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Research Article

# A G-type lectin receptor kinase negatively regulates *Arabidopsis* immunity against root-knot nematodes

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/General-Instructions) is Isgouhi Kaloshian.

## Abstract

Root-knot nematodes (*Meloidogyne* spp., RKN) are responsible for extensive crop losses worldwide. During infection, they penetrate plant roots, migrate between plant cells, and establish feeding sites, known as giant cells, near the root vasculature. Previously, we found that nematode perception and early responses in plants were similar to those of microbial pathogens and required the BRI1-ASSOCIATED KINASE1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE3 (BAK1/SERK3) coreceptor in *Arabidopsis* (*Arabidopsis thaliana*) and tomato (*Solanum lycopersicum*). Here, we implemented a reverse genetic screen for resistance or sensitivity to RKN using *Arabidopsis* T-DNA alleles of genes encoding transmembrane receptor–like kinases to identify additional receptors involved in this process. This screen identified a pair of allelic mutations with enhanced resistance to RKN in a gene we named *ENHANCED RESISTANCE TO NEMATODES1* (*ERN1*). *ERN1* encodes a G-type lectin receptor kinase (G-LecRK) with a single-pass transmembrane domain. Further characterization showed that *ern1* mutants displayed stronger activation of MAP kinases, elevated levels of the defense marker *MYB51*, and enhanced $H_2O_2$ accumulation in roots upon RKN elicitor treatments. Elevated *MYB51* expression and ROS bursts were also observed in leaves of *ern1* mutants upon flg22 treatment. Complementation of *ern1.1* with 35S- or native promoter–driven *ERN1* rescued the RKN infection and enhanced defense phenotypes. Our results indicate that *ERN1* is an important negative regulator of immunity.

## Introduction

The majority of plant parasitic nematodes (PPN) are soil-dwelling organisms that use a specialized mouth part or stylet to penetrate root tissues and establish an intimate relationship with their hosts. Plant–nematode signaling starts even before nematode penetration, as nematodes are attracted by root diffusates dispersed in the soil (Goverse and Smant 2014). After finding their host, different species of PPN deploy specific strategies to penetrate plant tissues and initiate feeding. Root-knot nematodes (*Meloidogyne* spp., RKN) are the most devastating group of PPN, causing considerable crop losses worldwide (Jones et al. 2013; Kaloshian and Teixeira 2019). RKN are sedentary endoparasites that penetrate plant roots behind the root tip and migrate between cells until they reach the vascular cylinder,

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where they establish a specialized feeding site (Wyss et al. 1992; Goverse and Smant 2014). RKN secretions, mainly from esophageal glands, contribute to massive reprogramming of plant cell processes, including cell division (Goverse et al. 2000; de Almeida and Gheysen 2013; Siddique et al. 2015). The outcome is the development of enlarged and multinucleated cells, termed giant cells, resulting from karyokinesis without cytokinesis (Vieira et al. 2014). Each nematode forms ~6 to 8 giant cells surrounded by enlarged neighboring endodermis and cortical cells, forming a root gall. This feeding site acts as a nutrient sink, nourishing the RKN during its entire life cycle. Once the feeding site is established, RKNs become sedentary, and as females mature, they become pearl shaped, with the reproductive organs occupying most of their bodies. Most RKN species reproduce parthenogenetically. A gelatinous sac develops at its posterior end, protruding onto the surface of the root, where a large number of eggs are laid.

Plant defense responses are triggered by elicitors derived from microbes. General elicitors or microbe-associated molecular patterns (MAMP) are perceived by cell surface–localized pattern recognition receptors (PRRs) and trigger an immune response known as pattern-triggered immunity (PTI) (Yu et al. 2017). A number of PRRs have been identified, and among them is the well-characterized receptor FLS2 (FLAGELLIN SENSING2). *Arabidopsis* (*Arabidopsis thaliana*) FLS2 recognizes a highly conserved stretch of 22 amino acids, flg22, present on the N-terminus of bacterial flagellin, which acts as a molecular glue that brings together FLS2 and the coreceptor BAK1/SERK3 (BRI1-ASSOCIATED KINASE1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE3), to elicit downstream signaling events, including phosphorylation events, transcriptional reprogramming, callose deposition, and ROS burst (Felix et al. 1999; Gomez-Gomez et al. 1999; Zipfel et al. 2004; Chinchilla et al. 2007). Notably, flg22-mediated defense elicitation is followed by transcriptional upregulation of *FLS2* and *BAK1*, as well as upregulation of negative regulators of immunity, such as *PBL13* (*AvrPphB SUSCEPTIBLE1-LIKE13*) (Lin et al. 2015). Additionally, after elicitation of defense responses, FLS2 is internalized from the plasma membrane to internal vesicles, likely for degradation (Salomon and Robatzek 2006; Ben Khaled et al. 2015).

Recently, processes involved in root perception of nematodes and early responses during nematode penetration have been unveiled. It was shown that RKN and cyst nematodes (*Heterodera* spp., CNs), infective-stage juveniles, are perceived by plant roots during their root migration phase, similar to the perception of microbial pathogens in aboveground tissues (Mendy et al. 2017; Teixeira et al. 2016). RKN perception required the coreceptor BAK1/SERK3 as *SERK3*-silenced tomato plants displayed enhanced susceptibility to RKN (Peng and Kaloshian 2014). BAK1/SERK3 is a coreceptor of multiple MAMPs coordinating perception with diverse PRRs to activate PTI. Enhanced susceptibility to both RKN and CN was also reported in the *Arabidopsis bak1-5* mutant (Mendy et al. 2017; Teixeira et al. 2016). Interestingly, a leucine-rich repeat (LRR) serine/threonine kinase, NILR1 (NEMATODE-INDUCED LRR-RLK 1), was shown to have similar characteristics as microbial PRRs and, therefore, could be a receptor of an, as yet, unidentified nematode-associated molecular pattern (NAMP) (Mendy et al. 2017). In addition, mutants in *Arabidopsis* BIK1 (BOTRYTIS-INDUCED KINASE1), which is associated with and is phosphorylated by BAK1, and the double mutant of the RBOH D/F (RESPIRATORY BURST NADPH OXIDASE HOMOLOG), which are phosphorylated by BIK1, also displayed enhanced susceptibility to RKN (Lu et al. 2010; Zhang et al. 2010; Lin et al. 2014; Li et al. 2014; Kadota et al. 2014; Teixeira et al. 2016). Taken together, these findings indicate that canonical PTI signaling is involved in RKN perception.

Despite the great potential of the broad and lasting defense mediated by PTI responses, nematode-related immunity research has largely focused on the characterization of resistance (*R*) gene-mediated defense and nematode effectors (Gheysen and Mitchum 2011; Goverse and Smant 2014; Kaloshian and Teixeira 2019). Nevertheless, a few investigations have characterized transcriptional changes in response to nematode infection at early time points. A study describing gene expression changes found 8 differentially expressed genes in response to RKN in both susceptible and resistant tomato (*Solanum lycopersicum*) plants only 12 h after inoculation, when nematodes were still migrating through plant tissues (Lambert et al. 1999). Interestingly, the transcriptome of tomato roots collected 24 h after RKN inoculation showed consistently more genes upregulated than downregulated among those that were differentially expressed, including defense-related genes (Bhattarai et al. 2008). More recently, using reporter GUS lines, activation of defense-related genes was shown in *Arabidopsis* roots exposed to crude RKN extracts, providing a valuable tool for evaluating defense responses in the absence of wound-induced responses due to RKN penetration (Teixeira et al. 2016).

To identify additional immune receptors involved in the PTI response against RKN, we used a reverse genetics approach to screen *Arabidopsis* T-DNA insertion alleles of receptor-like kinases (RLKs). Instead of a receptor acting as a positive regulator of immunity, we identified a recently discovered negative regulator of RKN immunity. Here, we present the characterization of a negative regulator of RKN immunity encoded by a lectin receptor kinase.

## Results

### Mutation of the *Arabidopsis* RLK ERN1 results in resistance to RKN

To identify putative receptors involved in immunity against RKN, we screened homozygous *Arabidopsis* T-DNA insertional mutants in genes encoding transmembrane receptor-like kinases for resistance or sensitivity to RKN. At least 2 mutant alleles for each gene were screened (Supplemental Table S1). Among the mutants, those with T-DNA insertions

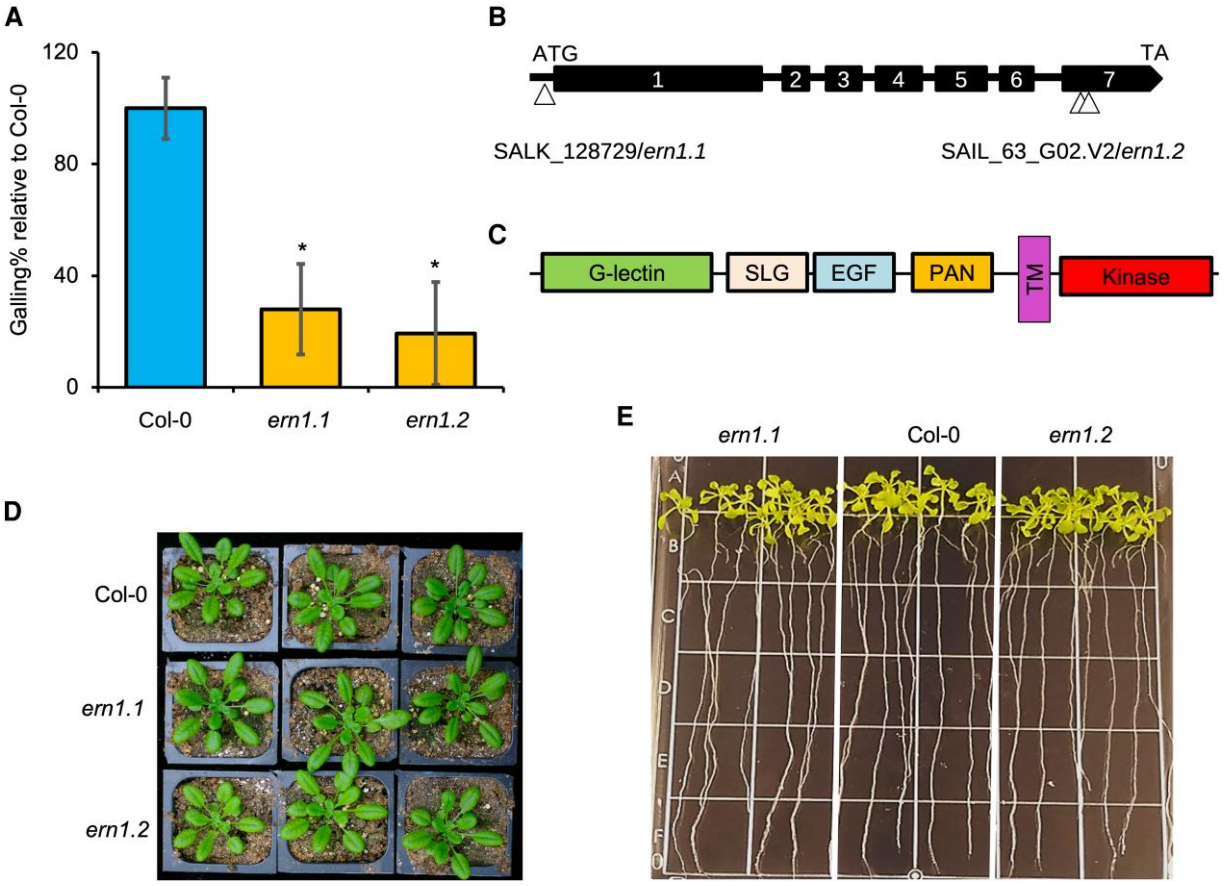


**Figure 1.** *Arabidopsis ern1* mutant resistance phenotype, *ERN1* gene/protein structure, and *ern1* morphological phenotypes. **A)** Root-knot nematode infection phenotype of *Arabidopsis* WT Col-0, *ern1.1* (SALK_128729) and *ern1.2* (SAIL_63_G02) T-DNA mutants, grown on Gamborg media and infected with 100 infective-stage juveniles. Bars are percentage of galls on roots normalized to WT ± SE ($n = 75$ per genotype). Asterisks indicate significance difference ($P < 0.001$, ANOVA). **B)** SALK_128729/*ern1.1* has an insertion in the 1st exon while the SAIL_63_G02/*ern1.2* mutant has at least 2 insertions in the 7th exon of the gene. **C)** ERN1-predicated protein structure: S-locus glycoprotein (SLG), epidermal growth factor–like (EGF), a plasminogen–apple–nematode (PAN), and transmembrane (TM) domains. **D)** Shoot growth phenotype of 3-wk-old *Arabidopsis* plants grown under a 16-h photoperiod at 22 °C. **E)** Root growth phenotype of 3-wk-old *Arabidopsis* plants grown on MS agar plates under a 12-h photoperiod at 22 °C.

in At1g61550, SALK_128729, and SAIL_63_G02, displayed significantly fewer galls on roots indicating enhanced resistance to RKN infection (Fig. 1A). Accordingly, the locus was named *ENHANCED RESISTANCE TO NEMATODES* (*ERN1*) and the mutants designated *ern1.1* (SALK_128729) and *ern1.2* (SAIL_63_G02) (Fig. 1A).

## Characterization of *ERN1* and its mutant alleles

Sequence analysis indicated that the *ERN1* genomic sequence is 3,629 base pairs (bp) with 7 exons and a cDNA of 2,982 bp (Fig. 1B). Protein domain examination using InterPro revealed that *ERN1* encodes a G-lectin receptor kinase (G-LecRK) with a G-type lectin domain, a single transmembrane domain, and an intracellular serine/threonine kinase domain (Fig. 1C). In addition, ERN1 contains a S-locus glycoprotein, an epidermal growth factor-like (EGF), and a plasminogen–apple–nematode (PAN) extracellular domain (Fig. 1C). Phylogenetic analysis of *Arabidopsis* G-LecRKs classified *ERN1* as G-LecRK-VIII.8 (Teixeira et al. 2018).

The T-DNA insertions in the *ern1.1* and *ern1.2* alleles were predicted to be in the 1st and 7th exons, respectively. We confirmed the locations of the T-DNA insertions in both mutants by PCR and sequencing. This analysis revealed that the insertion in *ern1.1* is located 49 bp upstream of the *ERN1* start codon, while *ern1.2* has a deletion of 61 bp and introduction of a premature stop codon in the predicted kinase domain (Fig. 1B). In addition, our results indicated that *ern1.2* has at least 2 T-DNA insertions in tandem and reverse orientation (Figs. 1B and S1A). Assessing the expression of *ERN1* in these mutants indicated that *ern1.1* is a null allele as no *ERN1* transcripts were detected in this mutant, while in *ern1.2*, we detected a deletion suggesting no that full-length transcript is made (Supplemental Fig. S1B).

The enhanced nematode resistance in the *ern1* mutants lead us to investigate whether the resistance phenotype is

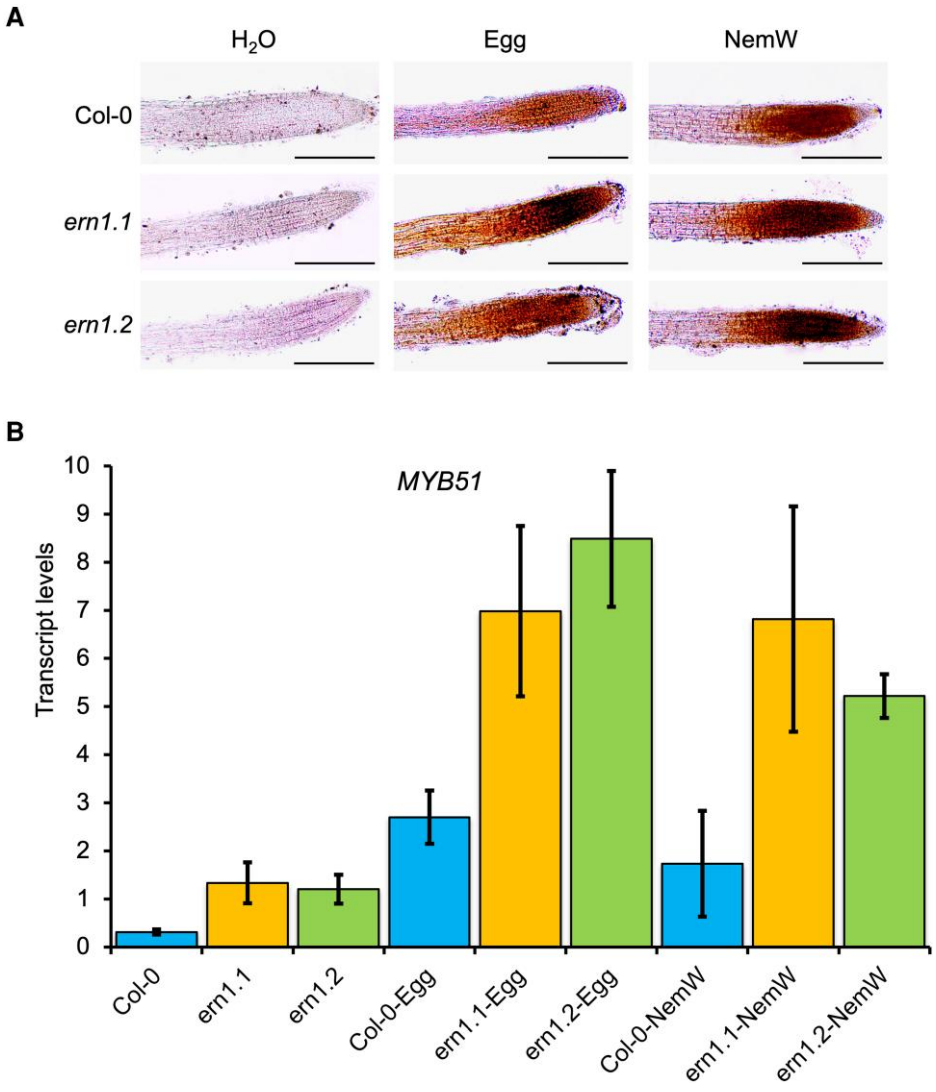


**Figure 2.** RKN egg extract and excretions induce enhanced immune responses in *ern1* roots. **A)** DAB-stained roots of 5-d-old *Arabidopsis* Col-0, *ern1.1*, and *ern1.2* seedlings treated with RKN egg extract (Egg) or NemaWater (NemW) for 1 h. The experiment was performed 4 times with similar results. Scale bars = 10 mm. **B)** *MYB51* expression in roots of 5-d-old *Arabidopsis* Col-0, *ern1.1*, and *ern1.2* mutants treated with egg extract or NemW for 1 h and used for RNA extraction. Gene expression analysis was performed by RT-qPCR and normalized to *UBQ22*. Bars are means of 3 biological replicates with 2 technical replicates ± SE.

due to morphological changes in plant or root growth phenotypes. Wild-type (WT; Col-0), *ern1.1*, and *ern1.2* mutants were grown in soil under short- (12 h/12 h L/D) and long- (16 h/8 h L/D) day photoperiods at 22 °C and under a short-day length at 19 °C. No difference in above-ground growth morphology was detected between Col-0 and either of the *ern1* mutants under all tested conditions (Figs. 1D and S2). Measurements of rosette leaf dimensions also did not reveal any leaf growth changes in the *ern1* mutants compared to WT (Supplemental Fig. S3). Since RKNs infect behind root tips and alterations in root development may affect nematode infection rates, we investigated root growth in the *ern1* mutants. Seedlings grown on agar plates were evaluated for root growth over a 4-d period (Days 4 to 8) and lateral root capacity at 8 d. No difference was detected in the root growth rate or lateral root capacity between WT and the *ern1* alleles (Figs. 1E and S4).

## The *ern1* mutants display higher levels of $H_2O_2$ accumulation and upregulation of immune genes induced by RKN elicitors

The enhanced resistance displayed by the *ern1* mutants and the lack of obvious root growth phenotypes suggests enhanced immunity in these mutants. To evaluate enhanced immune responses in roots, we stained the roots of the *ern1* mutants and WT plants with immunohistochemical 3,3′diaminobenzidine (DAB) to evaluate $H_2O_2$ accumulation detected as a brown-stained precipitate. No brown stains were detected in WT control or *ern1* mutant roots indicating

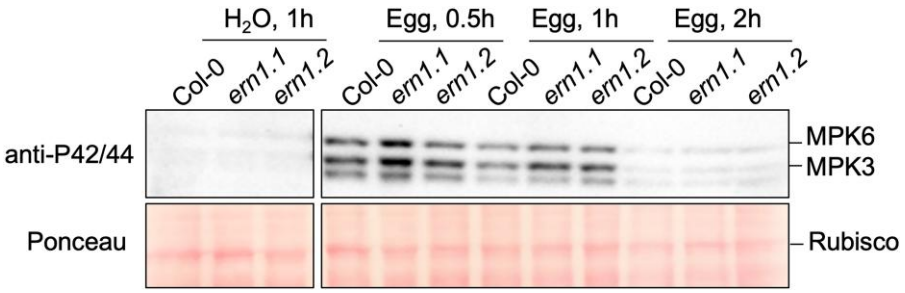


**Figure 3.** RKN egg extract–induced MPK activation is enhanced in *ern1* roots. Roots of 12-d-old *Arabidopsis* Col-0, *ern1.1*, and *ern1.2* seedlings were treated with RKN egg extract (Egg) for 0.5, 1, and 2 h or water control for 1 h and used for immunoblot analysis. Activated MPKs were detected using antiphospho-p42/44 MPK antibody. Ponceau-stained Rubisco was used for equal loading control.

no constitutive accumulation of $H_2O_2$ (Fig. 2A). To evaluate the induced immune responses in *ern1*, roots of *ern1* and WT plants were treated with RKN NemaWater, which is nematode-free water generated by incubating newly emerged infective-stage juveniles in water overnight. NemaWater from *Meloidogyne incognita* and the cyst nematode *Heterodera schachtii* have been shown to trigger immune responses in different plant species, including *Arabidopsis* (Mendy et al. 2017). NemaWater or water-treated roots were stained with DAB. While control roots treated with water did not display any color change, both *ern1.1* and *ern1.2* mutant roots treated with NemaWater consistently showed more intense dark-brown staining compared to WT roots (Fig. 2A). Previously, we found that crude extracts from infective juveniles also triggered immune responses in roots, including activation of the transcription factor *MYB51* (Teixeira et al. 2016). We wondered whether extracts of nematode eggs, which contain tissues of different embryonic stages as well as first- and second-stage juveniles, could also trigger similar responses as NemaWater. We first evaluated the ability of nematode egg extracts to trigger *MYB51* expression in *Arabidopsis* roots. *Arabidopsis* plants expressing the *MYB51pro::GUS* reporter were treated with nematode egg extract and evaluated for GUS activity. Infective-stage juvenile extract was used as a positive control. While no GUS activity was detected in control roots of *MYB51pro::GUS* reporter lines treated with PBS, GUS activity was detected in roots of *MYB51pro::GUS* reporter lines treated with either egg extract or infective juvenile extract indicating that nematode egg extract is able to trigger immune responses similar to that from infective juveniles (Supplemental Fig. S5). Similarly, *ern1* mutant roots treated with nematode egg extracts and stained with DAB also showed more intense brown staining in the roots compared to WT confirming increased $H_2O_2$ accumulation (Fig. 2A).

To quantitatively evaluate the enhanced resistance of *ern1* mutants, roots of *ern1* mutants and WT were treated with nematode egg extracts, NemaWater, or water and were used to examine *MYB51* expression by RT-qPCR. Both nematode egg extracts and NemaWater induced *MYB51* transcripts levels to higher levels in the *ern1* mutant roots compared to WT (Fig. 2B). Together, these results suggest that enhanced resistance of *ern1* mutants may be attributed to increased $H_2O_2$ accumulation and strong induction of *MYB51* expression, which are likely induced by different RKN elicitors.

## Egg-derived elicitor(s) activate MAP kinases at higher levels in *ern1* mutants

The mitogen-activated protein kinases MPK3 and MPK6 are activated transiently upon perception of many MAMPs and elicitors (Asai et al. 2002). Given the enhanced resistant phenotype of *ern1* mutants, we explored whether nematode egg-derived elicitors induce MPK3/6 activity and if differential levels of kinase activity could be detected in *ern1* mutants. Since MPK3/6 activity is known to be an early and transient response, we performed a time course experiment collecting tissues early after the egg extract treatment of *ern1* and WT roots and performed immunoblot analysis using antiphospho-p42/44 antibody. Egg extract treatment resulted in rapid activation of MPK3/6, and more importantly, stronger activation was detected in *ern1* mutants compared to WT (Fig. 3). MPK3/6 activation was transient reaching to nondetectable levels by 2 h after egg extract treatment. These results indicate that the egg-derived elicitors induce a transient activation of MAP kinases similar to observations upon elicitation by other MAMPs (Asai et al. 2002).

## ERN1 is localized to the plasma membrane and the endoplasmic reticulum

The ERN sequence predicts several protein domains, including a transmembrane domain suggesting that ERN1 spans the plasma membrane. To determine the subcellular localization of ERN1, we transiently expressed ERN1 fused with a C-terminal m-Scarlet red fluorescent protein (ERN1-mScarlet) in *Nicotiana benthamiana* leaves. To label the plasma membrane, we transiently coexpressed LT16b, a plasma membrane–localized marker, fused with N-terminal eGFP (eGFP-LT16b) (Cutler et al. 2000). Using confocal microscopy, ERN1-mScarlet was detected at the plasma membrane overlapping with eGFP-LT16b (Fig. 4). To determine whether ERN1 localized to membranes other than the plasma membrane, we also transiently coexpressed ERN1-mScarlet with the endoplasmic reticulum (ER) retention 4 peptide sequence KDEL, N-terminally tagged with GFP

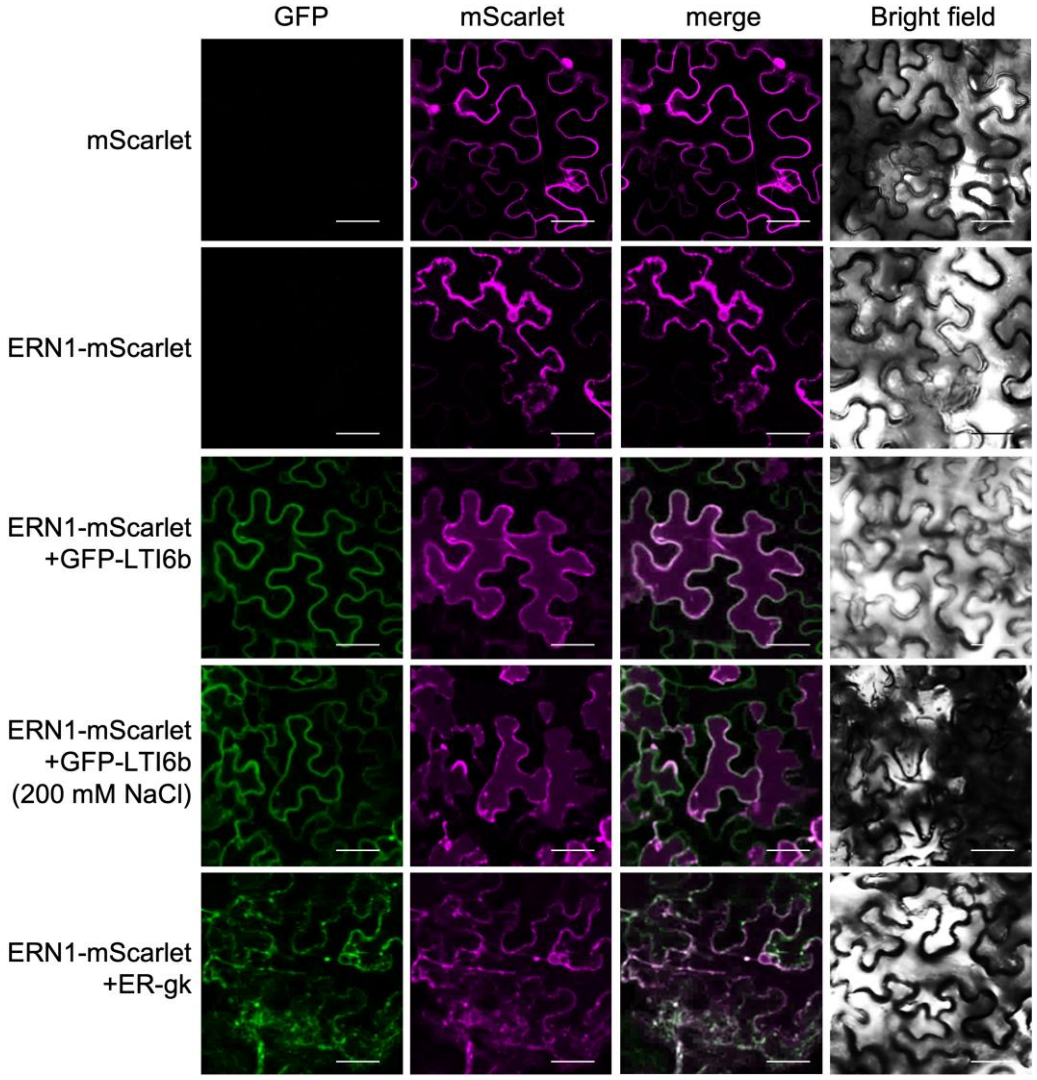


**Figure 4.** ERN1 is localized to the plasma membrane and ER in *N. benthamiana* leaf epidermal cells. mScarlet-tagged ERN1 (ERN1-mScarlet) was transiently coexpressed in *N. benthamiana* leaves with plasma membrane marker GFP-LTI6b and ER marker ER-gk, respectively. mScarlet and GFP signals were detected in leaf epidermal cells by a confocal microscope 48 h after infiltration with *A. tumefaciens* expressing the different constructs. Scale bars = 50 $\mu$m.

(GFP-KDEL). ERN1-mScarlet was colocalized with GFP-KDEL indicating that ERN1 is also localized to the ER (Fig. 4).

To confirm ERN1 subcellular localization, we generated transgenic *Arabidopsis*, stably expressing ERN1-GFP driven by the *ERN1* native promoter (*ERN1*$_{pro}$*::ERN1-GFP*) in the *ern1.1* mutant background. Confocal microscopy of the transgenic roots confirmed the localization of the ERN1 to both the plasma membrane and the ER *in planta* (Fig. 5). These results are consistent with ERN1's function as a transmembrane receptor kinase.

## Complementation of the *ern1.1* mutant results in enhanced plant growth and enhanced susceptibility to nematodes

To confirm that disruption of *ERN1* by the T-DNA insertion is the cause of the enhanced nematode resistance phenotype, we complemented the *ern1.1* mutant with 2 different *ERN1* constructs, 1 driven by CaMV 35S promoter (*35S::ERN1*) and the other by its native promoter (*ERN1pro::ERN1*). Transgenic plant expression of *ERN1* from its native or 35S promoter displayed enhanced plant growth phenotypes (Figs. 6A and S6). The enhanced growth phenotype was more profound at later stages of plant development (Fig. 6A). To assess the nematode resistance phenotype, *ern1.1* plants complemented with both *35S::ERN1* and *ERN1pro::ERN1* were used in nematode infection assays. Our data show that lines expressing either transgene were significantly more susceptible to RKN than untransformed *ern1* mutants (Fig. 6, B and C). In particular, the *35S::ERN1*-complemented lines consistently displayed enhanced RKN susceptibility compared to WT, in further support of ERN1 function as a negative regulator of immunity (Fig. 6B).

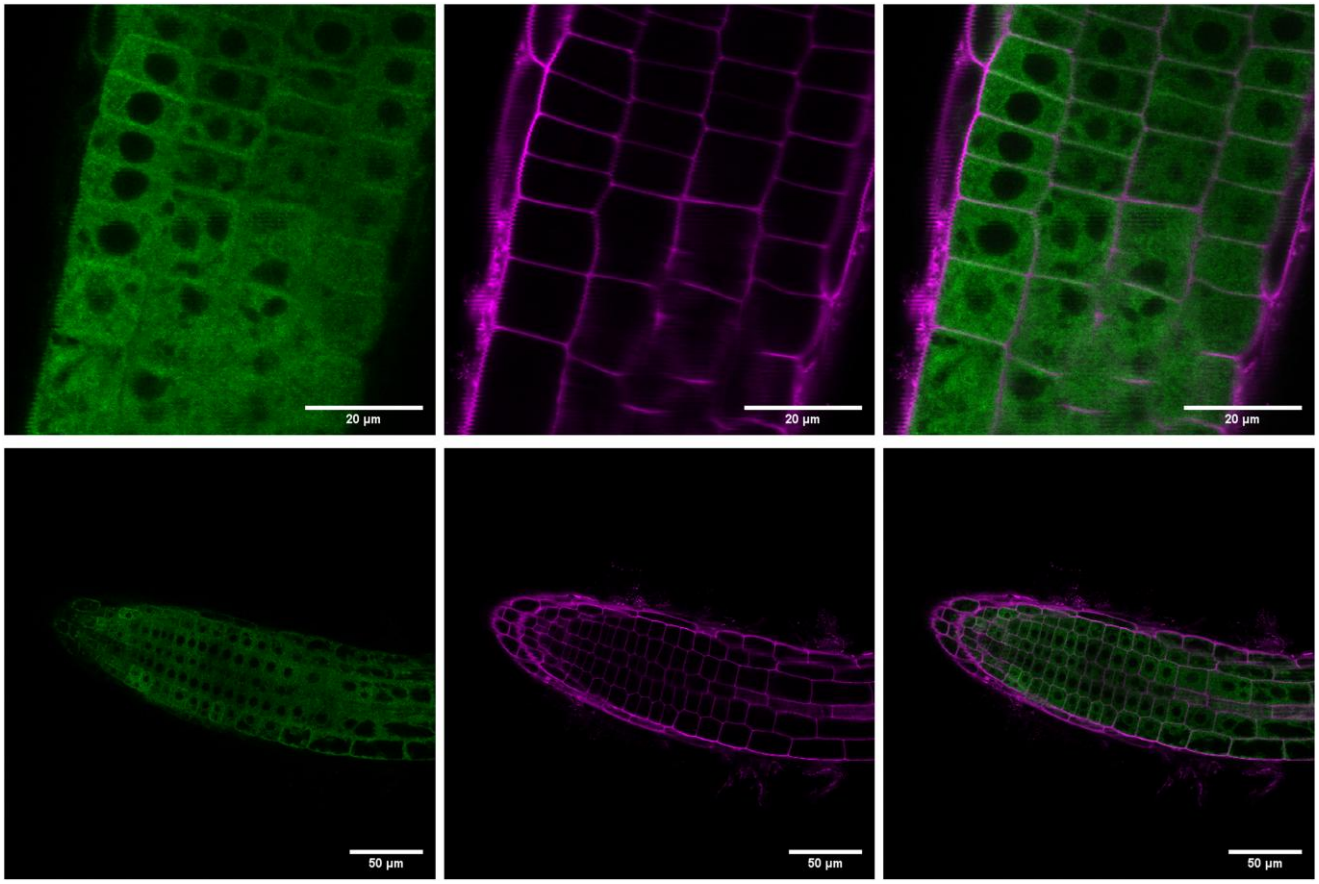


**Figure 5.** ERN1 is localized at the plasma membrane and ER in transgenic *Arabidopsis* roots. ERN1-eGFP fusion protein driven by ERN1 native promoter ($ERN1_{pro}$::ERN1-eGFP) was stably expressed in *ern1.1* plants. Roots were stained with propidium iodide (PI) to visualize cell outlines and imaged with GFP fluorescence by a confocal microscope. GFP alone (left panels), PI alone (center), and GFP + PI merged (right panels).

### ERN1 is a negative regulator of immunity

The enhanced resistance displayed by the *ern1* mutants to RKN combined with enhanced defense marker analyses leads us to propose that ERN1 acts as a negative regulator of immunity. To assess the role of *ERN1* in immunity, we evaluated the response of the *ern1* mutants to the well-known flg22 peptide elicitor by triggering a burst of reactive oxygen species (ROS). We found significantly higher levels of ROS burst in response to flg22 in *ern1.1* and *ern1.2* leaves compared to WT (Fig. 7, A and B). Because our nematode resistance phenotype was based on examination of roots, we also assessed $H_2O_2$ accumulation in roots upon flg22 treatment using DAB staining. Consistent with enhanced ROS burst in *ern1* leaves, darker staining was observed in the *ern1* mutant roots compared to WT (Fig. 7C). Further confirmation of the role of ERN1 as a negative regulator of immunity was obtained by examining expression of the defense marker *MYB51* as triggered by flg22 treatment in leaves. Both *ern1* alleles displayed significantly higher *MYB51* transcript levels compared WT (Fig. 7D). These results extend the function of ERN1 from resistance to nematode infection to a broader role in general pathogen response.

Overexpression of *ERN1* in *ern1.1* (*35S:ERN1* lines #19-2 and #21-8) displayed significantly enhanced susceptibility to RKN compared to the *ern1.1* mutant as well as to WT. To further assess this putative reduced immunity, we examined these complemented lines for flg22-triggered ROS burst and $H_2O_2$ accumulation in leaves and roots, respectively. Both overexpression lines showed lower levels of ROS burst in leaves and less intense brown staining in roots compared to WT and *ern1.1* mutants, which is consistent with overexpression of *ERN1* leading to reduced immune responses (Fig. 8).

## Discussion

Our quest to identify a cell surface–localized nematode immune receptor unexpectedly resulted in identification of a negative regulator of plant immunity. We named this immune regulator ERN1, and it is a G-LecRK belonging to a family with over 38 members in *Arabidopsis* (Teixeira et al. 2018). The G-LecRKs are proteins with an ectodomain that resembles the *Galanthus nivalis* agglutinin (GNA) mannose-binding motif and are also known as B-type LecRKs (Lannoo and Van Damme 2014; Vaid et al. 2012). The best-known members of this group are the S-locus (S-locus glycoprotein/SLG containing) RKs, known for their role in self-incompatibility in flowering plants

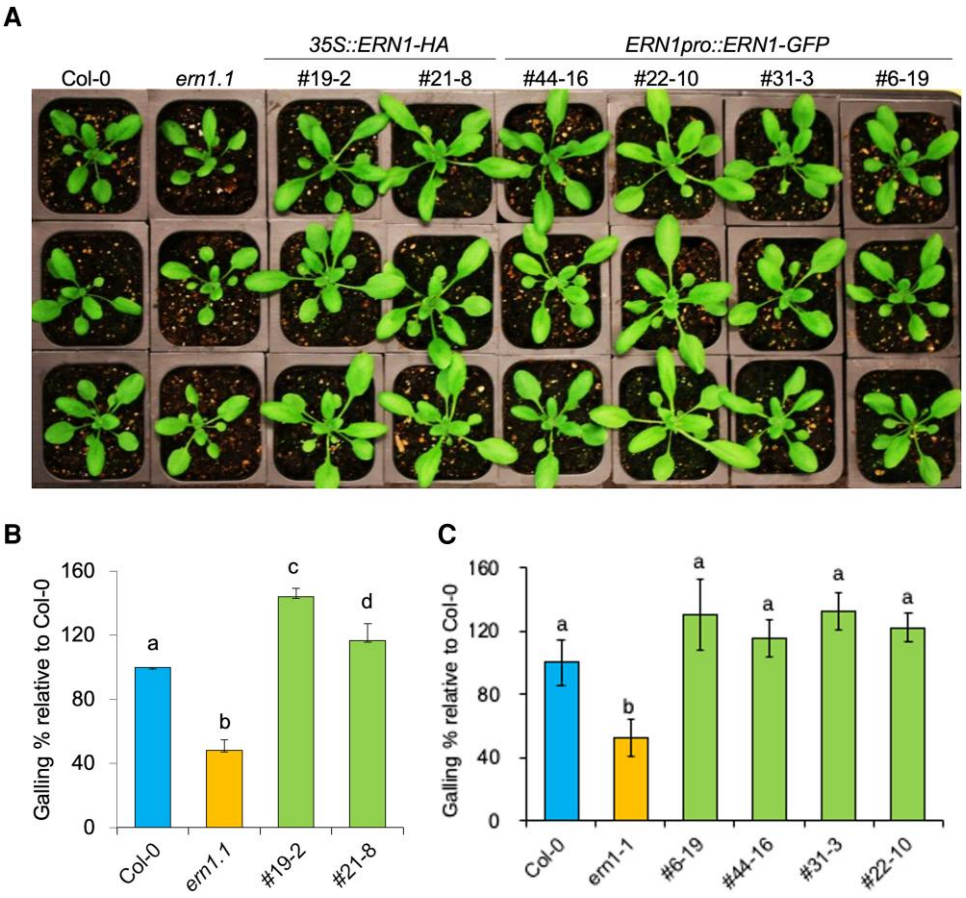


**Figure 6.** The *ern1.1*-complemented plants exhibit enhanced growth and increased susceptibility to RKN. **A)** *35S::ERN1-HA*- and *ERN1pro::ERN1-GFP* *ern1.1*-complemented 5-wk-old *Arabidopsis* lines grown at a 12-h photoperiod at 22 °C. **B)** RKN infection of *ern1.1* plants expressing *35S::ERN1-HA* and **C)** *ERN1pro::ERN1-GFP* relative to Col-0. *Arabidopsis* plants grown on Gamborg agar plates were infected with 100 J2s and evaluated for galling 3 to 4 wk later. **B**, **C)** Bars are means ± SE ($n = 48$ per genotype), and bars with different letters indicate significant differences ($P < 0.05$, ANOVA).

(Sherman-Broyles et al. 2007; Kusaba et al. 2001). While members of the L-LecRKs have been implicated in defense, the G-type LecRKs have not been much explored for their role in immunity and defense (Sun et al. 2020). Our domain analysis identified 6 distinct domains (G-lectin, SLG, EGF, PAM, TM, and kinase) in the ERN1 sequence, which is the largest number of domains identified among the members of this family. Besides lectin-binding and kinase functions, the cysteine-rich domain known as the EGF domain is thought to play a role in disulfide bond formation (Shiu and Bleecker 2001), while the PAN motif specifies protein–protein or protein–carbohydrate interactions (Tordai et al. 1999). This suggests that ERN1 likely partners with multiple types of proteins to carry out its function.

Both *ern1* mutants displayed a reduced number of nematode infections and consequently enhanced resistance to RKN infection. Root growth and branching are important characteristics when it comes to RKN infection. This group of nematodes infects behind the root tips, and the ability of roots to form branches with new root tips affects nematode infection. Our root growth measurements combined with lateral root capacity assays indicated that *ern1* mutants have similar root growth morphology as WT eliminating the possibility that the reduction in the RKN infection rate is due to altered root formation. Wild type root morphology, but an enhanced resistance phenotype suggests a specific role for ERN in immunity.

One of the challenges studying nematode immune responses, particularly the transiently induced responses, is synchronization of nematode penetration and infection without causing physical damage to the roots. To circumvent these challenges, nematode elicitors of immune responses have been identified, including NemaWater and infective-juvenile extracts (Teixeira et al. 2016; Mendy et al. 2017). Using RKN NemaWater, infective juvenile extracts, and egg extracts, we showed that several immune responses, including ROS bursts and defense gene expression, are induced at higher levels in the *ern1* mutants. Preparation of NemaWater and infective juvenile extracts require tedious and labor-intensive acquisition of clean infective juveniles. In contrast, RKN eggs can be easily extracted from infected plants used to maintain the nematode cultures and are far more abundant than infective-stage juveniles. Therefore, our work also identified a simpler and more abundant source of RKN immune elicitors. It is likely that

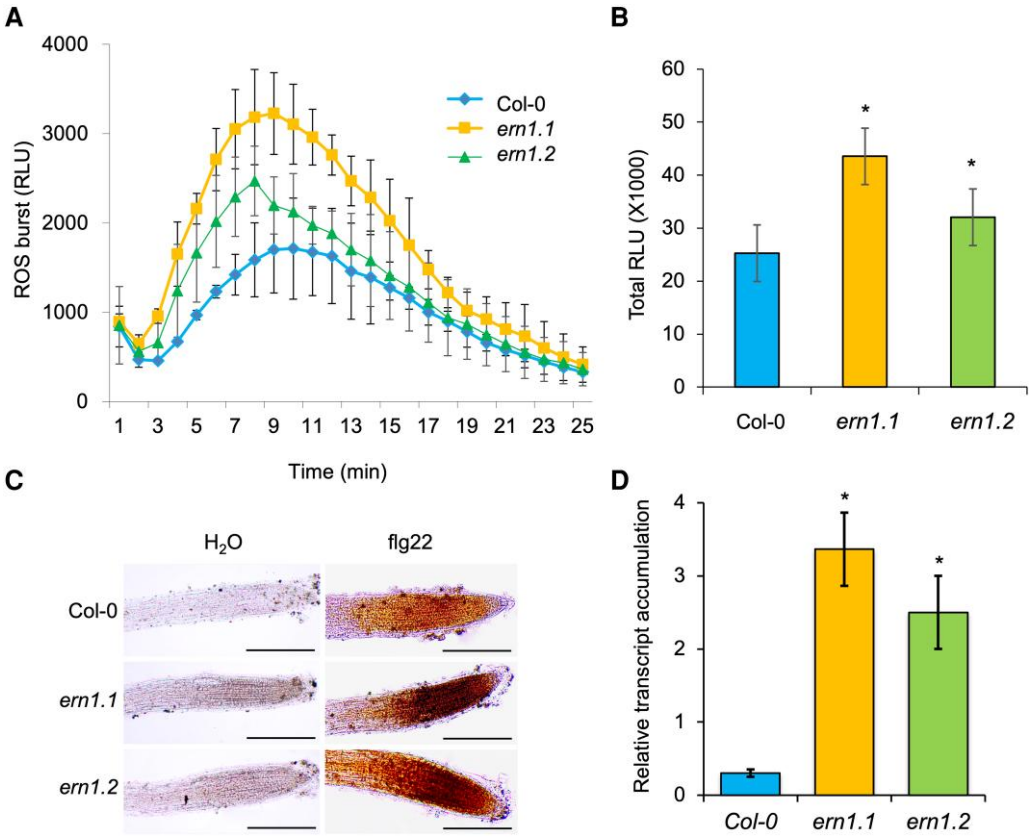


**Figure 7.** Flg22 triggered enhanced immune responses in the *ern* mutants. Flg22-induced ROS burst in leaves over time **A)** or cumulative **B)** and $H_2O_2$ accumulation in roots **C)** of *ern1.1* and *ern1.2* mutants. **A**, **B)** Leaf disks from 3-wk-old plants were treated with 1 $\mu$M flg22, and the production of ROS was measured by a chemiluminescence and expressed as relative light units (RLU). Values are means ± SD ($n = 4$ per genotype). DAB staining **C)** and *MYB51* expression by RT-qPCR **D)** in roots of 5-d-old *Arabidopsis* seedlings treated with 1 $\mu$M flg22 or water for 30 min. **D)** *MYB51* expression was normalized to *UBQ22*, and bars are means of 3 biological replicates with 2 technical replicates ± SE. **B**, **D)** Asterisk denotes a significant difference ($P < 0.05$, ANOVA). Scale bar = 10 mm.

eggs and infective-stage juveniles have overlapping but also stage-specific immune elicitors. For example, nematode egg shells are highly enriched in chitin, while only trace amounts of chitin are present in the nematode juvenile esophageal lumen (Holbein et al. 2016).

It is well documented that immune responses are tightly regulated to protect against runaway immunity and its lethal consequence. Different mechanisms for immune regulation have been identified, including negative regulators of immunity (Couto et al. 2016; Lin et al. 2015; Lu et al. 2018; Niu et al. 2016). Our data indicates that ERN1 is a negative regulator of immunity. ERN1 localization at the plasma membrane and its effect on immune responses, induced by both nematode and microbial elicitors, indicate a wide role for this regulator in immunity against pathogens. How ERN1 suppresses immune responses remains unknown and requires further investigation. While *ern1* mutants exhibit enhanced immunity, we did not observe any necrotic or cell death–associated lesions, on above-ground or below-ground tissues, as seen on other enhanced immunity phenotypes also known as lesion mimic mutants (Dietrich et al. 1994; Greenberg et al. 1994; Weymann et al. 1995). Such lesions were also absent from the RKN-infected plants indicating that while *ern1* immune responses are enhanced, it is likely a balanced immune response. This observation also suggests that more powerful negative immune regulators are still working in the *ern1* mutant background. Furthermore, these results reveal that manipulation of an appropriate negative immune regulator may yield plants with desirable enhanced immunity without adverse effects.

The enhanced immune response in *ern1* mutants and the absence of plant size and morphological phenotypes seem to contradict the expected balance between immune responses and plant growth. Elevated immune responses require more energy and are associated with a fitness cost. However, our observations do not detect a fitness cost in *ern1* mutants. Such fitness costs could exist but were not observed under our conditions or may be displayed in features we did not experimentally evaluate. Interestingly, while the *ern1* mutants were similar to WT, complementation of *ern1* mutants with an overexpression construct or with the native *ERN1* promoter yielded an altered plant growth phenotype with both complemented lines having larger rosettes than the WT (Fig. 6A). Despite this morphological similarity, complemented lines with the native promoter and WT had comparable levels of RKN susceptibility

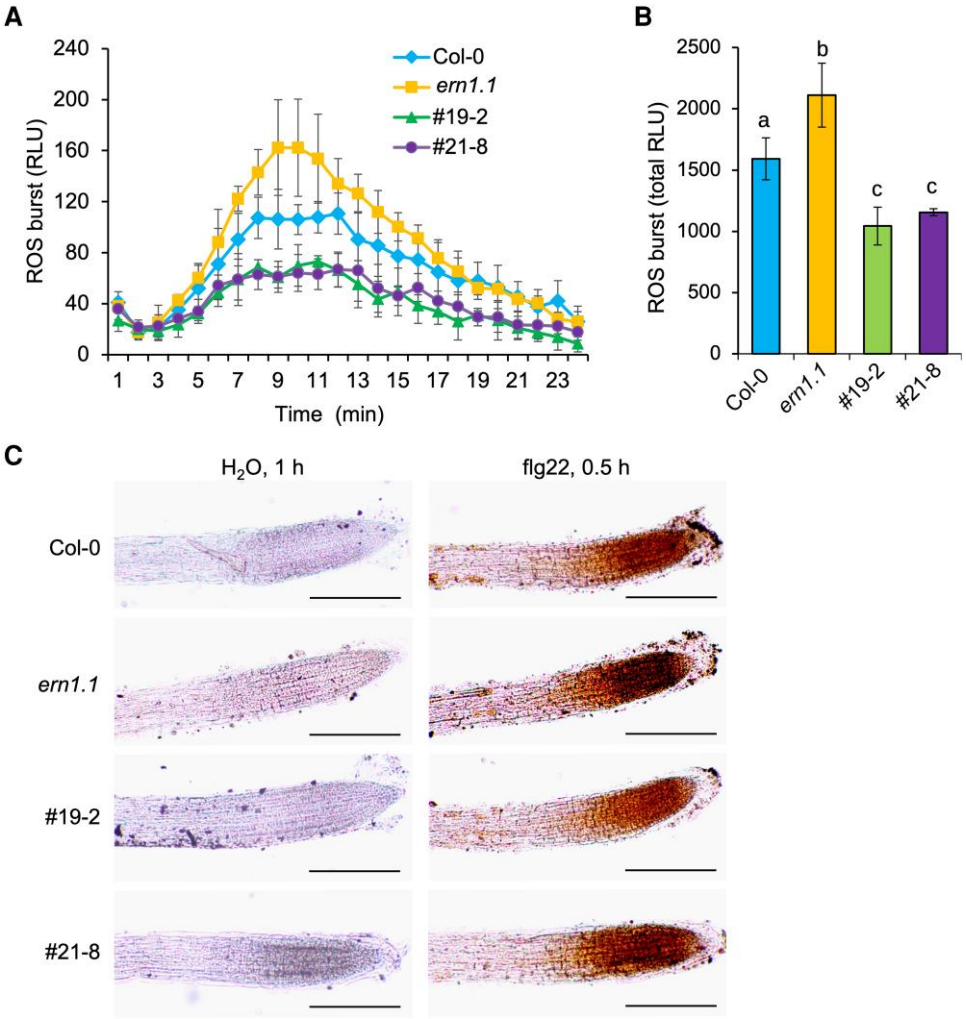


**Figure 8.** The *ern1*-complemented lines have reduced levels of $H_2O_2$ accumulation in leaves and roots after flg22 elicitor treatment. **A)** Time course and **B)** total flg22-induced ROS burst in leaves of 3-wk-old *Arabidopsis ern1.1* mutant and 35S::ERN1-HA *ern1.1*-complemented lines (#19-2 and #21-8). Values are means ± SE ($n = 4$ per genotype). Different letters indicate significant differences ($P < 0.05$, ANOVA). RLU, relative light units. **C)** DAB-stained 5-d-old *Arabidopsis* roots treated with 1 $\mu$M flg22 for 30 min. Scale bar = 10 mm.

(Fig. 6C), while the overexpression-complemented lines exhibited higher levels of RKN susceptibility (Fig. 6B). This enhanced susceptibility is associated with reduced levels of immune response (Fig. 7) and further confirms the role of ERN1 as a negative regulator of immunity.

Several members of the G-LecRK family have been implicated in abiotic stress responses, and evidence is starting to emerge as to their roles in biotic stresses (Sun et al. 2020). Both gene expression and mutant analyses indicate positive roles of G-LecRKs in immunity to microbial pathogens as well as to insect pests (Chen et al. 2006; Cheng et al. 2013; Gilardoni et al. 2011; Li et al. 2014; Ranf et al. 2015; Gouhier-Darimont et al. 2019; Sun et al. 2020). In contrast, here, we report that the ERN1 G-LecRK is a negative regulator of immunity. Compared to *Arabidopsis*, the G-LecRK family has expanded in crops, such as tomato, and more than 1 orthologous sequence to *AtERN1* is identified in crops (Teixeira et al. 2018). It is important to determine whether the phenotype displayed in the *Arabidopsis ern1* mutant can be replicated in tomato. Editing these orthologous genes with CRISPR gene editing technology should be prioritized to potentially develop tomato plants with enhanced resistance to RKN. Resistance to RKN in tomato exists and is conferred by the *Mi-1* resistance gene. However, the appearance of *Mi-1* resistance-breaking RKN isolates is a commonplace worldwide, due to reliance on this single source of RKN resistance, and necessitates the identification of alternate modes of resistance. Extending the insights gained here to *ERN1* homologs in tomato and other crops may provide additional sources of resistance to this harmful group of nematodes.

## Materials and methods

### Nematode culture and inoculum preparation

*M. incognita* isolate P77R3 was maintained on tomato (*S. lycopersicum*) cultivar 'Daniela' (Hazera). Plants were grown in

UC mix3 and sand (1:9, *v*/*v*), fertilized with MiracleGro (Scotts Miracle-Gro Co.), and kept in a glasshouse at 24 to 30 °C.

Nematode eggs were extracted from roots using 10% (*v*/*v*) bleach and sieving (Hussey and Barker 1973). Eggs and plant debris collected on a 500-mesh sieve were fractionated twice on 35% (*w*/*v*) sucrose and rinsed several times with sterile water. The eggs were sterilized by vortexing in 5% (*v*/*v*) bleach for 5 min, rinsed several times with sterile water, resterilized by shaking in 50 mg/L nystatin and 30 mg/L gentamicin for 5 min, and rinsed several times with sterile water. Sterilized eggs were either flash frozen in liquid $N_2$ or hatched under sterile conditions using a modified Baermann funnel (Martinez de Ilarduya et al. 2001). Two days later, infective-stage juveniles (J2s) were collected, counted, and suspended in a 0.5% (*w*/*v*) carboxymethylcellulose solution.

## Plant material, growth conditions, nematode inoculation, and infection evaluation

*Arabidopsis* (*A. thaliana*) Col-0 and mutants were obtained from the Arabidopsis Biological Resource Center (ABRC). Seeds were plated on ½ strength Murashige and Skoog (½ MS) basal salt media (Phytotechnology Laboratories) supplemented with 1% (*w*/*v*) sucrose and 0.8% (*w*/*v*) agar (Sigma-Aldrich) (pH 5.7) and maintained vertically in plant growth rooms with a 12-h photoperiod at 22 °C. For RKN infection assays, seeds were plated on Gamborg media (Sigma-Aldrich) supplemented with 3% (*w*/*v*) sucrose and 0.6% (*w*/*v*) daishin agar (Bioworld) (pH 6.0) and maintained at a 45° angle as described above. For each experiment, 2-wk-old seedlings, with 6 seedlings per square plate, and 8-d-old seedlings, with 20 seedlings per square plate, were used for galling assay and RKN-induced gene expression analysis, respectively. Seedlings were inoculated with 100 J2s per seedling and plates were kept horizontally for 24 h in the dark. Later, plates were maintained as described above. For pathogenicity assays, 5 plates per genotype were used and galls on roots were evaluated 4 wk after inoculation. To estimate the number of galls per root area, roots were photographed, the surface area determined using ImageJ (https://imagej.nih.gov/ij/), and the number of galls per cm root was calculated. The number of galls on Col-0 roots was defined as 100%, and number of galls on mutant roots was reported relative to Col-0. RKN infection of *Arabidopsis* mutant lines was repeated once while infection of *ern1.1* (SALK_128729) and *ern1.2* (SAIL_63_G02) lines was repeated 5 times. RKN-induced gene expression experiments were performed 3 times.

## Plant growth for phenotyping

Seeds for the different *Arabidopsis* genotypes were bulked at the same time for these experiments. For rosette leaf measurements, seeds were germinated on agar plates and 3-d-old seedlings were transplanted into UC soil mix 3 supplemented with Osmocote. Plants were maintained at a 12-h light photoperiod at 22 °C, unless otherwise stated, for 3 wk. Rosettes were photographed and measured using ImageJ. Four plants per genotype were used and the experiment was performed once.

Col-0 and *ern1* seedlings were grown side by side on agar plates for root length and lateral root capacity assays. For the root growth rate, the position of the root tip was marked starting 4 d after placement in the growth room and marked 24 h later until 8 d. The plates were scanned, and then lengths were measured with ImageJ from the hypocotyl rootward for Days 0 to 4 and between the consecutive marks for each time point thereafter. Lateral root capacity assays were performed as described in Moreno-Risueno et al. (2010). Briefly, plants were grown for 8 d, then the root tips were excised, and plants were returned to grow for 3 more days. At 11 d, all emerged lateral roots were counted per root per genotype. This process prevents the formation of new lateral roots from the primary root and promotes the outgrowth of existing primordia allowing one to assess how many lateral roots had been specified at 8 d. For both root growth rate and root capacity experiments, 7 seedlings per genotype were grown on a single plate and 4 plates per experiment were used. Root growth rate and lateral root capacity experiments were performed twice.

## T-DNA insertion localization

All *Arabidopsis* mutant SALK, SAIL, and GK lines were insertion mutants in the Col-0 background. They were genotyped using both antibiotic selection and PCR amplification with gene-specific primers and T-DNA border primers (Supplemental Table S2). To determine the T-DNA insertion site in SALK_128729 and SAIL_63_G02 lines, homozygous plants were evaluated by PCR along with left or right border primers and a gene-specific primer in various combinations (Supplemental Table S2). Amplicons were sequenced and obtained sequences were aligned to the At1g61550 sequence and to the T-DNA pROK2 and pCSA110 to precisely determine the T-DNA insertion sites in SALK_128729 (*ern1.1*) and SAIL_63_G02 (*ern1.2*) lines, respectively.

## Nematode juvenile/egg extracts and NemaWater

Surface-sterilized RKN eggs and J2s hatched under sterile conditions from these eggs were prepared as described for the inoculum preparation and frozen at −80. Frozen J2s or eggs were ground in a mortar and pestle, and the powder was suspended in PBS (pH 7.0) used immediately or frozen at −20 °C overnight. The suspension was centrifuged at 9,500 × *g* for 15 min at 4 °C, and the supernatant was used to treat *Arabidopsis* seedlings or roots.

For NemaWater preparation, J2s were hatched in small volume of water for 48 h as described earlier (Mendy et al. 2017). The hatched J2s were spun down by centrifugation at 2,000 rpm for 5 min and the supernatant was used as NemaWater.

## Gene expression analysis

RNA was isolated from *Arabidopsis* roots and leaves using the GeneJET Plant RNA purification kit (Life Technologies) or TRIzol (Life Technologies), respectively. Three micrograms

of RNA was DNase treated and used for cDNA synthesis using Superscript III reverse transcriptase enzyme (Invitrogen) and oligo-dT primers according to the manufacturer's recommendations.

RT-qPCR analysis was performed using gene-specific primers (Supplemental Table S2) and iQ SYBR Green Supermix (Bio-Rad) in iCycler5 IQ (Bio-Rad) l using the following program: 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a final cycle of 72 °C for 3 min. Three biological replicates, with 2 technical replicates each, were performed, and the generated threshold cycle ($\Delta\Delta C_T$) was used to calculate transcript abundance relative to *UBQ22* or *At*18S (Supplemental Table S2). All RT-qPCR experiments were performed twice.

## Elicitor experiments and DAB staining

Five-day-old seedlings grown on mesh-supported ½ MS plates were placed into 6-well plates with liquid ½ MS media for 24 h. Then, they were exposed to egg extract or water, for 1 h or to 1 $\mu$M flg22 for 30 min. The solutions were replaced with freshly prepared 1 mg/mL of DAB (3,3′-diaminobenzidine; pH of 5.5) in 200 mM $Na_2HPO_4$ buffer for 45 min (Thordal-Christensen et al. 1997). Seedlings were washed 3 times with 50% (*v*/*v*) ethanol, mounted in 50% (*v*/*v*) glycerol, and observed using a LEITZ DMRB Leica Microscope, objective 10×/0.65 mm N Plan. Ten seedlings were used per genotype/treatment and experiments were performed 4 times.

## Kinase activity

Roots of 12-d-old seedlings grown on ½ MS plates were floated in water overnight. Then, the roots were treated with 5 eggs/$\mu$L egg extract for 0.5, 1, and 2 h or water control for 1 h. Protein extracts were fractionated onto 10% SDS–PAGE gel and activated MPKs were detected using antiphospho-p42/44 MPK antibody. This experiment was performed twice.

## Vector constructs, complementation, and microscopy

The *ERN1* overexpression complementation construct (35S:ERN1-3xHA) was generated in the Gateway vector pGWB14 containing a 35S promoter. The coding sequence of *ERN1* without the stop codon was amplified and cloned into pENTR221 and recombined into pGWB14 using a 1-tube recombination protocol. Primers used for amplification and assembly of these constructs are listed in Supplemental Table S3. The *ERN1* native promoter–driven complementation construct (ERN1pro::ERN1-mEGFP) was generated in a single reaction, using the NEBuilder HiFi DNA Assembly Master Mix (NEB, E2621), to assemble 3 fragments into a modified pCAMBIA1300-MCS vector that lacks a promoter (Guo et al. 2022) (Supplemental Table S3). The promoter and coding sequence of *ERN1* without the stop codon were amplified from genomic DNA of WT *Arabidopsis* plants. Similarly, an overexpression *ERN1-mScarlet* construct was developed in *pCAMBIA1300-35S::ERN1-mScarlet* (Supplemental Table S3). All constructs were sequence verified before use.

Expression constructs were transformed into *ern1* mutant plants by the floral dip method (Clough and Bent 1998), and transformants were identified using standard methods. For each reporter or complementation transgene, T2 lines that exhibited a 3:1 ratio of resistant:sensitive seedlings, indicating that the transgene is inherited as a single locus, were selected for propagation and homozygous T3 lines were used in further analyses. For each reporter, at least 3 independent lines were examined.

For subcellular localization in *N. benthamiana* leaves, *ERN1* was transiently expressed in 3-wk-old plants by hand infiltrating *Agrobacterium tumefaciens* containing either 1 or 2 of the following constructs: *pCAMBIA1300-35S::mScarlet*, *pCAMBIA1300-35S::ERN1-mScarlet*, *35S::GFP-LTI6b* (Martiniere et al. 2012), or 35S::*ER-GFP* (ER-gk) (Nelson et al. 2007), using a needleless syringe at a final $OD_{600}$ = 0.5. Fluorescence in leaf tissues was detected after 48 h by a Leica SP5 confocal microscope. GFP and mScarlet were excited at 488 and 543 nm, respectively, and images were collected at 498 to 530 and 555 to 635 nm. For the plasmolysis treatment, leaves were treated with 200 mM NaCl for 5 to 10 min before visualization. This experiment was performed at least twice.

For subcellular localization in *Arabidopsis* roots, stable transgenic T3 homozygous lines expressing *ERN1pro::ERN1-GFP* were grown on ½ MS plates. GFP fluorescence in 5-d-old roots was detected by a Leica SP8 confocal microscope using the settings described above. This experiment was performed at least twice with at least 4 plants, and at least 3 independent transgenic lines were analyzed.

## ROS burst assay

ROS burst in leaves was evaluated using 3-wk-old *Arabidopsis* plants. Leaves were excised into 2-mm pieces using a blade and floated overnight on sterile water in a petri dish. Similar-size leaves were transferred to a white 96-well plate (Corning Costar) with 170 $\mu$L sterile water supplemented with 100 nM flg22, 20 $\mu$M luminol (Sigma), and 5 $\mu$g/mL horseradish peroxidase (Sigma). Luminescence was measured with a Tecan Infinite F200 plate reader. Four replicates were used per genotype treatment and experiments were performed 2 to 4 times.

## Statistical analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Comparisons were performed using Student *t* test or 1-way ANOVA.

## Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AT1G61550.

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## Author contributions

I.K., J.M.V.N., and L.W. planned and designed the experiments; D.Z., J.H., D.G.-V., M.T., J.G., and J.M.V.N. performed the experiments; I.K. and J.M.V.N. supervised the experiments and analyzed the data; I.K. wrote the manuscript with contributions from all authors. I.K. agrees to serve as the author responsible for contact and ensures communication.

## Supplemental data

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

**Supplemental Figure S1**. *ERN1* genomic structure with locations of T-DNA insertions and gene expression in the *ern1* mutants.

**Supplemental Figure S2**. Shoot growth is not compromised in the *ern1* mutants.

**Supplemental Figure S3**. Above-ground growth is not compromised in the *ern1* mutants.

**Supplemental Figure S4**. Root growth is not compromised in the *ern1* mutants.

**Supplemental Figure S5**. Nematode egg extract induces defense gene expression in *Arabidopsis* roots.

**Supplemental Figure S6**. Expression of *ERN1* and shoot phenotype of complemented plants.

**Supplemental Table S1**. List of *Arabidopsis* RLK mutants screened with root-knot nematodes.

**Supplemental Table S2**. List of primers used in PCR and RT-qPCR.

**Supplemental Table S3**. List of primers used in cloning.

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*Conflict of interest statement.* None declared.

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