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Identification of a damage-associated molecular pattern (DAMP) receptor and its cognate peptide ligand in sweet potato (*Ipomoea batatas*)

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Abstract

Sweet potato (*Ipomoea batatas*) is an important tuber crop, but also target of numerous insect pests. Intriguingly, the abundant storage protein in tubers, sporamin, has intrinsic trypsin protease inhibitory activity. In leaves, sporamin is induced by wounding or a volatile homoterpene and enhances insect resistance. While the signalling pathway leading to sporamin synthesis is partially established, the initial event, perception of a stress-related signal is still unknown. Here, we identified an *Ib*LRR-RK1 that is induced upon wounding and herbivory, and related to peptide-elicitor receptors (PEPRs) from tomato and Arabidopsis. We also identified a gene encoding a precursor protein comprising a peptide ligand (*Ib*Pep1) for *Ib*LRR-RK1. *Ib*Pep1 represents a distinct signal in sweet potato, which might work in a complementary and/or parallel pathway to the previously described hydroxyproline-rich systemin (HypSys) peptides to strengthen insect resistance. Notably, an interfamily compatibility in the Pep/PEPR system from Convolvulaceae and Solanaceae was identified.

KEYWORDS
DMNT, herbivory, LRR-RLK, plant defense, plant elicitor peptide receptor

1 | INTRODUCTION

Plants have evolved several mechanisms to cope with biotic and abiotic stresses. When encountering stresses, such as pathogen infection, insect feeding, and wounding, receptor kinases (RKs) or receptor-like proteins (RLPs) properly identify specific patterns derived either from the aggressors (microbe-associated molecular patterns, MAMPs; and herbivore-associated molecular patterns, HAMPs) or from the perturbation of cellular integrity (danger- or damage-associated molecular patterns, DAMPs). Subsequently, these pattern recognition receptors (PRRs) trigger signal transduction pathways to activate appropriate plant immune responses, leading

This article is dedicated to Prof. Dr. Kai-Wun Yeh, who is no longer with us. He passed away during the preparation of the final stages of this article. He will always be remembered as a passionate scientist and an excellent mentor.

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to pattern-triggered immunity (PTI) (Boller & Felix, 2009). PTI can reduce the damages caused by the invasion of many pathogens and insects (Böhm et al., 2014).

PRRs are usually composed of extracellular, transmembrane and intracellular domains. They are classified by their extracellular domains. The extracellular leucine-rich repeat domain, a single-pass transmembrane domain, and a cytoplasmic protein kinase domain characterize leucine-rich repeat receptor kinases (LRR-RKs). LRR-RKs and LRR-RLPs are sensors for proteinaceous immunogenic ligands, such as peptides and small proteins (Böhm et al., 2014). For example, the FLS2 receptor binds a 22-amino acid epitope (flg22) conserved in bacterial flagellins (Chinchilla et al., 2006), EFR recognizes a conserved N-terminal fragment of bacterial elongation factor Tu (Zipfel et al., 2006), and *Sl*Eix1 and *Sl*Eix2 bind *Trichoderma* cell wall-derived xylanase (Ron & Avni, 2004).

Peptide ligands play an important role in regulating the signal transduction of insect resistance and wound defense responses (Bartels & Boller, 2015; Huffaker, 2015). In *Arabidopsis thaliana*, eight plant elicitor peptides (*At*Pep1-*At*Pep8) are found to participate in damage-related defense responses after recognition by a pair of LRR-RKs, the PEP receptors 1 and 2 (*At*PEPR1 and 2) (Krol et al., 2010; Yamaguchi et al., 2010). Each of the *At*Peps is derived from the carboxy terminus of their precursor protein *At*PROPEP1-8 (Bartels et al., 2013; Huffaker et al., 2006), how and if the peptides are cleaved off is, however, mostly not known. However, a METACASPASE4 (MC4)-dependent maturation of *At*Pep1 was recently described. High levels of $[Ca^{2+}]_{cyt}$ that occur only in directly damaged cells bind to MC4, which in this activated form cleaves PROPEP1 and releases *At*Pep1 (Chen et al., 2020; Hander et al., 2019). *AtPROPEP2*, *AtPROPEP3* and the receptor genes *AtPEPR1/2* are strongly induced upon herbivore attack. Moreover, *pepr1 pepr2* double mutant plants display a reduced resistance to *Spodoptera littoralis* larvae (Huffaker, 2015; Klauser et al., 2015; Ross et al., 2014). In *Zea mays*, the precursor of *Zm*Pep3, an *At*Pep-ortholog, can be induced by insect oral secretion and insect HAMP. The application of *Zm*Pep3 can induce emission of some insect herbivory-related volatile organic compounds (VOCs), biosynthesis and accumulation of phytohormones and transcripts that are indirectly involved in defense against herbivores. *Zm*Pep3 also causes accumulation of proteinase inhibitor and contributes to the resistance to lepidopteran insects (Huffaker et al., 2013).

Systemin was the first peptide discovered in plants with signalling capacities. In *Solanum lycopersicum*, the injury-induced systemin can cause defense responses against insects (Orozcocardenas et al., 1993; Pearce et al., 1991). Tomato systemin is an endogenous peptide ligand composed of 18 amino acids, which is derived from a precursor protein by phytaspase-dependent cleavage at two aspartate residues (Beloshistov et al., 2018). Systemin induces proteinase inhibitors and activates phospholipase A2, thereby promoting the release of jasmonic acid precursors from the cell membrane. Induction of insect-resistance defense genes by jasmonic acid signalling pathways further contributes to the resistance of herbivore attack (Pearce et al., 1991), mediated by the LRR-RK receptor *Sl*SYR1, which, however, is not necessary for wound responses (Wang et al., 2018).

Hydroxyproline-rich systemins (HypSys) are systemin-like endogenous peptide ligands in *Solanaceae* plants. In addition to the hydroxyproline-rich conserved sequence, the HypSys precursor protein preproHypSys has a secretion sequence at the N-terminus, which is absent from Peps and systemin precursor proteins. Similar to systemin, HypSys induces the production of jasmonates and the expression of defense genes (Pearce, 2011). In petunia, HypSys is described to induce the expression of the immune gene *defensin1* (Pearce et al., 2007). The precursors of *Sl*HypSys I, II and III in tomato are synthesized and sequestered in the cell wall matrix of phloem parenchyma cells in response to systemin, wounding, and methyl jasmonate (Narváez-Vásquez et al., 2005). Moreover, the HypSys precursor gene *IbpreproHypSys* in sweet potato (*Ipomoea batatas*) can be induced by injury. The application of *Ib*HypSys in sweet potato induces downstream insect-resistance genes such as *sporamin* and *ipomoelin*, and improves the biosynthesis of lignin, to increase the ability to repel insects (Chen et al., 2008; Li et al., 2016). However, it is still unclear how HypSys binds to receptors and participates in defense responses.

Sweet potato is the fifth largest food crop in the world and has high nutritional and economic value. Several cultivars of sweet potato have higher insect resistance than others. For example, *I. batatas* cv. Tainong 57, which is widely cultivated in Taiwan, has strong insect resistance and represents a suitable model crop for studying insect resistance mechanisms (Meents et al., 2019). Sporamin, which was previously thought to be a unique storage protein in sweet potato tuberous roots, was recently described to be regulated by herbivore attack, injuries, jasmonic acid, and the homoterpene (*E*)-4,8–dimethyl–1,3,7-nonatriene (DMNT) in sweet potato leaves (Meents et al., 2019; Rajendran et al., 2014). Functional studies revealed that sporamin is a serine-type trypsin inhibitor, which acts in the insect intestine and retards insect growth and development (Imanishi et al., 1997; Yeh, Chen, et al., 1997). Transgenic *Nicotiana benthamiana* and *Brassica rapa* subsp. *chinensis* plants overexpressing sporamin demonstrated a strong pest resistance capacity (Chen et al., 2006; Yeh, Lin, et al., 1997), as did transgenic sweet potato plants overexpressing *IbNAC1*, which is a transcription factor (TF) binding to the sporamin wounding response element region of the *sporamin* promoter (Chen, Lin, et al., 2016). *Ib*NAC1 also regulates the jasmonic acid response and ROS signalling (Chen, Kuo, et al., 2016) and is regulated by TFs *Ibb*HLH3 and 4 (basic helix–loop–helix TF; aka MYC2, acting downstream of jasmonates), and *Ib*EIL1(ethylene-insensitive-like TF) as well as by *Ib*WIPK1 (wound-induced protein kinase) and *Ib*JAZ2 (jasmonate-ZIM domain protein, repressing MYC2), upon injury (Chen, Lin, et al., 2016). The MAPK pathway is also part of the signal transduction from wounding stress to *sporamin* expression (Chen, Lin, et al., 2016). However, the molecular connection between danger perception (ligands, receptors) and downstream defense responses is still elusive.

To discover the key players upstream of the intracellular signalling cascade leading to induced resistance against herbivores,

we isolated candidates for both, a cell surface receptor and endogenous peptide ligands from sweet potato. Among the wound- and herbivore-induced genes in *I. batatas*, we detected a gene encoding a leucine-rich receptor kinase related to the plant elicitor peptide (Pep) receptor (PEPR) family, *Ib*LRR-RK1. When heterologously expressed in *N. benthamiana*, this receptor candidate did not provide responsiveness to HypSys but to extracts of damaged sweet potato leaf tissue. Finally, we identified the cognate peptide ligand, *Ib*Pep1, characterized the specificity and sensitivity of the new receptor/ligand-pair and compared the signalling capacities of the newly identified peptide with the previously described HypSys peptides.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

Sweet potato scions (*I. batatas* Lam.; cultivar Tainong 57) were grown in phytochambers under long-day conditions (16 h light: 8 h dark) at 28°C (day) and 25°C (night) in 70% relative humidity for 3 weeks as previously described (Meents et al., 2019). When growing for 4–5 weeks, sweet potato and *N. benthamiana* plants were maintained in a greenhouse with a 16 h photoperiod and a 25°C/20°C day/night programme. *A. thaliana* ecotype Columbia-0 (Col-0) was grown at 22°C with an 8 h photoperiod in growth chambers for 4–5 weeks.

2.2 | Peptides

Peptides were ordered from GenScript Biotech (Leiden, Netherlands). They were dissolved before each experiment in BSA/NaCl (10 mg/mL, 0.1 M) solution. The list of peptides and their sequences can be found in Supporting Information: Table 1.

2.3 | RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analyses

Harvested sweet potato leaves were processed and used for qRT-PCR as described in (Meents et al., 2019) with the additional primer pairs for *IbLRR-RK1-5*, *sporamin*, *IbWIPK1*, *IbNAC1*, *IbCML1* (Supporting Information: Table 3) on a Bio-Rad CFX96 RT-PCR Detection System (Bio-Rad Laboratories).

2.4 | RNA-Seq analysis and processing

RNA from single third leaves treated for 1 h with *Ib*HypSysIV, *Ib*Pep1 and water (control) was extracted according to (Meents et al., 2019) using TRIzol Reagent (Invitrogen). Four biological replicates per treatment were used for RNA-Seq experiments conducted by Novogene Europe. RNA quality was monitored using NanoPhotometer® spectrophotometer (IMPLEN) and RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies). A total of 1 μg of RNA per sample was used as template material for further sample preparations. Sequencing libraries were generated via NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB) following manufacturer's instructions. A total of 20 M paired end reads of 150 bp per sample were generated, sequenced on an Illumina NovaSeq. A total of 6000 instrument. Raw reads were trimmed by in-house scripts. The clean reads were mapped onto *Ipomoea trifida* reference genome (http://sweetpotato.uga.edu/), using HISAT2 V2.0.5 with default parameter. HTSeq V0.6.1 software was used with the union mode to count read numbers mapped of genes for each sample.

R package from Bioconductor, DESeq. 2 V1.22.2 was used to estimate gene abundance and detect differentially expressed genes (DEGs) among the sample groups. A model based on the negative binomial distribution was carried out to determinate DEGs with an adjusted *p* value cutoff of 0.05 using the Benjamini-Hochberg correction. Genes with a log2-fold change ≥1 and *p*adj < 0.05 were considered as significantly DEGs. Gene ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analyses of DEGs were implemented by the GOseq V1.34.1 R package and KOBAS V3.0 software.

2.5 | Wounding, insect feeding and peptide spray treatments

I. batatas and *N. benthamiana* plants with six to eight fully developed leaves were used in the study. For wound treatment, the third or fourth fully expanded leaves were wounded using tweezers and the wounded leaves samples were collected at different time points. For insect feeding treatment, starved *Spodoptera litura* larvae (second instar) were placed on the third or fourth fully expanded leaves and the treated leaves samples were collected at intervals.

To study the local effects of peptide solutions on DMNT emission and gene expression, whole sweet potato plants with six to eight fully expanded leaves were evenly sprayed with peptide solution or double-distilled water (control) until all leaves were fully covered in liquid. After a 1 h incubation period, single plants were placed for 24 h in 2.4 L glass desiccators (VWR international) for headspace volatile collection. For RNA-Seq, qRT-PCR and phytohormone analyses each third fully expanded leaf was locally sprayed with peptide solution or ddH_2O (control) and harvested together with the adjacent fourth leaf (systemic) after the indicated time points.

2.6 | VOC collection and quantification

Volatiles were collected over 24 h from peptide- or water-treated sweet potato plants enclosed in 2.4 L desiccators using the closed-loop stripping technique (Kunert et al., 2009). Throughout the headspace collection, each desiccator was connected to an air circulation pump (Fürgut GmbH) containing a charcoal trap with

1.5 mg absorption material (CLSA filter, 6 cm long, 0.5 cm diameter, Gränicher & Quartero). After collection, volatiles were eluted and measured as described (Meents et al., 2019) with minor modifications. In this study, samples were eluted with 2 × 20 µL of dichloromethane containing 10 µg mL^{-1} *n*-bromodecane as internal standard used for further relative quantification.

2.7 | Cloning of receptor and propeptide gene candidates

*Ib*LRR-RK1 and *Ib*PROPEP1 genes were identified using blastn as well as tblastn on various databases using the receptor and propeptide sequences for Solanaceae plants from (Lori et al., 2015). Used databases included Sweet Potato Genomic Resource database (http://sweetpotato.plantbiology.msu.edu/index.shtml), *I. batatas* cv. TN57 transcriptome database (Rajendran et al., 2014), *I. batatas* database: Ipomoea Genome Hub (https://ipomoea-genome.org/), and NCBI (https://www.ncbi.nlm.nih.gov/). The *Ib*LRR-RK1 and *Ib*PROPEP1 coding sequences were amplified from sweet potato leaf cDNA using gene-specific primers (*Ib*LRR-RLK1_FL_F, *Ib*LRR-RLK1_FL_R, *Ib*PROPEP1_FL_F, *Ib*PROPEP1_FL_R, as shown in Supporting Information: Table 3) in a PCR reaction with Q5 High-Fidelity DNA Polymerase (NEB), respectively. The coding sequence encoding the tomato *Sl*PEPR1 (XP_004235511) was amplified using the primers *Sl*PEPR1_FL_F and *Sl*PEPR1_FL_R (Supporting Information: Table 3). All full-length coding sequences were cloned into the pCR8/GW/TOPO vector (Invitrogen). LR clonase (Invitrogen) was used to transfer these coding sequences from PCR8 to pMDC83 vectors (Curtis & Grossniklaus, 2003), generating C-terminal fusions with green fluorescent protein (GFP).

2.8 | Generation of chimeric receptors

Gene-specific level I modules for SYR1 (Wang et al., 2018) and *Ib*LRR-RK1 (see above) were generated by proofreading PCR (Phusion High Fidelity DNA Polymerase, ThermoFisher Scientific) from existing templates using the oligonucleotide primers listed in Supporting Information: Table 3, subcloned, and verified by sequencing. GoldenGate cloning was used to assemble the receptor expression constructs with general level I modules (A-B p35S (G005), D-E GFP (G011), E-F nos-T (G006) and dy F-G (BB09)) into the vector backbone LIIα F 1-2 (BB10) as described (Binder et al., 2014).

2.9 | Transient expression of receptor constructs and bioassays

Transient expression in *N. benthamiana* was performed as described (Albert et al., 2010). The oxidative burst was measured with leaf pieces floating on 100 µL water containing 20 µM L-012 (Wako) and 2 µg/mL horseradish peroxidase (Applichem), after addition of peptides, with a luminescence plate reader (Mithras LB 940, Berthold, or Infinite M200 PRO plant reader, TECAN). The amount of ethylene was measured by GC in the headspace of four leaf pieces floating on 500 µL water, treated for 4 h with the peptides or controls. Transient co-expression of the pFRK1:Luciferase reporter (Yoo et al., 2007) with the receptor expression constructs in mesophyll protoplasts of *A. thaliana* Col-0 wild-type was performed as described (Wang et al., 2016). Luminescence was recorded for up to 6 h in W5-medium containing 200 µM firefly luciferin (Synchem UG) after overnight incubation for 14 h and subsequent treatment with peptides or control solution.

2.10 | Subcellular localization

The *Ib*LRR-RK1-GFP, *Ib*PROPEP1-GFP, the tonoplast localization marker protein fusion γ-Tip-mCherry (Nelson et al., 2007) were transiently expressed in *N. benthamiana* leaves and *A. thaliana* mesophyll protoplast as described above. The plasma membrane marker PIP2A-mCherry was expressed in *N. benthamiana* leaves. Plasmolysis was induced by infiltration of 1.0 M mannitol before fluorescence images were taken. Fluorescence images were taken using a TCS SP5 Confocal microscope (Leica) and analyzed by LAS AF Lite application software (Leica) or a Zeiss Axio Zoom.V16.

2.11 | Crude endogenous ligand extraction

According to (Chien et al., 2015), 10 g injured and noninjured sweet potato leaves were harvested, respectively. Samples were homogenized with 1% cold trifluoroacetic acid (TFA) in a blender for 2 min. After filtering the extracts through four layers of Miracloth to remove plant debris and centrifuging at 8500 rpm for 20 min at 4°C, the supernatant was slowly pressed through a customized Sep-Pak C18 solid phase extraction cartridge (Waters) and eluted with 60% (v/v) methanol/0.1% (v/v) TFA. The eluate-containing peptides were dried in a speed vac and resuspended in 200 µL double-distilled H_2O.

2.12 | Phytohormone extraction and quantification

Local and systemic leaves collected after 1 h peptide treatment were extracted and measured as described (Meents et al., 2019) using an Agilent 1200 HPLC system (Agilent) with subsequent API 5000 tandem mass spectrometer (Applied Biosystems) equipped with a Turbo spray ion source employed in negative ionization mode.

2.13 | Statistical analysis

Data generated using qRT-PCR was analyzed as described in (Meents et al., 2019) followed by a Shapiro–Wilk normality test with

subsequent t-test or Mann–Whitney rank sum test based on the data distribution. Phytohormone levels were analyzed using a two-way analysis of variance with initial Shapiro–Wilk-normality and equal variance test. For all analyses, phytohormone content was set as the dependent variable with treatment and leaf type as independent variables. For identification of significant differences between groups, pairwise multiple comparison procedure via the Holm–Sidak method was implemented with a significance level of $p < 0.05$. All statistical analyses were conducted in SigmaPlot (V 11.0).

3 | RESULTS

3.1 | Sweet potato encodes putative DAMP receptors

We based our search for receptors of sweet potato which are involved in responses to wounding and herbivore attack on published sequences for DAMP-related receptors in Arabidopsis (*At*PEPR1/2: AT1G73080/AT1G17750) and tomato, *S. lycopersicum* (*Sl*SYR1/2: *Solyc*03g082470/Solyc03g082450.2.1; *Sl*PEPR1: XP_004235511). Several closely related receptor genes, designated *ItLRR-RK1–ItLRR-RK13*, were mined from the *I. trifida* 'Sweet potato Genomic Resource database' (http://sweetpotato.plantbiology.msu.edu/index.shtml) (Supporting Information: Figure 1). Next, two transcriptomic databases, that is, the *I. batatas* cv. Tainong 57 transcriptome database (Rajendran et al., 2014) and *I. batatas* database, *Ipomoea* Genome Hub (https://ipomoea-genome.org/) were accessed to explore putative *LRR-RK* genes with sequence homology to *ItLRR-RKs*. Five putative *I. batatas* RK genes (*IbLRR-RK1* to *IbLRR-RK5*) were identified. Analysis by qRT-PCR experiments revealed that wound treatment did not induce the upregulation of *IbLRR-RK2* - *IbLRR-RK5* while both wounding and insect herbivory rapidly induced *IbLRR-RK1* (MT210638) (Figure 1a,b). Upon wounding and treatment with *Spodoptera* larvae-derived oral secretion, the relative expression level of *IbLRR-RK1* increased nearly 30-fold at 15 min, 12-fold at 30 min, and returned to normal levels at 60 min. Herbivory feeding also increased *IbLRR-RK1* expression level 5.4-fold at 15 min and 1.7-fold at 30 min (Figure 1b). These data demonstrate that mechanical wounding and herbivory induce the receptor-like kinase *Ib*LRR-RK1, suggesting that this receptor might be involved in perception of a wound-related signal.

3.2 | The receptor candidate *Ib*LRR-RK1 is related to PEPRs

The gene *IbLRR-RK1* from *I. batatas* cv. Tainong 57 encodes a typical member of the PEPR family. It consists of an ectodomain composed of a signal peptide, an N-terminal cap region typically found in plant LRR-RKs, 26 repetitions of the plant-specific version of the LRR motif, and an outer juxtamembrane; this is followed by a transmembrane domain; the cytosolic part contains the inner juxtamembrane domain and a serine/threonine kinase domain (Supporting Information: Figure 2). As expected, the GFP-tagged *Ib*LRR-RK1 protein, transiently expressed in either *A. thaliana* protoplasts or *N. benthamiana* leaves, localized to the plasma membrane (Figure 1c), like other plant LRRs such as *Sl*SYR1-GFP or *At*EFR-GFP, which were used as positive controls. *Ib*LRR-RK1 is most likely related to PEPRs from tomato (XP_004235511, *Sl*PEPR1) and Arabidopsis (*At*1g73080, *At*1g17750; PEPR1 and PEPR2; Supporting Information: Figure 3). *Ib*LRR-RK1, which is 97% identical with *It*LRR-RK1, shared 65% or 50% identical amino acid residues to *Sl*PEPR1 or *At*PEPR1, respectively, and 35% identity to *Sl*SYR1, while other putative RLK members selected from the Sweet Potato Databases never shared more than 36% identity to either of the mentioned receptors (Table 1). In addition, comparing the extracellular domains of different receptors also showed that *Ib*LRR-RK1 has a highly similar ligand-binding surface when compared with *Sl*PEPR1 and *At*PEPR1 (60% and 48% identity, respectively), and shares only 36% identical residues with tomato *Sl*SYR1 (Table 2). Thus, *Ib*LRR-RK1 is part of the plant elicitor peptide receptor (PEPR) group.

3.3 | *Ib*LRR-RK1 is a functional receptor

Establishing the functionality of new receptor candidates for which the ligands are not known is challenging, and can be overcome by approaches in which chimeric versions are ectopically expressed in suitable plants (Albert et al., 2010; Butenko et al., 2014). To test if the kinase domain of the putative receptor from sweet potato is able to feed into the immune response pathway, we generated a chimeric version with the ectodomain of tomato SYR1 a receptor with known ligand (Supporting Information: Figure 4a). The chimeric receptor SYR1-*Ib*K as well as the original *Ib*LRR-RK1 and SYR1 were transiently expressed in leaves of *N. benthamiana*. The GFP-tagged recombinant proteins localized to the cell surface, as predicted (Figure 1c, Supporting Information: Figure 4b). Treatment with the ligand of SYR1 resulted in the induction of an oxidative burst for the SYR1-*Ib*K expressing leaf pieces (Figure 2a, Supporting Information: Figure 4c), proving the functionality of the kinase domain of *Ib*LRR-RK1. Several other defense-related peptides from various plants such as *Sl*Pep6, *Sl*HypSysIII, *Ib*HypSysIV or *At*Pep1 were applied in addition to systemin (Supporting Information: Table 1) in bioassays with leaves expressing either the original *Ib*LRR-RK1 or SYR1-*Ib*K. Interestingly, *Sl*Pep6 triggered the defense pathway in the presence of *Ib*LRR-RK1, leading to ROS production and ethylene accumulation (Figure 2b,c). We then verified the recognition of *Sl*Pep6 by *Ib*LRR-RK1 in protoplasts, generated from *A. thaliana* Col-0 mesophyll cells. *FRK1* (flg22-induced receptor-like kinase 1) is a PTI marker gene of early defense responses in Arabidopsis (Asai et al., 2002) and its promoter is widely used in combination with a luciferase reporter gene to monitor PAMP activity (Yoo et al., 2007). The co-expression of *Ib*LRR-RK1 with p*FRK1:LUC* resulted in *Sl*Pep6-dependent induction of the reporter (Figure 2d), confirming the previous experiments

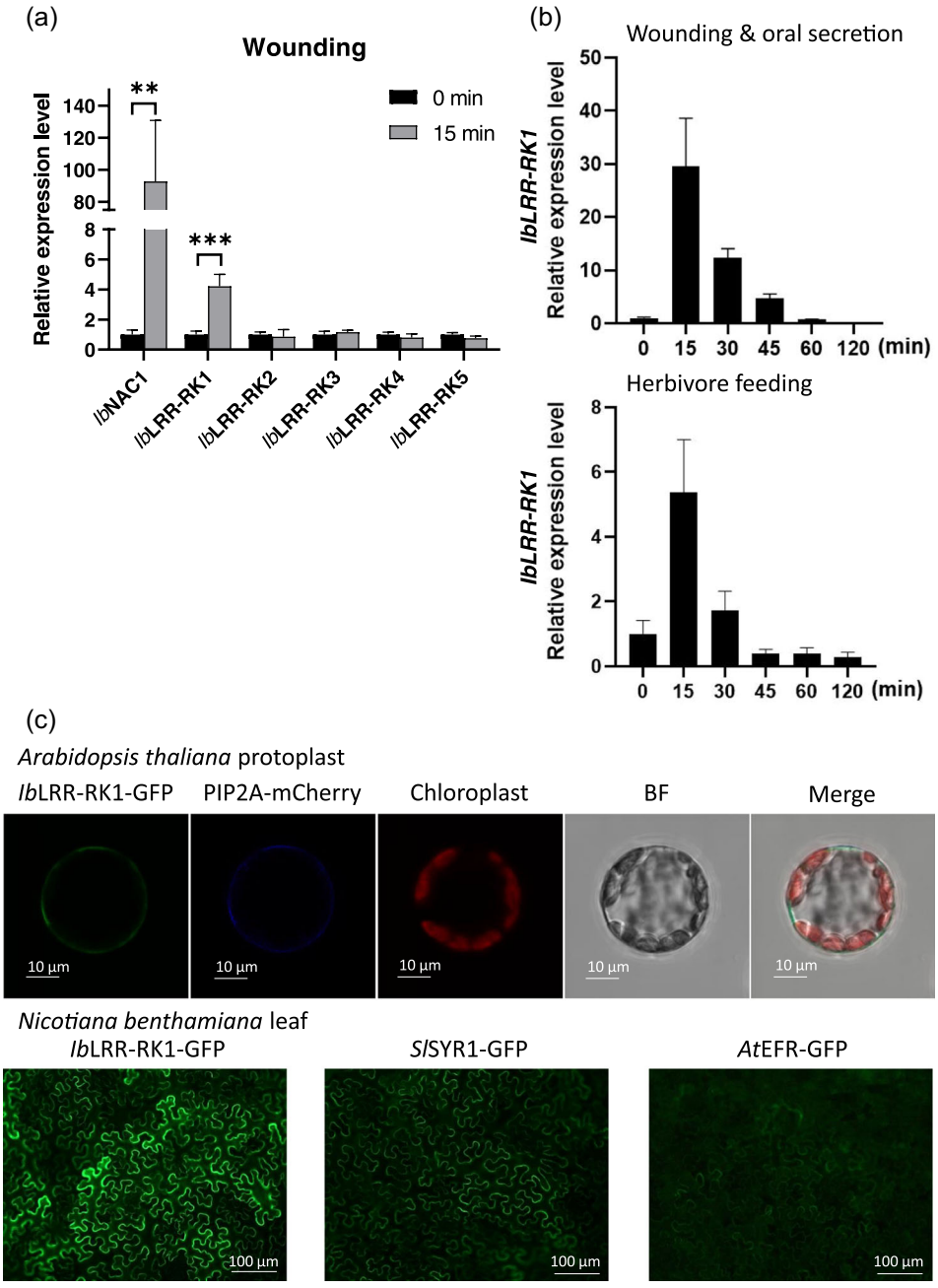


FIGURE 1 Receptor kinase *Ib*LRR-RK1 is induced by wounding and herbivory in sweet potato leaves. (a) The expression pattern of *IbLRR-RK1*–*IbLRR-RK5* receptor-like genes in response to wounding in sweet potato leaves. *IbActin1* expression was used as internal control, and *IbNAC1* was used as positive control of wounding by quantitative real-time polymerase chain reaction (RT-PCR). Bars and error bars represent mean ± SD of $n = 4$. (b) Sweet potato leaves were wounded and treated with *Spodoptera litura* larvae oral secretion, or exposed to feeding *S. litura* larvae. Expression of *Ib*LRR-RK1 was analyzed by qRT-PCR. Bars and error bars represent mean ± SD of $n = 4$. (c) Cell surface localization of the green fluorescent protein (GFP)-tagged *Ib*LRR-RK1 in transiently transformed *Arabidopsis thaliana* protoplasts or *Nicotiana benthamiana* leaf samples, observed by confocal microscope (TCS SP5 Confocal; Leica); PIP2A (plasma membrane-intrinsic protein 2A) and well-studied plasma membrane-localized receptor proteins (SYR1 from tomato and EFR from *A. thaliana* (Wang et al., 2018; Zipfel et al., 2006) were used as positive controls in *A. thaliana* and *N. benthamiana*, respectively.

in *N. benthamiana*, while the chimeric receptor SYR1-*Ib*K recognized systemin, but not *Sl*Pep6 (Figure 2e). Taken together, we demonstrated that the activation of *Ib*LRR-RK1 can trigger plant immune responses such as ROS burst, ethylene biosynthesis, and defense gene expression and identified a heterologous ligand.

3.4 | *Ib*LRR-RK1 perceives an endogenous peptide

For the molecular identification of the cognate ligand of *Ib*LRR-RK1 we took advantage of the fact that *Sl*Pep6 was functional in activating sweet potato *Ib*LRR-RK1. We hence used the sequence of *Sl*Pep6 and

TABLE 1 Comparison of the full-length amino acid sequence of *Ib*LRR-RK1 with related sequences, calculation of % identity by Vector NTI.

	*It*LRR-RLK1	*Sl*PEPR1	*At*PEPR1	*At*PEPR2	*Sl*SYR1	*Sl*SYR2
*Ib*LRR-RK1	97	65	50	49	36	35
*It*LRR-RK1		64	50	49	36	35
*Sl*PEPR1			51	48	35	35
*At*PEPR1				66	36	34
*At*PEPR2					36	35
*Sl*SYR1						79

Abbreviations: At, *Arabidopsis thaliana*; Ib, *Ipomoea batatas*; It, *Ipomoea trifida*; Sl, *Solanum lycopersicum*.

TABLE 2 Comparison of the amino acid sequence of the extracellular domain of *Ib*LRR-RK1 with *At*PEPR1, *Sl*PEPR1 and *Sl*SYR1, calculation of % identity by Vector NTI.

	*Sl*PEPR1	*At*PEPR1	*Sl*SYR1
*Ib*LRR-RK1	60	48	36
*Sl*PEPR1		49	36
*At*PEPR1			36

Abbreviations: At, *Arabidopsis thaliana*; Ib, *Ipomoea batatas*; Sl, *Solanum lycopersicum*.

other Peps from the Solanaceae family as probes to search for endogenous peptides and applied scanning sequence pattern and tBlastn strategies on the sweet potato genomic resource database and the *Ipomoea* genome hub database (Supporting Information: Figure 5). Two putative Peps were selected from the *I. trifida* genomic resource database. The 23 C-terminal residues of the precursor protein *It*PROPEP1 (itf01g30920.t1) were selected according to the general length of Peps (Lori et al., 2015) and named *It*Pep1 (LSSRPPRP GLGNSGDPQTNDTSS) (Supporting Information: Figure 5b). The putative *It*Pep2 (RRGRTPPRPENLKLNLRARKHSLEDQ), containing a typical, conserved peptide motif of Peps (RRGRXP), was derived from the C-terminus of *It*PROPEP2 (itf07g21780.t1) (Supporting Information: Figure 5c). Both candidate peptides, *It*Pep1 and *It*Pep2, were synthesized and applied to *N. benthamiana* leaf discs transiently expressing *Ib*LRR-RK1. *It*Pep1, but not *It*Pep2, activated an *Ib*LRR-RK1-dependent ROS burst (Figure 3a), and neither peptide elicited a response in the SYR1-*Ib*K or p19 controls (Supporting Information: Figure 6a,b). Next, the cDNA of the *PROPEP1* gene from *I. batatas* cv. TN57 was cloned by RT-PCR using oligonucleotides deduced from *ItPROPEP1*. The CDS encompasses 378 bp and 125 deduced amino acid residues with a calculated molecular weight of 13.25 kDa, and a pI of 4.44. The putatively bioactive 23-mer peptide, *Ib*Pep1, corresponds to the C-terminus of the precursor protein and is 100% identical to the one from *It*PROPEP1 (Supporting Information: Figure 6c). As expected, the *I. batatas* PROPEP as well as the Pep amino acid sequences are more closely related to those from solanaceous plants than to those of Arabidopsis (Supporting Information: Figure 6d, Supporting Information: Table 2).

3.5 | *Ib*LRR-RK1 perceives *Ib*Pep1 with high sensitivity and specificity

Exploiting the same heterologous expression system described above we interrogated the sensitivity and the specificity of the putative ligand/receptor-pair. The dose-dependent induction of ROS by *Ib*Pep1 was clearly detectable in the subnanomolar range and the half-maximal activation of this output was estimated at 1 nM (Figure 3b). The tomato Pep (*Sl*Pep6) was 10-times less efficient in this bioassay with *Ib*LRR-RK1 (Figure 3b). In the reciprocal approach, we cloned the Pep receptor of tomato (*Sl*PEPR1, (Lori et al., 2015)), expressed it in *N. benthamiana* and compared the efficiencies of the Peps for the induction of ROS. The tomato PEPR/*Sl*Pep6 pair showed the same efficiency as the corresponding sweet potato pair, with an EC_{50} value of 1 nM. Interestingly, *Sl*PEPR1 also recognized the peptide from sweet potato, albeit with a much lower sensitivity, and an estimated EC_{50} value above 100 nM (Figure 3d).

N-terminal and C-terminal truncated versions of *Ib*Pep1 were synthesized to investigate the specificity for the predicted sweet potato peptide on *Ib*LRR-RK1 (Figure 3c). Deleting up to three N-terminal residues did not have a major impact on the perception, the loss of arginine at position 4, however, led to a severe increase of the EC_{50} value (either when deleted as in *Ib*Pep1 (5–23) or when changed to an alanine as in *Ib*Pep1 (A4)). In contrast, the C-terminus needs to be present for a sensitive perception although the last two serine residues can be replaced by alanine (*Ib*Pep1 (A22A23)).

3.6 | *Ib*PROPEP1-GFP is mainly localized with the tonoplast

We expressed *IbPROPEP1* (OP311829) as a C-terminal fusion with GFP in *N. benthamiana* and *A. thaliana* protoplasts and observed the localization of the protein by confocal microscopy. *Ib*PROPEP1-GFP not only localized with the tonoplast (Figure 4) as reported for *At*PROPEP1-YFP (Hander et al., 2019), but also aggregated into bright small globular structures, resembling bulbs (Saito et al., 2002), inside the vacuole of Arabidopsis mesophyll protoplasts. Similarly, in *N. benthamiana*, *Ib*PROPEP1-GFP mainly accumulated in the tonoplast and aggregated into several small globular structures, which

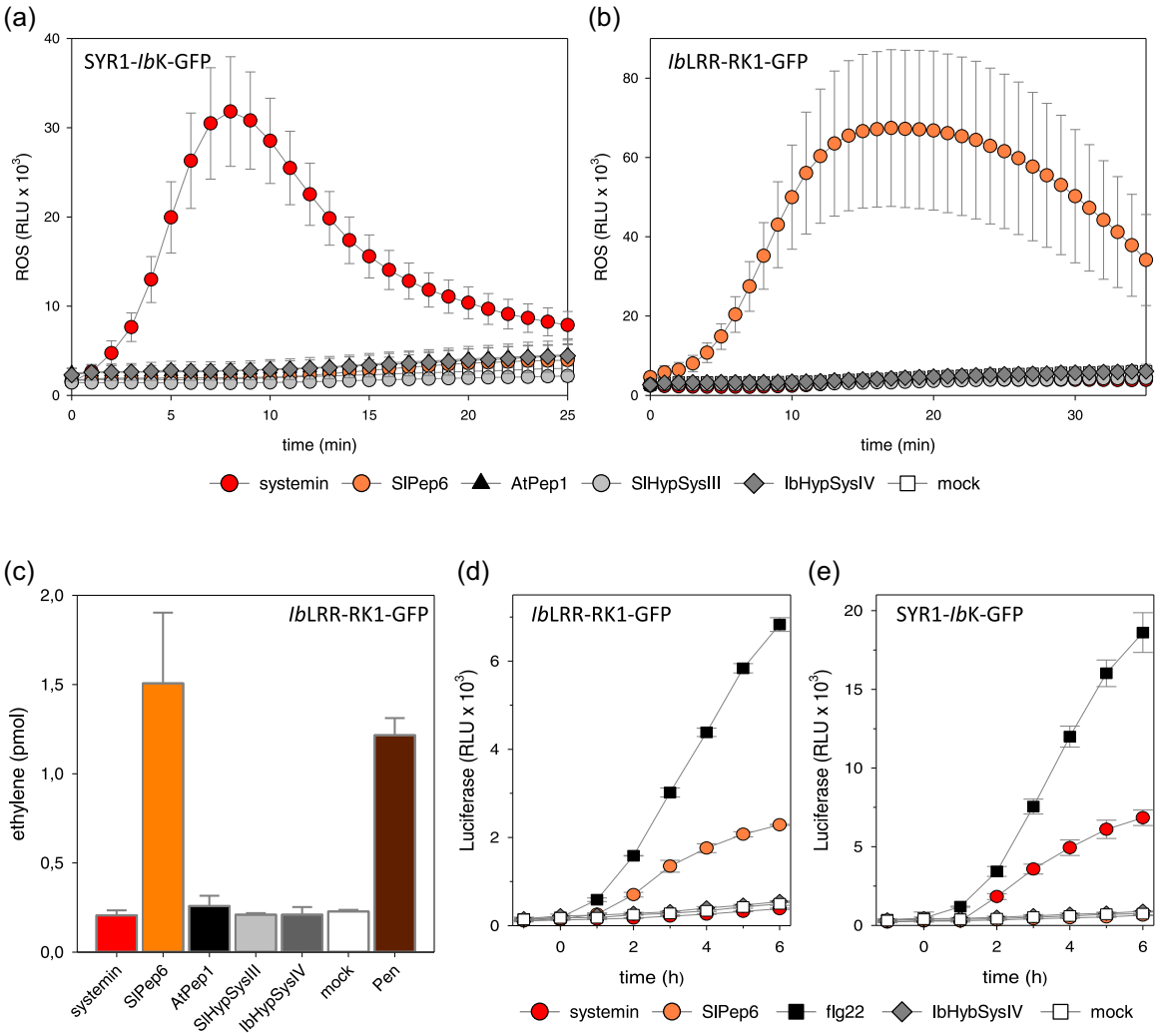
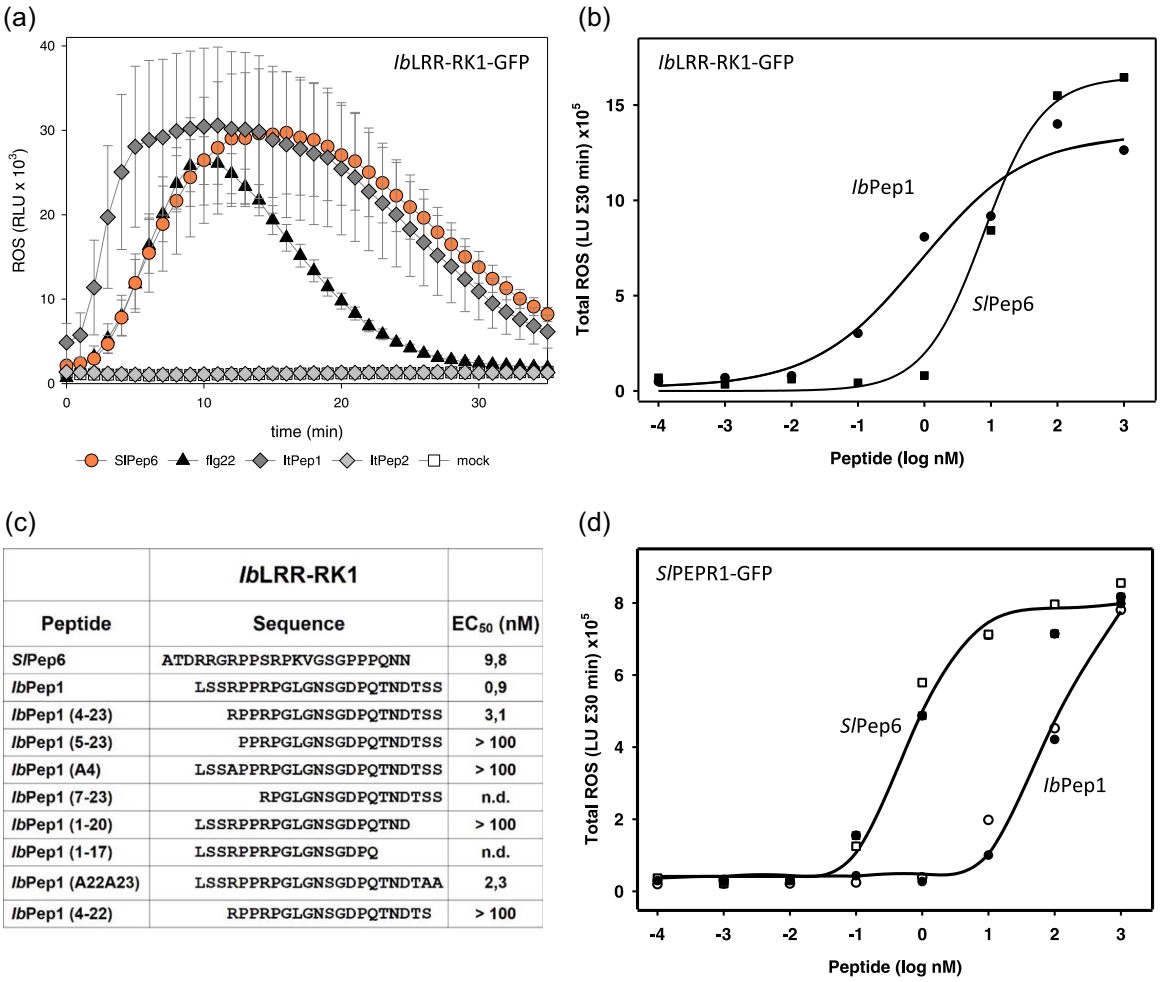


FIGURE 2 Activation of *Ib*LRR-RK1 by *Sl*Pep6 from tomato induces various immune responses. ROS burst in *Nicotiana benthamiana* leaves transformed with either SYR1-*Ib*K (a) or *Ib*LRR-RK1 (b) was induced with 1 μM systemin (red circles), *Sl*Pep6 (orange circles), *At*Pep1 (black triangles), *Sl*HypSysIII (grey circles), *Ib*HypSysIV (dark grey diamonds) or the control (BSA/NaCl, open squares). Values and error bars represent mean ± SE of $n = 4$ replicates. (c) Ethylene production in *Ib*LRR-RK1-expressing leaf discs of *N. benthamiana* was induced with the same selection of peptides at 1 μM, 90 ng/μL Pen extract (Thuerig et al., 2005) was used as a positive control. Values and error bars represent mean ± SD of $n = 3$ replicates. Mesophyll protoplasts from *Arabidopsis thaliana* Col-0 were co-transformed with either SYR1-*Ib*K (d) or *Ib*LRR-RK1 (e) and the reporter construct (pFRK1:luciferase), or with pFRK1:luciferase only (Supporting Information: Figure 4e). Induction of luminescence was monitored after treatment with either 10 nM flg22 (black squares, positive control), systemin (red circles), *Sl*Pep6 (orange circles) or *Ib*HypSysIV (dark gray diamonds) at time point 0, mock control is shown with white squares. Values and error bars represent mean ± SD of $n = 2$. [Color figure can be viewed at wileyonlinelibrary.com]

moved inside the vacuole (Supporting Information: Figure 7, Supporting Information: Movie 1).

3.7 | An *Ib*LRR-RK1-activating DAMP is present in sweet potato leaves

It has been demonstrated that PROPEPs are cleaved by wounding-activated proteases to release immunomodulatory Peps (Bartels & Boller, 2015; Hander et al., 2019). To simulate a corresponding scenario, we first prepared an extract from *I. batatas* cv. TN57 leaves and applied it on transiently *Ib*LRR-RK1-expressing *N. benthamiana* leaves. Leaf discs expressing the receptor responded to the treatment with the partially purified leaf extract with a ROS burst, which was not detectable in control leaves transformed with p19 only (Figure 5a). Next, in addition to tissue disruption to get the extract, we damaged the sweet potato leaves beforehand by squeezing them with tweezers and waiting for 10 min. This material was then harvested, in parallel to tissue from nontweezer-treated control plants. Interestingly, the elicitor activity was higher in extracts from wounded leaves in comparison to the directly extracted leaves. This activity clearly depended on the expression of *Ib*LRR-RK1



(c)

	IbLRR-RK1	
Peptide	Sequence	EC_{50} (nM)
*Sl*Pep6	ATDRRGRPPSRPKVGSGPPPQNN	9,8
*Ib*Pep1	LSSRPPRPGLGNSGDPQTNDTSS	0,9
*Ib*Pep1 (4-23)	RPPRPGLGNSGDPQTNDTSS	3,1
*Ib*Pep1 (5-23)	PPRPGLGNSGDPQTNDTSS	> 100
*Ib*Pep1 (A4)	LSSAPPRPGLGNSGDPQTNDTSS	> 100
*Ib*Pep1 (7-23)	RPGLGNSGDPQTNDTSS	n.d.
*Ib*Pep1 (1-20)	LSSRPPRPGLGNSGDPQTND	> 100
*Ib*Pep1 (1-17)	LSSRPPRPGLGNSGDPQ	n.d.
*Ib*Pep1 (A22A23)	LSSRPPRPGLGNSGDPQTNDTAA	2,3
*Ib*Pep1 (4-22)	RPPRPGLGNSGDPQTNDTS	> 100

FIGURE 3 *Ib*LRR-RK1 recognizes sweet potato *Ib*Pep1 with high sensitivity and specificity. ROS burst in *Nicotiana benthamiana* leaves transformed with p19 plus *Ib*LRR-RK1-GFP, (a) in response to 1 μM of *Sl*Pep6, *It*Pep1, *It*Pep2, 10 nM flg22 or BSA/NaCl (mock), respectively, and, (b) in response to the indicated concentrations of *Ib*Pep1 (●) and *Sl*Pep6 (■). Values and error bars in (a) represent mean ± SE of $n = 4$. (c) Sequences and specific ROS-inducing activities of various peptide derivatives of *Ib*Pep1 used in this study. EC_{50} values indicate concentrations required for induction of half-maximal ROS production in *N. benthamiana* leaves expressing *Ib*LRR-RK1-GFP. (d) Dose-response curves for *Sl*PEPR1-GFP treated with of *Ib*Pep1 (●) and *Sl*Pep6 (■), filled and open symbols correspond to independent experiments. Data in (b) and (d) correspond to the integrated ROS response over 30 min. Curve fittings and calculation of EC_{50} values were performed by nonlinear regression. [Color figure can be viewed at wileyonlinelibrary.com]

(Figure 5b). These data indicated that crude extracts of *I. batatas* leaves contain ligands for *Ib*LRR-RK1 that might accumulate upon wounding stress.

3.8 | *Ib*Pep1 and *Ib*HypSysIV activate complementary signalling cascades

We next addressed the question whether *Ib*Pep1 is involved in herbivore resistance responses in sweet potato or in other processes. Therefore, whole sweet potato plants were sprayed with 25 μM *Ib*Pep1 and analyzed for the induction of *sporamin* and other defense related genes well-known from former studies (Chen, Lin et al., 2016). For comparison, the synthetic hydroxyproline-rich glycopeptide *Ib*HypSysIV, which was shown to activate *sporamin* expression (Chen et al., 2008) was tested at 25 μM as well. The qRT-PCR analyses after 30 min and 1 h of incubation revealed HypSys-dependent transient increases of sporamin (X60930.1) (16-fold), *Ib*NAC1 (GQ280387.1) (22-fold) and *Ib*WIPK1 (HQ434622) (68-fold) transcript levels (Figure 6a), confirming the ability of HypSys peptides to rapidly trigger sporamin-related signalling cascades. In contrast, *Ib*Pep1 transiently induced *sporamin* only 3.5-fold after 30 min, while *Ib*NAC1 and *Ib*WIPK1 were induced to higher and longer-lasting expression levels compared to *Ib*HypSysIV treatment (Figure 6b). Moreover, when analyzing other defense-related genes we also found that *Ib*Pep1 and *Ib*HypSysIV treatments increased the expression of *Ib*LRR-RK1 and *Ib*CML1 (calmodulin-like protein1; OP311828) (Figure 6a,b). Further, compared to water controls and *Ib*Pep1, the application of *Ib*HypSysIV resulted in a significantly increased emission of the wound-inducible volatile DMNT (Figure 6c) (Meents et al., 2019). Scions incubated with the tomato-derived peptide

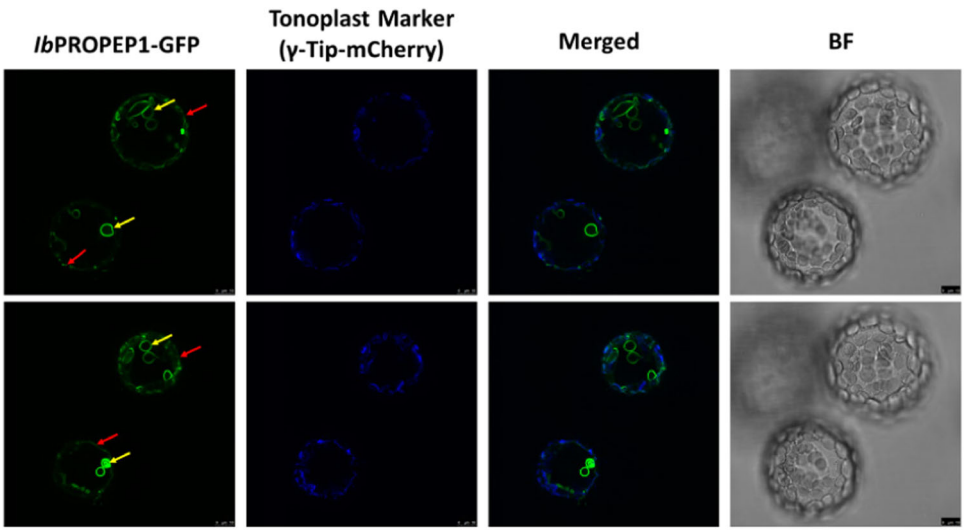


FIGURE 4 *Ib*PROPEP1 mainly localizes to the tonoplast. *Ib*PROPEP1-GFP was transiently transformed in *Arabidopsis thaliana* protoplasts, and protoplasts were observed to monitor the subcellular localization of *Ib*PROPEP1 by confocal microscopy. Partial colocalization with the tonoplast marker γ-Tip-mCherry was observed. The red arrows indicate the position of the tonoplast and the yellow arrows indicate the bulb structures. See Supporting Information: Movie 1 for the observation of moving green fluorescent protein (GFP)-labeled vesicles.
[Color figure can be viewed at wileyonlinelibrary.com]

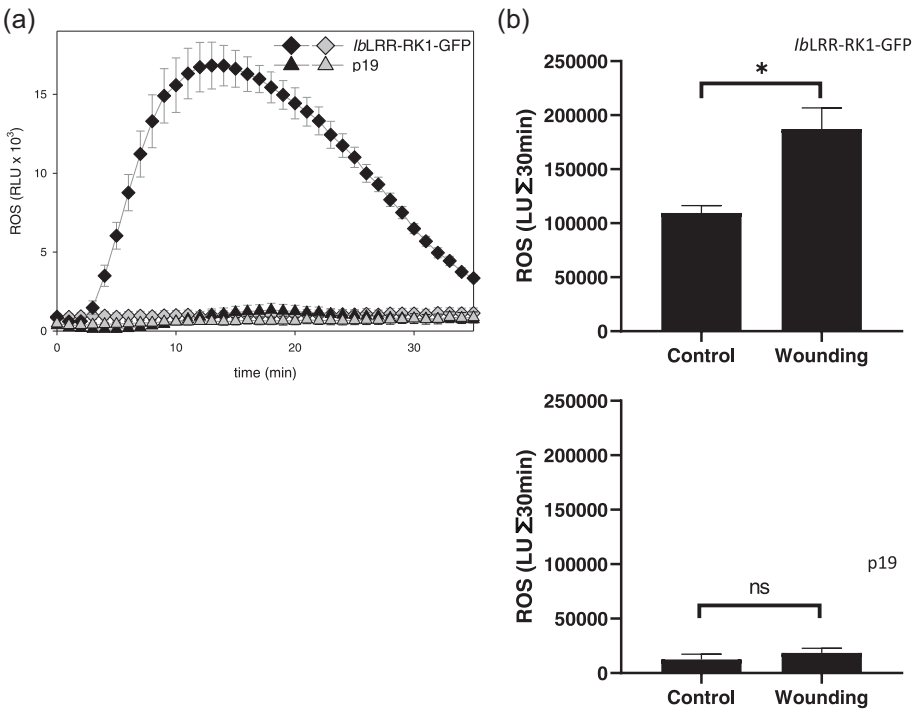


FIGURE 5 *Ib*LRR-RK1 recognizes an endogenous compound. (a) A partially purified extract from *Ipomoea batatas* leaves induces a ROS burst in *Nicotiana benthamiana* leaves expressing *Ib*LRR-RK1 (♦). The extract (black symbols, 1 µL) did not induce a response in leaf pieces transformed with p19 only (▲). Mock treatments are shown in gray symbols. Values and error bars represent mean ± SE of $n = 4$. (b) ROS burst (integrated over 30 min) in *N. benthamiana* control leaves (p19) or leaves expressing *Ib*LRR-RK1 in response to 1 µL partially purified extract from unwounded (control) or 10 min wounded sweet potato leaves. Bars and error bars represent mean ± SE of $n = 4$.

*Sl*Pep6 or an inactive scrambled peptide only displayed basal DMNT levels comparable to the control treatment, confirming thereby the functionality of the peptide application method and the (species-) specificity of the *Ib*HypSysIV elicitor.

To elucidate which role peptides play within the *Ipomoea* defense framework, local and systemic TN57 leaves were analyzed for phytohormone levels after peptide treatment. In comparison to water-treated controls, no significant differences in local and

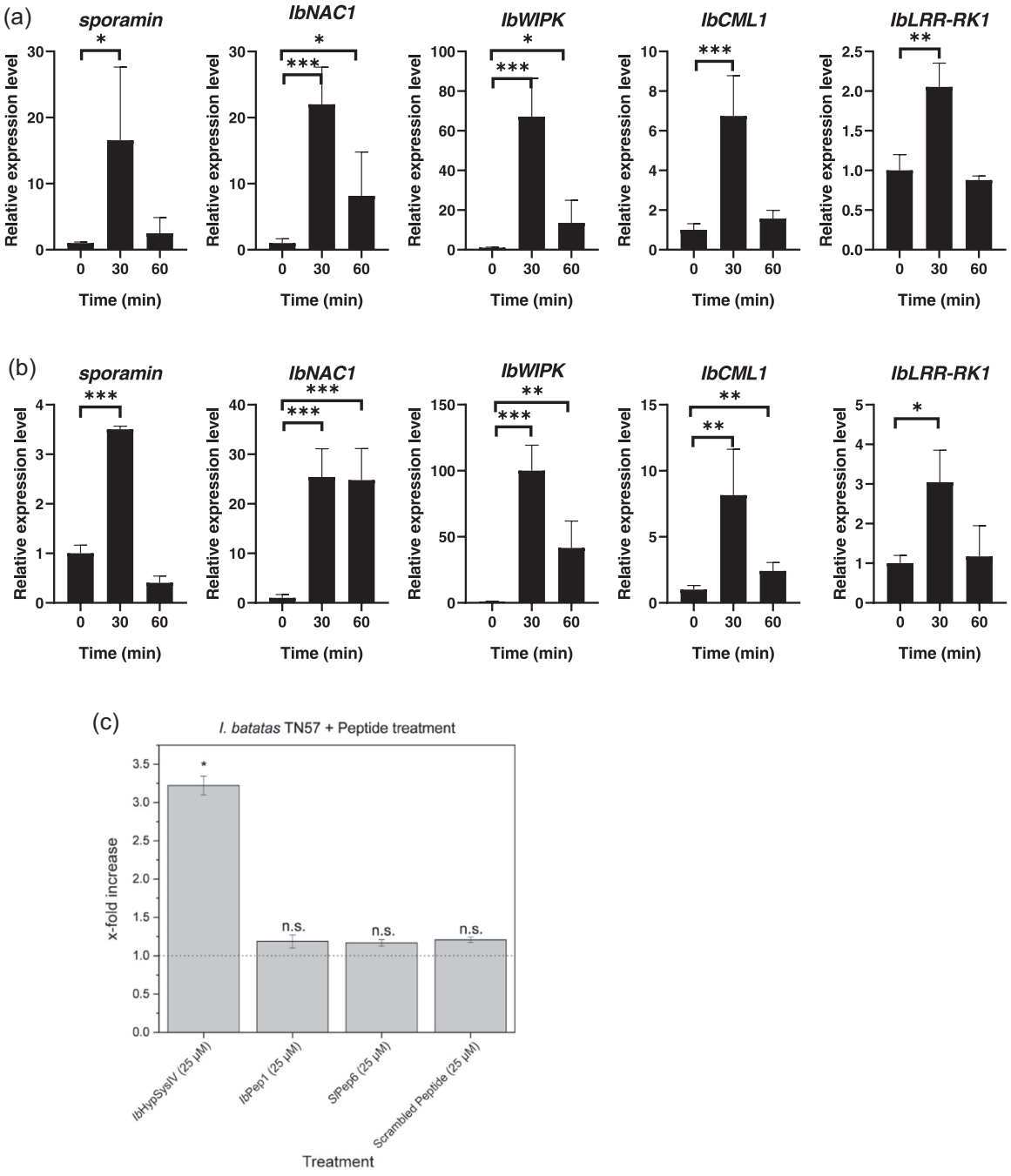


FIGURE 6 Induction of defense-related genes and volatiles in response to *Ib*Pep1 and *Ib*HypSysIV in sweet potato. Sweet potato leaves were treated with 25 µM of *Ib*HypSysIV (a) or *Ib*Pep1 (b), respectively, and tested for the expression level of herbivore defense-related genes. Expression of *sporamin*, *IbNAC1*, *IbWIPK1*, *IbCML1* and *IbLRR-RK1* were analyzed by quantitative real-time polymerase chain reaction (RT-PCR). Bars and error bars represented mean ± SE of $n = 4$. Significance levels are $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, respectively, according to one-tailed *t*-test. (c) The induced emission of (*E*)-4,8–dimethyl–nonatriene (DMNT) in *Ipomoea batatas* TN57 was evaluated after treatment of whole plants with 25 µM *Ib*HypSysIV ($n = 10$), *Ib*Pep1 ($n = 10$), *Sl*Pep6 ($n = 11$), or the scrambled peptide ($n = 7$), data are shown as fold-induction in comparison to the respective water controls. Bars represent the mean ± SE of DMNT emission. Significance levels are indicated by the asterisks (n.s. = not significant; $^{*}p < 0.05$) and are based on a Shapiro–Wilk normality test followed by a Mann–Whitney rank sum test.

systemic jasmonic acid concentrations could be observed after *Ib*HypSysIV treatment (Supporting Information: Figure 8a). Interestingly, *Ib*HypSysIV-treated leaves showed a significantly increased amount of bioactive JA-Ile, however, only locally (Supporting Information: Figure 8b). For the stress-related hormones SA and ABA, no significant differences to control treatments were detected, except for a local decrease in SA concentrations upon contact with *Ib*HypSysIV (Supporting Information: Figure 8c). Treatment with *Ib*Pep1 did neither alter jasmonate nor SA levels although low concentrations might mask possible effects. However, exposure to

*Ib*Pep1 resulted in decreasing amounts of ABA, mainly observed in the local leaf (Supporting Information: Figure 8d). Although no tremendous changes in phytohormone levels were overall visible, we noted a clear tendency that for phytohormones regulated by *Ib*HypSysIV, no response would occur during exposure to *Ib*Pep1 and vice versa.

3.9 | RNAseq of *I. batatas* reveals DEGs upon *Ib*Pep1 and *Ib*HypSysIV treatment

To better understand the similarities between *Ib*Pep1 and *Ib*HypSysIV and their particular functionalities, RNAseq experiments were conducted on single leaves treated with either peptide or water (control), respectively, for 1 h (Figure 7). Overall, 29385 expressed genes were detected based on mapping onto the *I. trifida* reference genome from which 27521 were shared among all treatments including control samples. A total of 261 genes were exclusively detected upon *Ib*HypSysIV treatment, while 383 transcripts were detected only after *Ib*Pep1 treatment. An additional number of 356 common transcripts was found in both peptide treatments but not in the control (Figure 7a). Strikingly, 253 expressed genes were mapped onto the *I. trifida* genome but found only in control plants, suggesting that expression of these genes is reduced upon peptide treatments.

Further, spraying of *Ib*HypSysIV induced significant upregulation of 261 genes, whereas 294 genes were significantly downregulated, compared to water-treated control leaves (data not shown). Upon *Ib*Pep1 incubation, an even stronger response was observed with 769 genes up- and 706 downregulated (data not shown). A comparison of both peptide treatments revealed that 1923 genes were significantly differentially regulated due to these different treatments, 889 up- and 1034 downregulated, when *Ib*HypSysIV versus IbPep1 was compared (Figure 7b). These results support the idea that the two sweet potato peptides have distinct functions, which may be based on their ability to regulate different genes. To support this hypothesis, further confirmation with KEGG and GO pathway analyses and qPCR of selected genes is necessary and will be performed. All original RNAseq data are available (NCBI; accession GSE227409).

4 | DISCUSSION

Recent evidence has shown that sweet potato exhibits DAMP-mediated activation of defenses. The volatile homoterpene, DMNT, has been demonstrated to activate resistance mechanisms in leaves leading to protection against herbivore feeding (Meents & Mithofer, 2020; Meents et al., 2019). Peptide-based activation of defense reactions also has been observed in sweet potato (Chen et al., 2008). However, the biological significance and interconnection with induced resistance against insects remained unclear. This study provides evidence for the existence of a Pep/PEPR-like system in sweet potato and investigates the input- and output conditions.

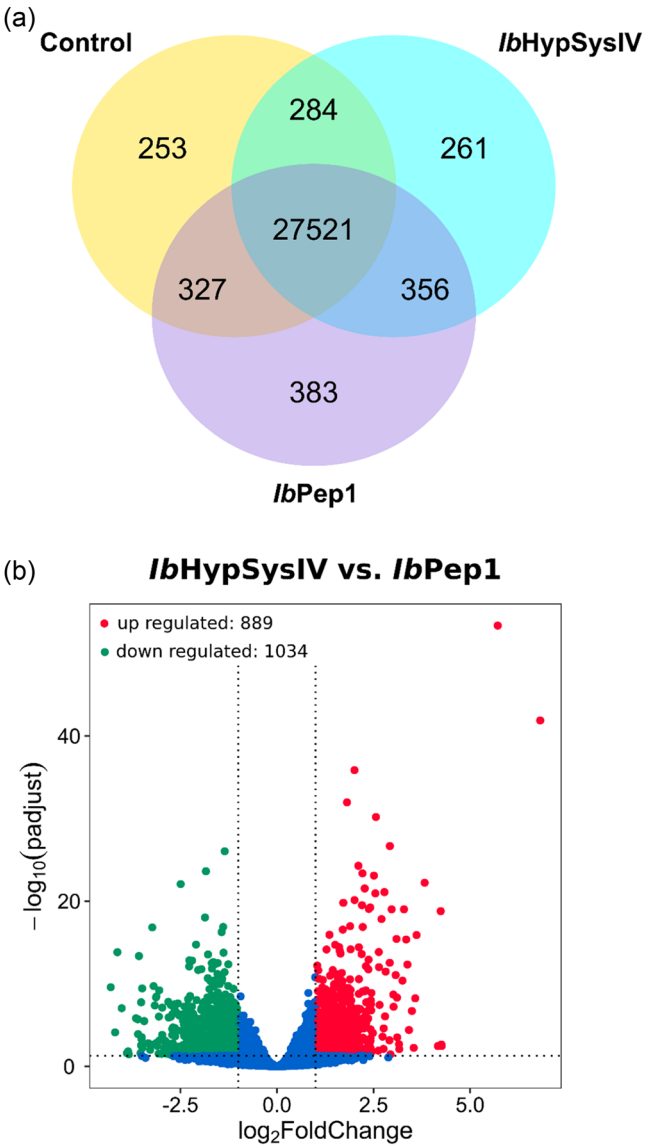


FIGURE 7 *Ib*Pep1 and *Ib*HypSysIV peptides differentially alter gene expression patterns in sweet potato leaves. (a) RNAseq data from *Ipomoea batatas* leaves, treated with 25 µM *Ib*HypSysIV, *Ib*Pep1 or ddH2O (control) for 1 h were mapped onto the *Ipomoea trifida* genome. The Venn diagram shows the numbers of identified, expressed genes in each treatment (Σ 29385 genes). Overlapping circle parts represent the shared expressed genes between the treatments. (b) Volcano plot of statistical significance (-log10 adj. $p > 1.3$, corresponds to adj. $p < 0.05$) against differentially expressed genes (DEGs, log2-fold change ≥1 and $p$adj < 0.05), by comparing both peptide treatments (25 µM each peptide, 1 h). The number of significantly upregulated genes (*Ib*HypSys vs. *Ib*Pep1) is indicated in red with the downregulated ones highlighted in green. DEGs not meeting significance thresholds are depicted in blue. [Color figure can be viewed at wileyonlinelibrary.com]

Briefly, we show that the system can be activated by a damage-amplified endogenous elicitor, provide indirect evidence that this elicitor might be *Ib*Pep1, the product of *Ib*PROPEP1 cleavage, and that it functions in parallel and complementary to a HypSys-dependent signalling pathway. Mining the sweet potato genome

databases, we identified a wound- and herbivory-induced gene encoding a canonical leucine-rich repeat-containing receptor kinase, *Ib*LRR-RK1 (Figure 1a,b and Supporting Information: Figure 2). Using a chimeric receptor approach, in which we combined the cytosolic kinase domain of *Ib*LRR-RK1 with the extracellular recognition domain of *Sl*SYR1 (Supporting Information: Figure 4a), we were able to generate a functional receptor after heterologous expressions in both *N. benthamiana* and *A. thaliana* (Figure 2, Supporting Information: Figure 4c). Phylogenetic analysis suggested that *Ib*LRR-RK1 might be a member of the PEPRs. Indeed, *Sl*Pep6 from tomato, but not *At*Pep1 from *A. thaliana*, was recognized by the native *Ib*LRR-RK1, and triggered the activation of typical defense responses after expression of *Ib*LRR-RK1-GFP in both *N. benthamiana* and *A. thaliana* (Figure 2). Of note, the sweet potato peptide *Ib*HypSysIV, which is described to be involved in the wound response (Chen et al., 2008) was not recognized by *Ib*LRR-RK1.

Based on the above findings, sequences of Peps and their precursor proteins (PROPEPs) from tomato and other Solanaceae plants were used to search for the related putative peptide in the sweet potato genome. We identified a 23-amino acids long peptide ligand, *Ib*Pep1, which is derived from the C-terminus of its precursor protein *Ib*PROPEP1. *Ib*Pep1 is capable to initiate the ROS burst in transgenic *Ib*LRR-RK1-expressing *N. benthamiana* with a 10-fold-higher sensitivity in comparison to *Sl*Pep6 (Figure 3b). However, the fact that the tomato peptide was recognized by sweet potato prompted us to investigate the reciprocal scenario. Indeed, *Sl*PEPR1, the tomato receptor for *Sl*Pep6 (Lori et al., 2015) recognized *Ib*Pep1, providing here for the first time data on interfamily (Solanaceae and Convolvulaceae) compatibility of Peps.

The structure–activity characterization of the ligand of *Ib*LRR-RK1 using various synthetic *Ib*Pep1 derivatives unraveled some structural requirements for the interaction with the corresponding receptor (Figure 3c). As for other Peps, the C-terminus of the peptide is of utmost importance, since the C-terminaly truncated peptide (*Ib*Pep1(1-20)) is at least 100-fold less efficient compared to the 23-mer *Ib*Pep1 (Figure 3c). Unlike the Peps from other plant families, however, the identity of the residues at the C-terminus seems not to be as important since the replacement of the last two residues with alanine residues only marginally decreased the affinity. Peps from sweet potato share 5 of the 12 highly conserved residues with the family-specific Pep-motif of the Solanaceae (Lori et al., 2015) in the overlapping 20-mer core region (Figure 3c, Supporting Information: Figure 9a). Testing one of these highly conserved residues (*Ib*Pep1[A4]) confirmed the importance of the arginine at that position. As illustrated in a composite consensus sequence for Peps of Solanaceae and Convolvulaceae, conserved arginine and proline residues are clustered at the N-terminus of the peptides, whereas, proline and asparagine residues are conserved at the C-termini (Supporting Information: Figure 9b).

PROPEPs have been reported to distribute to distinct subcellular localizations in Arabidopsis (Bartels et al., 2013). While *At*PROPEP3 is present in the cytosol, *At*PROPEP1 and *At*PROPEP6 are positioned at the tonoplast. Our findings show that *Ib*PROPEP1-GFP is localized at the tonoplast as well (Figure 4, Supporting Information: Figure 7). In addition, *Ib*PROPEP1-GFP also appeared in vesicle-like structures attached to the tonoplast that dynamically fuse with the vacuole (Supporting Information: Movie 1). Whether these structures correspond to bulbs, which have been described as cytoplasmic projections into the vacuole, surrounded by a tonoplast-derived double membrane (Madina et al., 2018; Saito et al., 2002), or are artefacts of dimerizing GFP with which the overexpressed PROPEP is tagged (Segami et al., 2014) remains to be investigated. However, to the best of our knowledge, this localization has never been reported for other PROPEPs. We hypothesize that the purpose of *Ib*PROPEP1 enrichment in bulbs could be to store sufficient amounts of the precursor and release it rapidly after cell and vacuole injury to allow cleavage into active *Ib*Pep1. *In planta*, we demonstrated the release of a specific agonist of *Ib*LRR-RK1. Incubation of only 10 min of wounded sweet potato leaves increased the amount of the elicitor in a partially purified fraction, in comparison to nonincubated leaf material (Figure 5).

The inherent trypsin inhibitory activity of sporamin provides strong protection against herbivory in sweet potato and other, transgenic plants species expressing sporamin (Chen et al., 2006; Meents et al., 2019; Yeh, Chen, et al., 1997). Strongly induced expression of *sporamin* was detected in sweet potato leaves during pest attack and injury stress (Yeh, Lin, et al., 1997). The 18 amino acid hydroxyprolinated peptide *Ib*HypSysIV, which can be extracted from sweet potato leaves was amplifying the wounding signal and activated the expression of *sporamin* (Chen et al., 2008). In the present study, we found that spraying with either peptide, *Ib*HypSysIV or *Ib*Pep1, rapidly induced the expression of wound-induced defense response genes including *IbWIPK1*, *IbNAC1*, *sporamin* and even *IbLRR-RK1*, in sweet potato leaves (Figure 6). However, *Ib*HypSysIV treatment induced the expression of *sporamin* much more strongly than *Ib*Pep1 treatment. Previous studies have revealed that application of *At*Peps and the activation of *At*PEPR1/2 lead to increased jasmonate accumulation and induced jasmonate responses in Arabidopsis (Huffaker, 2015). We found that the application of *Ib*Pep1 did not increase the amount of jasmonates, in contrast to *Ib*HypSysIV, which induced the accumulation of JA-Ile in sweet potato leaves slightly (Supporting Information: Figure 8), suggesting that *Ib*HypSysIV may trigger the jasmonate pathway and associated responses in contrast to *Ib*Pep1. A clear discrepancy between the two peptides lies in their ability to regulate the synthesis and release of the homoterpene DMNT. This volatile danger signal is induced in sweet potato upon wounding and herbivory (Meents et al., 2019). Only treatment with *Ib*HypSysIV but neither *Ib*Pep1 nor *Sl*Pep6 nor a scrambled control peptide were able to induced DMNT, indicating the specificity of this response (Figure 6c). Overall, our study suggests that in addition to the *Ib*Pep1/*Ib*LRR-RK1 pair described here for the first time, there is another, as yet unidentified, DAMP receptor that specifically interacts with the *Ib*HypSysIV ligand in sweet potato. The latter system appears to be more active than the Pep/PEPR pair in the jasmonate pathway regulating sporamin expression. A summarizing model of both peptide-induced pathways

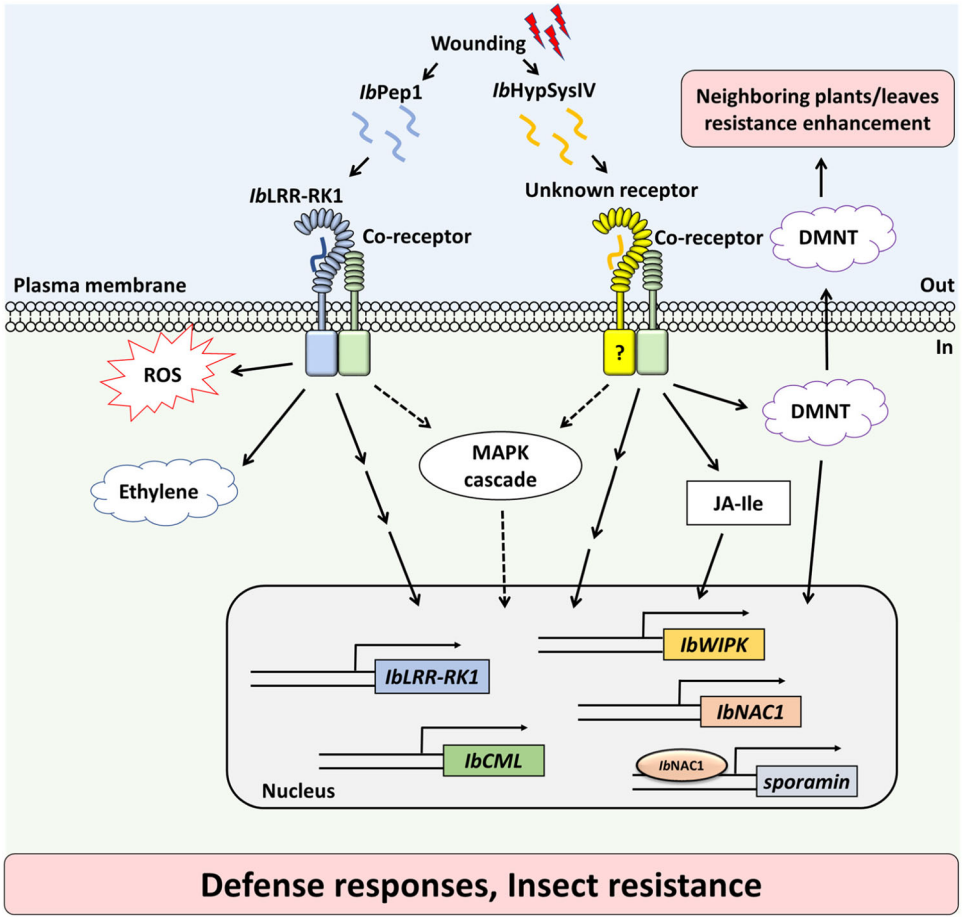


FIGURE 8 Proposed model of *Ib*Pep1 and *Ib*HypSysIV triggered resistance in sweet potato leaves. Upon wounding, peptide ligands *Ib*Pep1 and *Ib*HypSysIV are activated/induced in the treated leaf (Chen et al., 2008; Li et al., 2016). *Ib*Pep1 activates the *Ib*LRR-RK1 receptor leading to the generation of defense responses including ROS and ethylene production. Different from *Ib*Pep1, *Ib*HypSysIV induces DMNT emission as a volatile antiherbivore defense signal and the accumulation of JA-Ile (Meents et al., 2019). Both *Ib*Pep1 and *Ib*HypSysIV induce the expression of several wounding/defense-related genes such as *IbWIPK*, *IbNAC1*, *IbCML*, *IbLRR-RK1* and trypsin inhibitor gene *sporamin* (Chen, Kuo, et al., 2016; Chen, Lin, et al., 2016; Yeh, Chen, et al., 1997; Yeh, Lin, et al., 1997). In summary, *Ib*Pep1 and *Ib*HypSysIV might work in a complementary and/or parallel pathway to strengthen plant resistance against biotic threats. Dashed arrows: yet unproven pathways (here MAPK cascade).
[Color figure can be viewed at wileyonlinelibrary.com]

is shown in Figure 8. Having shown that both, *Ib*Pep1 and *Ib*HypSysIV, have a certain ability to regulate defense responses against herbivory attack and wounding, albeit with different efficacies, we have yet to define the key signalling pathway(s) regulated by *Ib*Pep1. Preliminary analyses of RNAseq data suggest that *Ib*Pep1 and *Ib*HypSysIV control partly distinct pathways, which will need to be further investigated in combination with real infestation and infection assays in the future.

5 | CONCLUSIONS

Previous studies have shown that Peps/PEPR ligand-receptor systems are widespread in plants. Here, we identified a novel peptide ligand and its corresponding receptor from sweet potato. This adds another ligand/receptor pair to the growing list of DAMP perception systems. Understanding how the downstream gene responses to different ligands are coordinated in the genetic network is a topic that needs to be addressed in the future. Although *Ib*Pep1 was not able to induce the emission of DMNT, the trypsin protease inhibitor sporamin and its TF *Ib*NAC1 were upregulated, hinting at a modular way to increase insect resistance. Peps vary widely from species to species, conserved family-specific Pep-motifs are sufficient for Pep recognition by PEPRs from different species of the same plant family (Lori et al., 2015). In our experiment, we found that the peptide ligand *Sl*Pep6 of tomato belonging to Solanaceae family did interact with *Ib*LRR-RK1 from sweet potato belonging to Convolvulaceae family and activated downstream responses. *Vice versa*, the reciprocal combination was functional as well. To our knowledge, this is the first example that a peptide ligand does not follow the rule of family-specific incompatibility of Peps but suggests the conservation of a plant order-specific peptide ligand structure in two families of Solanales, Solanaceae and Convolvulaveae.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. All RNAseq data have been deposited in NCBI (National Center for Biotechnology Information) and are available under the accession number GSE227409.

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SUPPORTING INFORMATION

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