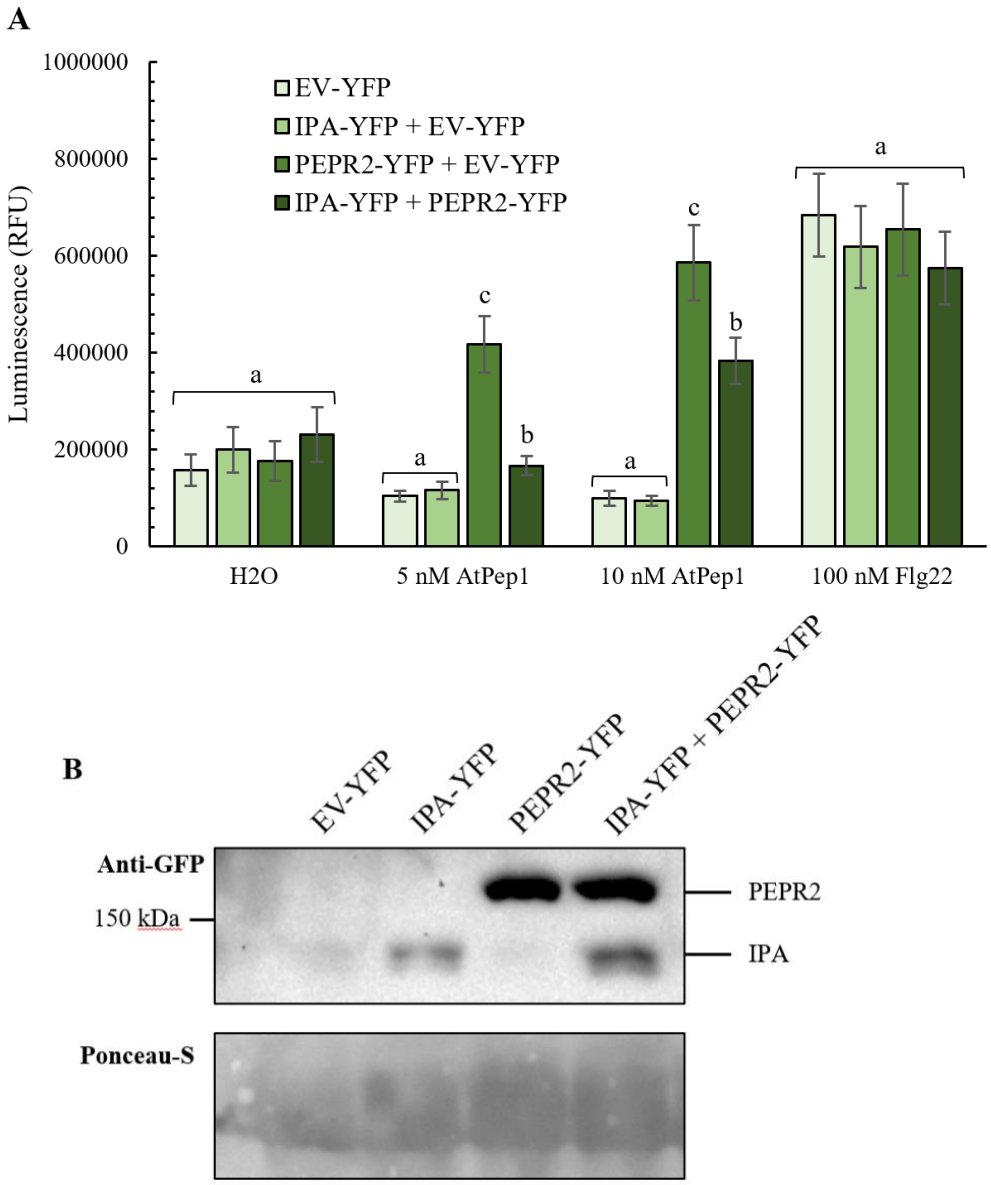

Figure 5. Effects of IPA on activation of PEPR2. (A) ROS production of *N. benthamiana* leaves transiently expressing combinations of YFP, IPA-YFP, and PEPR2-YFP 24 hours post infiltration. Excised leaf disks were treated with water, 5 nM AtPep1, 10 nM AtPep1, or 100 nM flg22 and ROS production was measured over the course of 30 minutes. For each set of constructs, 2 disks from each of 8 leaves were used (n=16). Error bars represent SEM. (B) Western blot analysis of PEPR2 and IPA protein expression in transgenic *N. benthamiana* leaves. Extracted protein was detected using an anti-GFP antibody as indicated. Ponceau-S stain is used to indicate even protein loading.

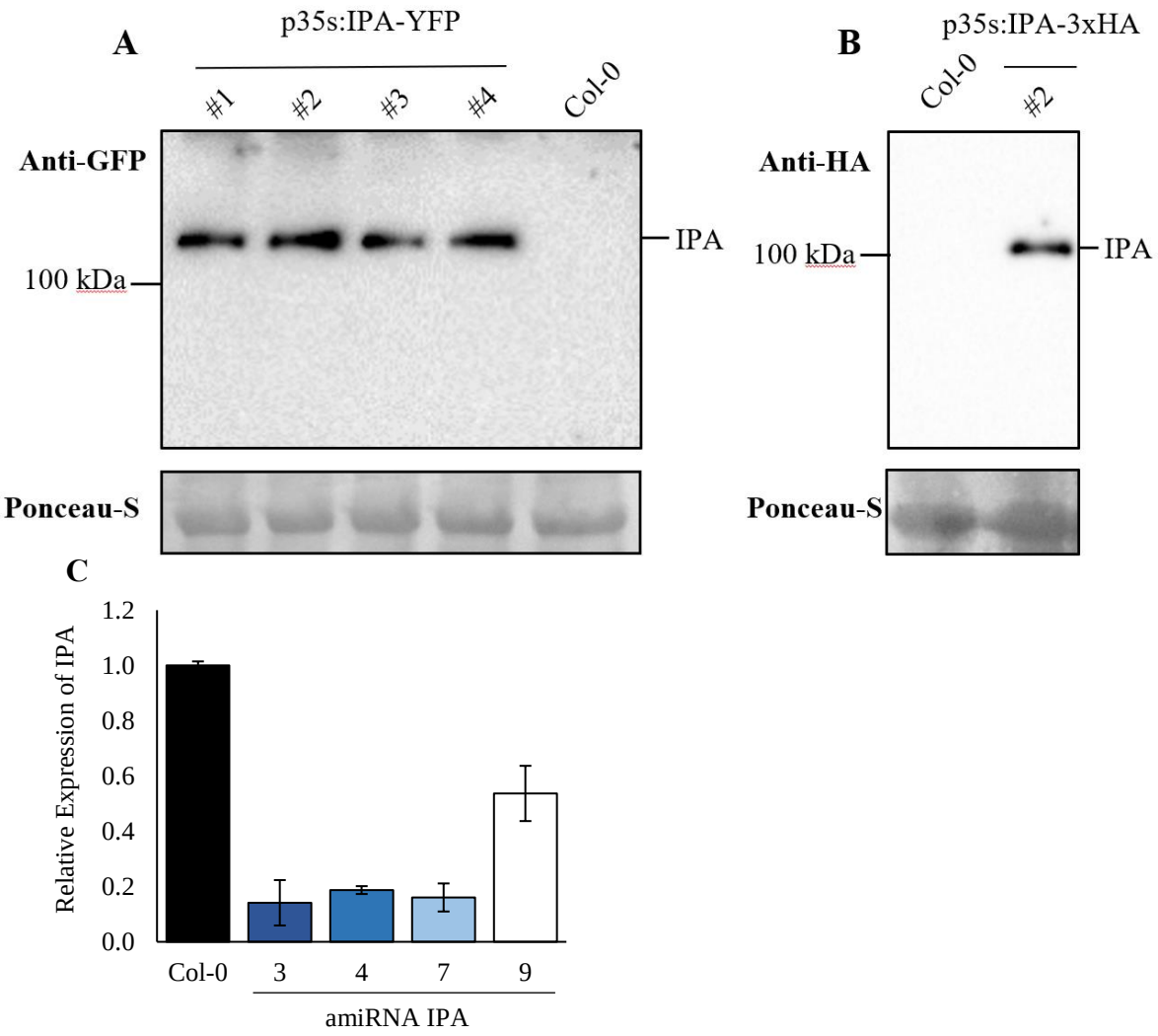

Figure 6. Generation of transgenic *IPA* lines. (A,B) Western blot analysis of IPA protein expression in 5-week-old Arabidopsis. Extracted protein was detected using an anti-GFP antibody or an anti-HA antibody, respectively, as indicated. Ponceau-S stain is used to indicate even protein loading. (C) Gene expression analysis of T_2 amiRNA IPA lines. *IPA* expression was quantified relative to expression of a reference gene, *GAPDH*. Error bars represent standard deviation (n=3).

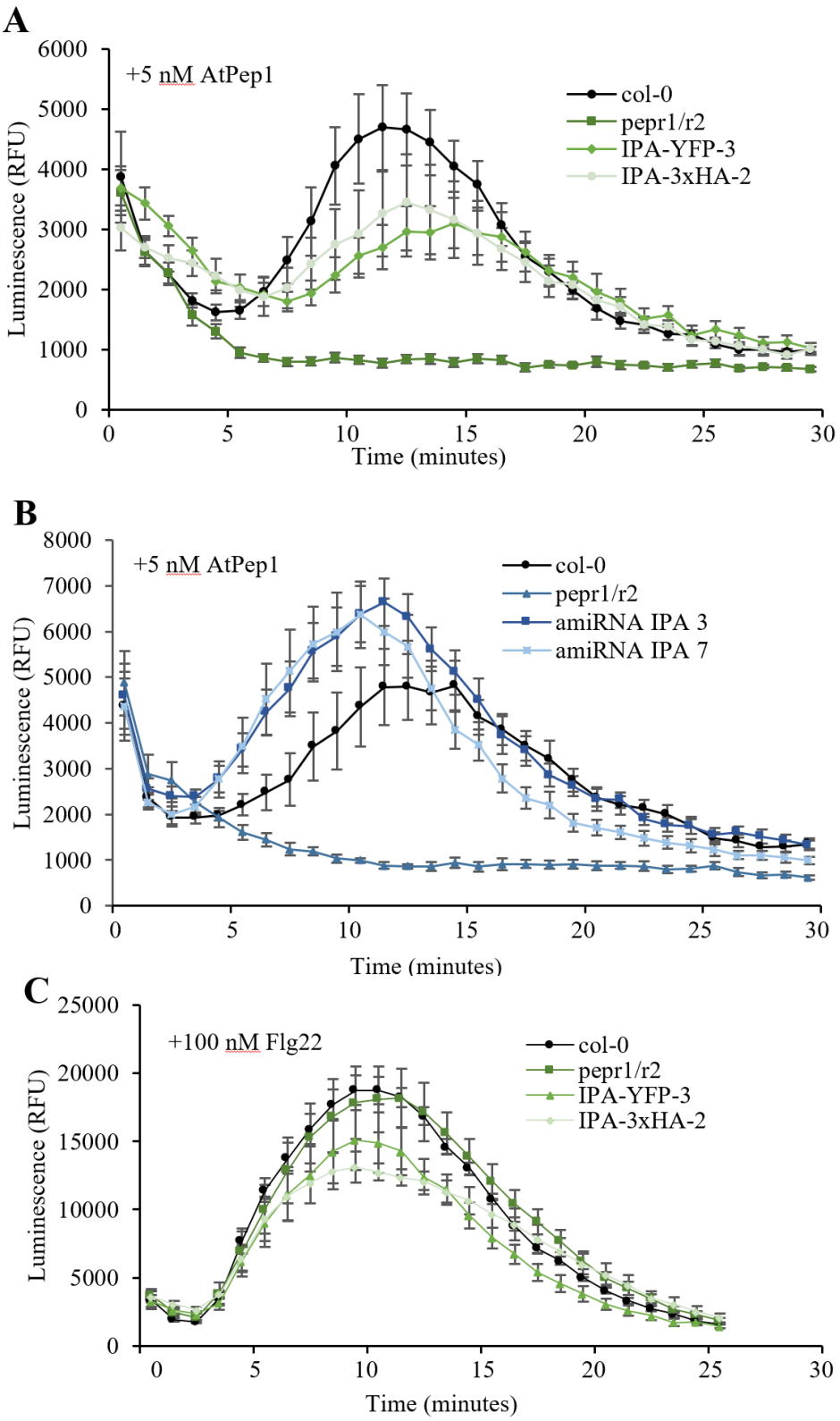

Figure 7. IPA negatively regulates ROS production. Leaf disks harvested from (A, C) p35s:IPA or (B) amiRNA IPA transgenic *A. thaliana* plants were elicited with 5nM AtPep1. ROS production was measured over the course of 30 minutes. Error bars represent SEM (n=8).

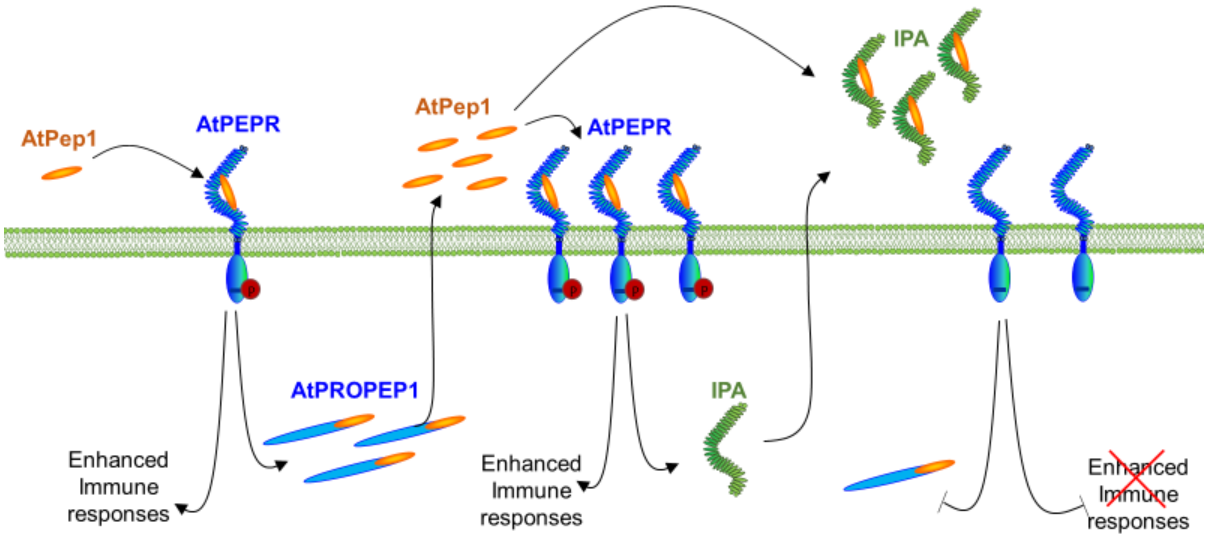

Figure 8. Proposed model for IPA regulation of Pep signaling. IPA is produced in the presence of high concentrations of AtPep1. IPA then binds to AtPep1 secreted into the apoplastic space, preventing the peptide from interacting with PEPR1 and PEPR2. This negatively regulates immunity by inhibiting the initial activation of the receptors.

Table 1. Primers utilized in this work. A list of primers used in gene expression analysis, cloning, and creation of transgenic constructs.

Name	Locus	Primer Sequence	Purpose
IPA gene	At1g73066	5'- TTTCAGGATTCGTTCCGCCC-3' Fw	Gene expression analysis
		5'- CGGCAATAAGAATGGTGAAAAGGT-3' Rv	
ACTIN2 gene	At3g18780	5'- TCCCTCAGCACATTCCAGCAGAT-3' Fw	Gene expression analysis
		5'- AACGATTCCTGGACCTGCCTCATC-3' Rv	
PEPR1 gene	At1g73080	5'- CAACAACAATGTGGAGGATA-3' Fw	Gene expression analysis
		5'- AACGAGATTACCGAACTGAA-3' Rv	
PEPR2 gene	At1g17750	5'- GGGTACATTGCACCAGAAAATG-3' Fw	Gene expression analysis
		5'- TCTGTCCAGTGCTCTCTTTCC-3' Rv	
GAPDH gene	At1g13440	5'- TCTCGATCTCAATTTCGCAAAA-3' Fw	Gene expression analysis
		5'- CGAAACCGTTGATTCCGATTC-3' Rv	
PDF 1.2 gene	At5g44420	5'-CTTATCTTCGCTGCTCTTGT-3' Fw	Gene expression analysis
		5'- CGTAACAGATACACTTGTGTGC-3' Rv	
PEPR2 gene	At1g17750	5'- CACCATGAGGAATCTTGGGT-3' Fw	Cloning into pENTR-D-TOPO
		5'- GTGAACTGAACCCGAAGTG-3' Rv	
IPA gene	At1g73066	5'-CACCATGGAGAATCTTGGGTTGTTCCAA-3' Fw	Cloning into pENTR-D-TOPO
		5'-AGAGTGGCGATCGGGCGGAAC-3' Rv	
amiRNA IPA	At1g73066	5'-gaTTACAAATCGGCCAAGCGCTTtctctctttgtattcc-3'	amiRNA IPA construct
		5'-gaAAGCGCTTGGCCGATTTGTAAtcaaagagaatcaatga-3'	
		5'-gaAAACGCTTGGCCGTTTTGTATtcacaggtcgtgatatg-3'	
		5'-gaATACAAAACGGCCAAGCGTTTtctacatatatattcct-3'	

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