Molecules and Cells

The *Ralstonia pseudosolanacearum* Type III Effector RipL Delays Flowering and Promotes Susceptibility to *Pseudomonas syringae* in *Arabidopsis thaliana*

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The plant defense responses to microbial infection are tightly regulated and integrated with the developmental program for optimal resources allocation. Notably, the defense-associated hormone salicylic acid (SA) acts as a promoter of flowering while several plant pathogens actively target the flowering signaling pathway to promote their virulence or dissemination. *Ralstonia pseudosolanacearum* inject tens of effectors in the host cells that collectively promote bacterial proliferation in plant tissues. Here, we characterized the function of the broadly conserved *R. pseudosolanacearum* effector RipL, through heterologous expression in *Arabidopsis thaliana*. RipL-expressing transgenic lines presented a delayed flowering, which correlated with a low expression of flowering regulator genes. Delayed flowering was also observed in *Nicotiana benthamiana* plants transiently expressing RipL. In parallel, RipL promoted plant susceptibility to virulent strains of *Pseudomonas syringae* in the effector-expressing lines or when delivered by the type III secretion system. Unexpectedly, SA accumulation and SA-dependent immune signaling were not significantly affected by RipL expression. Rather, the RNA-seq analysis of infected RipL-expressing lines revealed that the overall amplitude of the transcriptional response was dampened, suggesting that RipL could promote plant susceptibility in an SA-independent manner. Further elucidation of the molecular mechanisms underpinning RipL effect on flowering and immunity may reveal novel effector functions in host cells.

Keywords: basal defense, flowering genes, susceptibility, transcriptional reprogramming, virulence

INTRODUCTION

Plants possess elaborate systems to detect pathogenic microbes in their environment and adjust their behavior accordingly (Cook et al., 2015). Two large families of surface-localized and intracellular immune receptors sense microbe-derived molecules and activate specific signaling pathways leading to robust transcriptional and hormonal changes, and to the production of diverse arrays of antimicrobial compounds, effectively restricting the pathogen invasion (Cam-

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pos et al., 2018; Ngou et al., 2022; Pieterse et al., 2012). These responses are costly and therefore tightly regulated and integrated with the developmental program (Hulsman et al., 2016; Huot et al., 2014). Different lines of evidence suggest associations between disease resistance and the floral transition in maize, rice or in the model species *Arabidopsis thaliana* (hereafter, Arabidopsis) (Kazan and Lyons, 2016). Several flowering time regulators, such as FLOWERING LOCUS D and LEAFY (LFY) have been shown to affect plant defense responses (Singh et al., 2013; Winter et al., 2011), while one of the major defense-associated hormone salicylic acid (SA) can function as an inducer of flowering (Banday and Nandi, 2015; Villajuana-Bonequi et al., 2014). The regulation of the floral transition or flower development is also directly targeted by pathogens as a virulence strategy. For instance, the phytoplasma effector SAP54 promotes the degradation of key transcription factors from the MADS-domain family, resulting in the conversion of flowers into leaf-like structures, more attractive for the phytoplasma insect vector (MacLean et al., 2011; 2014).

Advances in our understanding of the molecular mechanisms employed by microbial pathogens to colonize plant tissues contribute to the development of sustainable strategies to minimize annual losses due to crop diseases (Koseoglou et al., 2022). Bacterial pathogens rely on virulence factors termed effectors that are directly injected in the host cell through the type III secretion system (Büttner and He, 2009; Mansfield et al., 2012). In the plant cell, these effectors play diverse roles to promote bacterial proliferation, notably suppressing defense responses or manipulating the host metabolism and physiology (Macho, 2016; Perez-Quintero and Szurek, 2019; Xin et al., 2018). Effector repertoires consist of tens of different proteins, some with characterized catalytic activity such as proteases, acetyltransferases, or ubiquitin-ligases, while a substantial number presents novel/unknown features (Peeters et al., 2013). Although highly polymorphic, some effector genes are broadly conserved across strains, species and even genera of bacterial pathogens, highlighting an essential role for pathogenicity (Dillon et al., 2019; Peeters et al., 2013; Schwartz et al., 2015; Wroblewski et al., 2009). The characterization of such conserved effectors offers the possibility to probe the host components that are targeted during the disease establishment hence delineating susceptibility factors in plants.

Ralstonia pseudosolanacearum (*Rps*) is the soil-born causal agent of bacterial wilt disease in major solanaceous crops such as potato, tomato, pepper and eggplants (Genin and Denny, 2012). Throughout the infection process, *Rps* mobilizes around 70 type III-secreted effectors, termed Ralstonia-injected proteins (Rip), whose functions are mostly unknown (Landry et al., 2020; Peeters et al., 2013). RipL is a conserved *Rps* effector, present in most strains and in the closely related species *Ralstonia solanacearum* and *Ralstonia syzygii* (Peeters et al., 2013; Prior et al., 2016; Prokchorchik et al., 2020). RipL protein harbors 4 predicted pentatricopeptide repeats (PPR) in the N-terminus and a predicted nuclear localization signal at the C-terminus, surrounding a large region (>1,000 AA) with no known homology that is structured by alpha helices. The PPR motifs, involved in specific RNA-binding in eukaryotic proteins, may have been acquired by *Rps* through horizontal gene transfer (Lurin et al., 2004; Salanoubat et al., 2002). However, a possible RNA-editing function for RipL is not directly supported by the small number of motifs and the divergence of the penultimate residue that determines the base-binding specificity (Manna, 2015). Moreover, using transient expression in *Nicotiana benthamiana*, we previously observed that RipL-YFP co-localized in lipid bodies with these organelle marker, LIPID DROP-ASSOCIATED PROTEIN 3-INTERACTING PROTEIN, rather than in the chloroplasts or mitochondria as generally observed for plant PPR-containing proteins (Jeon et al., 2020; Lurin et al., 2004; Pyc et al., 2017).

To gain insights into the possible contribution of this effector to virulence and the underlying molecular mechanism, we characterized developmental, physiological and transcriptional responses to bacterial pathogens in Arabidopsis transgenic lines expressing RipL. Our work provides evidence of dampened transcriptional reprogramming and enhanced susceptibility to infection in the presence of RipL. Moreover, we show that RipL expression caused a delayed flowering, which correlated with a lower expression of the main flowering regulator genes. Unexpectedly, both effects appeared to be independent from SA accumulation, suggesting a novel mechanism of effector function in promoting pathogen virulence.

MATERIALS AND METHODS

Molecular constructs

RipL coding sequence was split into three modules of ~1 kb in length. Each fragment was amplified from the *Rps* reference strain GMI1000 genomic DNA with BsaI site-flanking primers (Supplementary Table S1). The resulting blunt PCR products were ligated in the Golden Gate compatible entry vector pICH41021 linearized with SmaI (Engler et al., 2008; Salanoubat et al., 2002). Using the Golden Gate "one-pot" BsaI restriction/T4 DNA ligase ligation (Engler et al., 2008), the RipL modules were then assembled in frame with a C-terminal 3xFLAG epitope under the control of the cauliflower mosaic virus 35S promoter in the binary vector pICH86988 or under the control of the dexamethasone-inducible promoter in the modified binary vector pTA7002-GG for Arabidopsis transformation; in a broad-host range vector (pBBR1) in fusion with AvrRps4 promoter (128 bp) and AvrRps4 N-terminus (1-136 AA) for delivery from *Pseudomonas syringae* (Kovach et al., 1995; Sohn et al., 2007); and in a tobacco rattle virus (TRV2)-based binary vector under the control of the pea early brown virus CP promoter for virus-based expression in *N. benthamiana* (Macfarlane and Popovich, 2000). GFP coding sequence was assembled with N-terminal 3xFLAG fusion in the binary vectors and used as control for TRV2-based expression in *N. benthamiana*.

Bacterial strains

The binary vector constructs were mobilized into the *Agrobacterium tumefaciens* AGL1 strain by electroporation. The AGL1 strains were grown at 28°C on LB supplemented with 100 μg ml^{-1} carbenicillin and 50 μg ml^{-1} kanamycin. Cells were collected by centrifugation, resuspended in infiltration

medium (10 mM $MgCl_2$, 100 mM MES-KOH pH 5.6) and the optical density at 600 nm (OD_{600}) was adjusted to 0.5 for leaf infiltration. The broad-host range vector constructs were mobilized into the *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 strain by triparental mating. The *Pst* DC3000 strains were grown at 28°C on King's B medium with appropriate antibiotics (DC3000: 50 μg ml^{-1} rifampicin; DC3000ΔAvrPtoΔAvrPtoB: 50 μg ml^{-1} rifampicin, 100 μg ml^{-1} spectinomycin and 50 μg ml^{-1} kanamycin; DC3000(pBBR1) and DC3000(pBBR1:RipL): 50 μg ml^{-1} rifampicin, 20 μg ml^{-1} gentamycin). Cells were collected by centrifugation, resuspended in 10 mM $MgCl_2$ and the OD_{600} was adjusted to 0.01 or 0.0001 for leaf infiltration.

Plant materials

All the Arabidopsis lines used in this study are in the Col-0 background. RipL-expressing lines were obtained by floral dip transformation. Homozygous lines were selected on Murashige and Skoog (MS) basal medium (Sigma, USA) supplemented with 50 μg ml^{-1} kanamycin after selfing of independent primary transformants. The *soc1-2* line was kindly provided by Dr. Ilha Lee (Seoul National University, Republic of Korea) (Lee et al., 2000). The *fls2 efr cerk1* triple mutant line is described by Gimenez-Ibanez et al. (2009a). All the plants were grown in controlled environment cabinet or chamber. Arabidopsis plants were grown on soil or MS medium at 23°C under long-day (16-h light/8-h dark) or short-day (10-h light/14-h dark) conditions. *N. benthamiana* plants were grown on soil at 23°C in long-day conditions. For the dexamethasone treatment in RipL-2 and RipL-3 lines, 3-day-old seedlings grown on MS medium at 23°C under long-day were transferred to liquid MS medium. After 7 days, the seedlings were treated with 10 μM dexamethasone for 24 h and flash-frozen for RNA and protein extraction.

Protein detection

Total proteins were extracted using GTEN buffer (10% glycerol, 50 mM Tris-HCl pH 7.5, 2 mM EDTA pH 8, 150 mM NaCl) supplemented with 5 mM DTT, 1% IGEPAL (Sigma), PVPP and antiprotease cocktail (cOmplete mini; Sigma). Proteins were separated by SDS-PAGE, transferred onto PVDF membrane (Merck Millipore, USA) and probed with monoclonal anti-FLAG M2-peroxidase antibody (A8592; Sigma).

Morphological measurements

Flowering time was estimated by scoring the maximum rosette diameter, days to bolting and total leaf number of 10 individuals of each Arabidopsis line grown in long-day or short-day conditions in each of three independent repeats. The rosette diameter was measured and days to bolting were measured when the floral bud appeared. Total leaf number was measured when the height of the flowering shoot reached 10 cm. For *N. benthamiana* expressing 3xFLAG-GFP or RipL, the total leaf number at the first appearance of floral buds was scored in 12 individuals in each of four independent repeats. Days to bolting were scored when the first flower appeared in 10 individuals in each of two independent repeats.

Gene expression analysis

RNA was isolated from Arabidopsis seedlings grown on MS in long-day conditions collected 1 h after treatment with water or elf18 peptide (Peptron, Korea) or leaf discs from 5-week-old plants collected 24 h after infiltration with 10 mM $MgCl_2$ or *Pst* DC3000 suspension adjusted at OD_{600} 0.01. Total RNA was extracted using TRI reagent (Invitrogen, USA) according to the manufacturer's instructions. Residual genomic DNA was removed by treatment with DNAseI (Sigma). One microgram of RNA was used as template for cDNA synthesis using Maxima First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA). The qRT-PCR analysis was conducted with GoTaq qPCR Master Mix (Promega, USA) using a CFX Connect real-time system (Bio-Rad, USA). The reference gene *AtEF1α* (AT1G18070) was used for normalization of the samples. Specific primers are listed in Supplementary Table S1.

RNA sequencing

Fully expanded leaves of 5-week-old wild-type (WT), RipL-1 and RipL-2 Arabidopsis plants were infiltrated with 10 mM $MgCl_2$ or *Pst* DC3000 suspension at OD_{600} 0.01. Seven infiltrated leaves from different plants were pooled per genotype/condition 24 h after infiltration and immediately frozen in liquid nitrogen. Three independent biological repeats were collected before total RNA from the 18 samples (3 genotypes, 2 conditions, 3 repeats) was isolated using TRI reagent and treated with DNAse I. RNA purity was assessed by chromatography (Agilent, USA). The mRNA libraries were generated from 1 μg total RNA using TruSeq Stranded mRNA LT Sample Prep Kit and sequenced on an Illumina platform by Macrogen (Korea). The sequencing reads have been deposited in the National Center for Biotechnology Information Sequence Read Archive, https://www.ncbi.nlm.nih.gov/sra (BioProject accession No. PRJNA887206). Low quality reads were trimmed using Trimmomatic. The trimmed reads were mapped to Arabidopsis reference genome TAIR10 using HISAT2. Expression profile values, read count and FPM were obtained from transcript assembly using StringTie. Differentially expressed genes (DEGs) were called using DESeq2 with log2 fold-change threshold of 2 and nbinomWald test raw *P* value < 0.05. The Gene Ontology over-representation analysis was performed using the PANTHER classification system and visualized with R.

Bacterial enumeration in planta

Fully expanded leaves of 5-week-old Arabidopsis plants were infiltrated with bacterial suspensions adjusted at OD_{600} 0.0001. Leaf discs were collected in the infiltrated area at 2 days post-inoculation for *Pst* DC3000 and *Pst* DC3000 ΔAvrPtoΔAvrPtoB and at 2 and 5 days post-inoculation for *Pst* DC3000(pBBR1) and *Pst* DC3000(pBBR1:RipL). The samples were ground in 10 mM $MgCl_2$, and serial dilutions plated on King's B medium supplemented with the appropriate antibiotics. Colony-forming units were enumerated after 48 h incubation at 28°C.

Quantification of SA content

Fully expanded leaves of 5-week-old Arabidopsis plants were infiltrated with 10 mM $MgCl_2$ or *Pst* DC3000 suspension

adjusted at OD_{600} 0.01, collected 24 h after infiltration and ground in liquid nitrogen. SA content was determined using the modified method of Kim et al. (2006). Briefly, the ground samples (200 mg) were mixed with acetonitrile and HCl for 2 h before centrifugation at 2,000 *g* for 10 min. The supernatant was transferred to a fresh tube and evaporated under nitrogen gas. The residue was diluted in 1 ml 80% methanol, filtered through a 0.45 μm polyvinylidene fluoride membrane and subjected to high-performance liquid chromatography (Ultimate 3000; Thermo Scientific Dionex, USA) on an Inno C-18 column (YoungJin Biochrom, Korea) alongside SA standards.

Measurement of reactive oxygen species (ROS) production

Leaf discs from 5-week-old WT, RipL-expressing lines and *fls2 efr cerk1* plants were collected with a biopsy punch and floated on distilled water overnight. The water was replaced with 100 μl of assay solution containing 100 μM luminol (Sigma), 2 μg horseradish peroxidase (Sigma) and 10 nM efl18 (Peptron). Luminescence was measured in relative light unit for 1 h using GloMax microplate luminometer (Promega).

Seedling growth inhibition

Seedling growth inhibition assay was performed as described by Zipfel et al. (2006). Briefly, WT, RipL-expressing lines and *fls2 efr cerk1* seedlings were germinated on MS medium and transferred in liquid medium supplemented or not with 10 nM elf18. Fresh weight was determined after 12 days.

Statistical analysis

All the experiments were repeated at least two or three times, the measurements from independent repeats were merged, one-way variance was calculated by ANOVA and significance of difference assessed with Dunnett's multiple comparison test using the Prism 10.0.0 software (GraphPad Software, USA).

RESULTS

RipL expression correlates with reduced plant growth

To investigate the possible function of RipL *in planta*, we obtained independent Arabidopsis transgenic lines that expressed the effector constitutively. Arabidopsis Col-0 was transformed with constructions of the RipL-3xFLAG coding sequence under the control of the cauliflower mosaic virus 35S promoter (RipL-1 line) or under the control of a dexamethasone-inducible promoter (RipL-2 and RipL-3 lines). The transgene expression and protein accumulation were confirmed in homozygous plants (Figs. 1A and 1B). Using quantitative reverse transcription-PCR, we detected a clear accumulation of specific RipL transcripts in the three lines compared to the Col-0 line (hereafter named WT), even in absence of dexamethasone treatment in RipL-2 and RipL-3 lines. As the dexamethasone treatment did not enhance the accumulation of RipL in these lines (Supplementary Fig. S1), we considered RipL-2 and RipL-3 as constitutively expressing RipL, although to a lesser extent than the RipL-1 line (Fig. 1A). We could also detect a specific accumulation of the RipL-3xFLAG protein in seedling extracts by immunoblotting with anti-FLAG antibodies (Fig. 1B). In accordance with the higher accumulation of RipL transcripts in the RipL-1 line, a stronger accumulation of the RipL protein was observed in this line compared to RipL-2 and RipL-3.

The 3 independent homozygous lines were grown in two different day length conditions for further characterization. We noticed that RipL-expressing plants developed normally but were smaller than the WT plants in both short-day and long-day conditions (Fig. 1C). The quantification of the maximum rosette diameter supported this observation for RipL-1 line in both conditions (Figs. 1D and 1E). However, the rosette size was not significantly different from WT for RipL-2 in long-day and RipL-3 in short-day conditions, respectively.

RipL expression delays flowering time

While we processed to fix and amplify each line, we noticed that the RipL-expressing plants were late flowering. To confirm these observations, the 3 independent lines were grown alongside the WT and the late-flowering mutant *suppressor of overexpression of constans 1* (*soc1*) in short-day and long-day conditions and the days to bolting and total leaf number at bolting were measured (Fig. 2, Supplementary Fig. S2). As expected, the *soc1-2* plants were late flowering and bolted significantly later than the WT plants in both conditions (Lee et al., 2000). In long-day condition, RipL-1 were late flowering (Figs. 2A and 2B). The 3 RipL lines were significantly slower to flower in the short-day conditions (Figs. 2A and 2C). Of note, the floral development was not affected, and a normal number of viable seeds was obtained, allowing us to pursue the characterization of these lines.

As the flowering delay induced by RipL was unexpected, we attempted to evaluate its specificity by expressing RipL in another model plant, *N. benthamiana* (Supplementary Fig. S3). To allow for systemic and long-lasting expression of the effector, we used a virus-based expression vector containing the same RipL-3xFLAG coding sequence. *N. benthamiana* plants infected with the virus containing the 3xFLAG-GFP or the RipL-3xFLAG constructs were kept in long-day conditions until bolting. We could measure a higher total leaf number at the bolting stage in the RipL-expressing plants compared to the control, while the significant delay in flowering time was slight (Supplementary Fig. S3). Nonetheless, this experiment supported our finding that RipL expression correlates with a noticeable delay in the plant flowering time.

We hypothesized that the observed delay in flowering stemmed from mis-regulation of the flowering regulator genes. We measured the expression of the flowering integrator *FLOWERING LOCUS T* (*FT*) at its peak in 10-day-old long-day grown seedlings (Lee et al., 2007) (Fig. 3). In these conditions, FT transcript accumulation was reduced to a similar level in *soc1* and the 3 RipL-expressing lines compared to WT. Accordingly, the expression of the FT-regulated floral meristem identity genes *LFY* and *APETALA 1* (*AP1*) was also significantly lower in plants expressing RipL (Fig. 3), indicating that changes in *FT* expression could be the principal cause of the delayed flowering in presence of RipL in Arabidopsis.

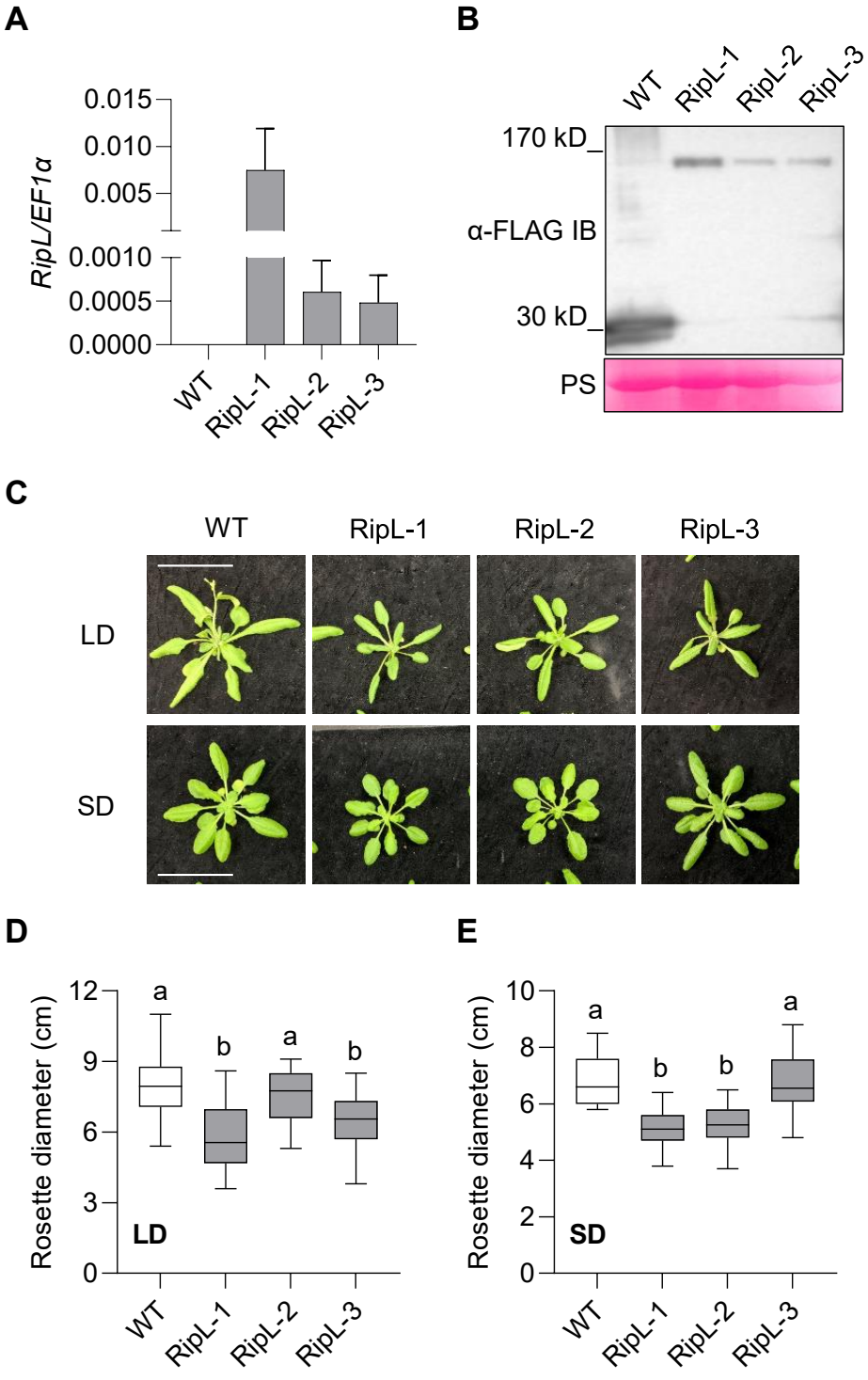

Fig. 1. Molecular and morphological characterization of RipL-expressing lines. Wild-type (WT) Col-0 and homozygous transgenic seedlings expressing RipL-3xFLAG (RipL-1, RipL-2, and RipL-3) were grown *in vitro* for 10 days before mRNA and protein extraction. (A) *RipL* transcript accumulation relative to *EF1α*. The mean value of two biological repeats ± SD is presented. (B) RipL-3xFLAG protein accumulation detected by immunoblotting with anti-FLAG antibodies on total protein extracts. Ponceau red staining (PS) of the large subunit of Rubisco is shown as loading control. (C-E) WT and *RipL*-expressing homozygous plants were grown in soil for 24 and 30 days under long day (LD) or short day (SD) conditions, respectively. (C) Photographs of representative plants. Scale bar = 2.5 cm. Maximum rosette diameter in plants grown under long day (D) and short day conditions (E). Measurements from 3 independent biological repeats are shown in boxes, whiskers indicate the minimum and maximum values. Different letters indicate statistical significance of difference with WT as tested with ANOVA followed by Dunnett's test ($P < 0.05$).

RipL promotes susceptibility to virulent bacterial strains

We have previously shown that when transiently expressed in *N. benthamiana*, RipL does not affect the pattern-triggered immunity signaling initiated by the perception of the bacterial epitope-peptide flg22 (Jeon et al., 2020). We tested the responsiveness of the RipL-expressing lines to elf18, another bacterial peptide that is detected by the EF-Tu receptor (EFR) in Arabidopsis (Kunze et al., 2004; Zipfel et al., 2006). The 3 independent RipL-expressing lines displayed similar sensitivity to elf18 treatment as the WT plants regarding the expression of the marker genes *FRK1* and *CYP81F2*, the production of ROS and the inhibition of seedling growth (Supplementary

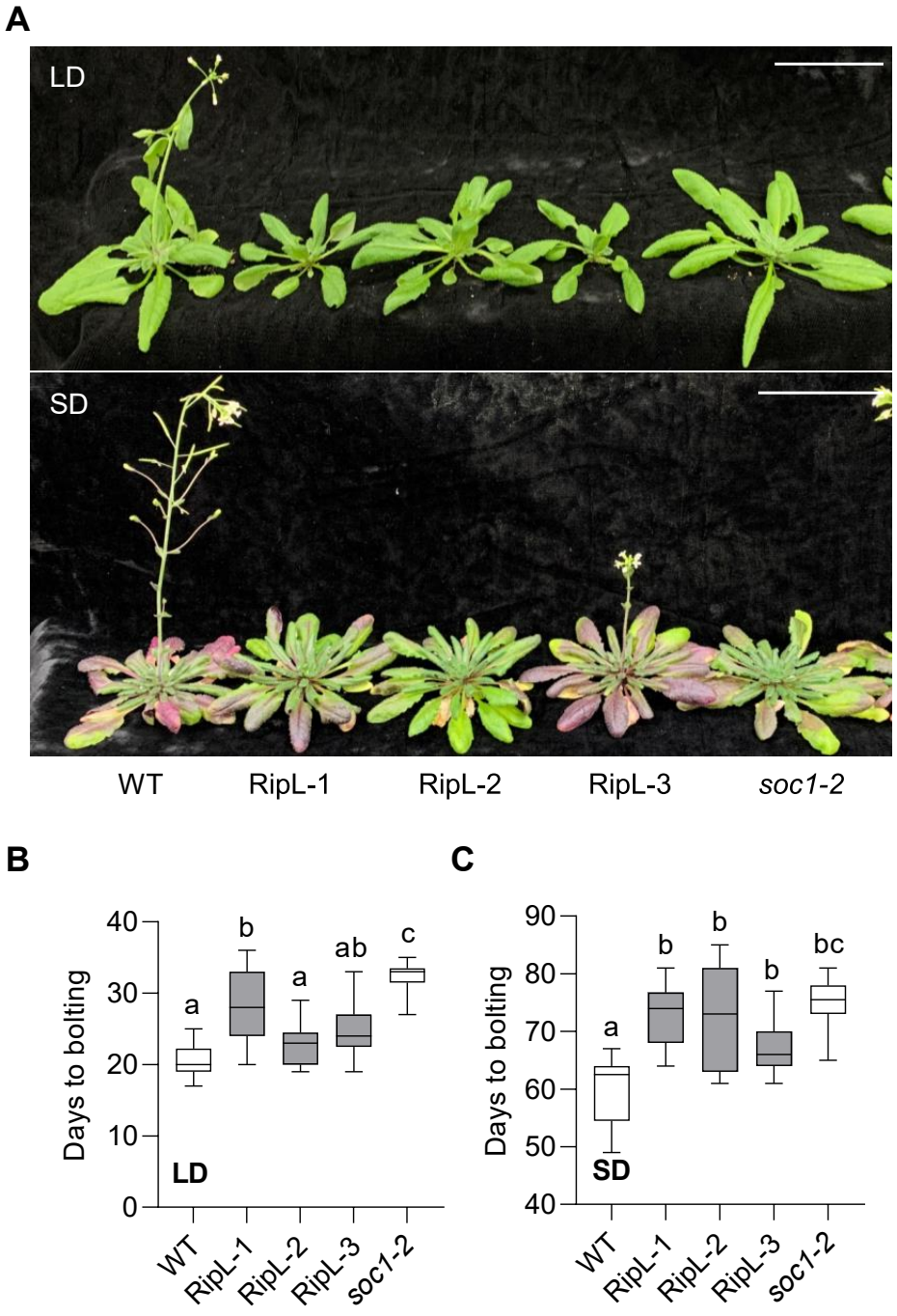

Fig. 2. Flowering time in RipL expressing lines. Wild-type (WT) Col-0, RipL-expressing plants (RipL-1, RipL-2, RipL-3) and delayed-flowering mutant *soc1-2* were grown in soil in long day (LD) or short day (SD) conditions. (A) Photographs of representative plants at bolting. Scale bars = 5 cm. Days to bolting in plants grown under long day (B) and short day condition (C). Measurements from 3 independent biological repeats are shown in boxes, whiskers indicate the minimum and maximum values. Different letters indicate statistical significance of difference with WT as tested with ANOVA followed by Dunnett's test ($P < 0.05$).

Fig. S4). These results indicate that pattern-triggered immunity signaling is not significantly affected by RipL expression in Arabidopsis.

We then evaluated the growth of a virulent strain of the leaf pathogen *Pst* DC3000 (Fig. 4A). Two days after inoculation, the bacterial titer in the infiltrated leaves of RipL-1 and RipL-2 plants was significantly higher than in the WT plants, indicating that the bacterial growth was enhanced in presence of RipL. Interestingly, the bacterial numbers were similar in WT and the late-flowering *soc1-2* mutant (Fig. 4A), suggesting that the enhanced susceptibility observed in RipL-expressing lines was not correlated with the late-flowering phenotype.

Next, we assessed whether RipL could promote the growth of the mildly virulent mutant strain *Pst* DC3000ΔAvrPtoΔAvrPtoB (Lin and Martin, 2005). This strain lacks two effectors known to inhibit pattern-recognition receptors and to block early defense signaling in Arabidopsis (Gimenez-Ibanez et al., 2009b; Lin and Martin, 2005; Zipfel and Rathjen, 2008). As expected, the *Pst* DC3000ΔAvrPtoΔAvrPtoB strain multiplied

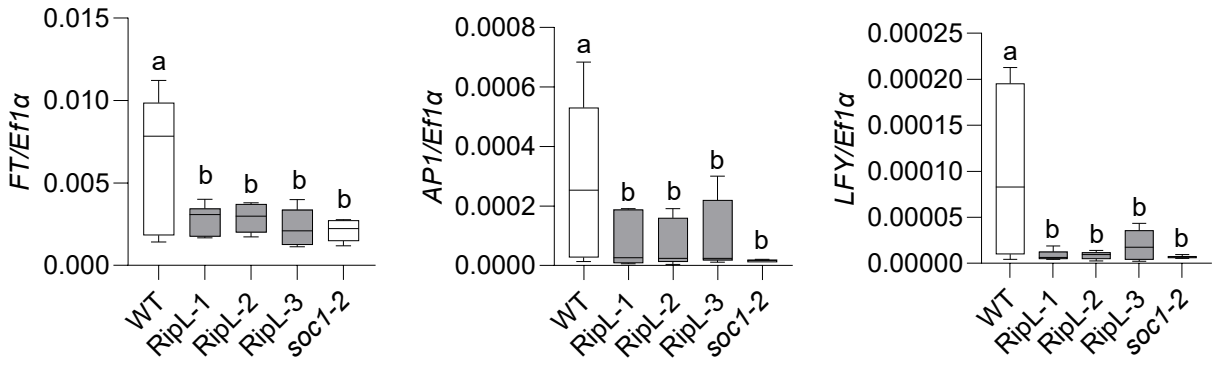

Fig. 3. Expression of the floral regulator genes in RipL-expressing lines. Flowering time gene expression relative to *EF1α* measured in 10-day-old Arabidopsis seedlings grown on MS under long day conditions. Measurements from 3 independent biological repeats are shown in boxes, whiskers indicate the minimum and maximum values. Different letters indicate statistical significance of difference with wild-type (WT) Col-0 as tested with ANOVA followed by Dunnett's test ($P < 0.05$).

less than the *Pst* DC3000 strain in WT plants (Figs. 4A and 4B). However, the bacterial titers were significantly higher in the RipL-1 and RipL-2 lines (Fig. 4B). This suggests that RipL expression altered the plant susceptibility, creating a more favorable environment even for mildly virulent pathogens.

To confirm the contribution of RipL to the promotion of susceptibility in plants, we also measured the bacterial growth of a strain of *Pst* DC3000 carrying a fusion of RipL with the N-terminal domain of the *P. syringae* effector AvrRps4 to allow a natural delivery of RipL through the type III secretion system (Sohn et al., 2007). In Col-0 plants, the *Pst* DC3000(RipL) strain grew significantly more than the control *Pst* DC3000(EV) strain 2 and 5 days post-inoculation (Fig. 4C). This result provided further evidence that RipL could contribute to the pathogen virulence, through promotion of the plant susceptibility.

SA biosynthesis and signaling are not impaired by RipL expression

Considering that RipL expression in plants improved the growth of the virulent *Pst* DC3000 strain, we hypothesized that SA-mediated defense—a key limiting factor for *P. syringae* growth in Arabidopsis (Zhang and Li, 2019)—was impaired. We quantified leaf SA content in WT and RipL-expressing lines 24 h after *Pst* DC3000 inoculation (Fig. 5A). The bacterial infection led to a sharp increase of SA in leaves compared to the mock treatment. However, a similar accumulation of SA was observed in RipL-expressing and WT lines. This result was in accordance with a similar accumulation of the transcript of the SA-biosynthesis gene *ISOCHORISMATE SYNTHASE 1* (*ICS1*) after *Pst* infection in WT and RipL-expressing lines (Fig. 5B).

SA-mediated defense responses are coordinated by two key transcription factors, SAR DEFICIENT 1 (SARD1) and CALMODULIN-BINDING PROTEIN 60g (CBP60g) in Arabidopsis (Zhang et al., 2010). The expression of *SARD1* and *CBP60g* was strongly upregulated 24 h after the *Pst* infection (Figs. 5C and 5D). A similar level of *SARD1* and *CPB60g* transcript accumulation was observed in RipL-expressing lines and WT. Altogether, these results suggest that the hypersusceptibility to *Pst* infection and the delayed flowering observed in presence of RipL were not imputable to an impairment of SA biosynthesis or SA-mediated signaling.

RipL modifies the expression of photosynthesis-related and transcription factor genes

To determine the cause of hypersusceptibility to pathogen infection of RipL-expressing lines, we performed a whole-genome scale analysis of gene expression in WT, RipL-1 and RipL-2 lines at 24 h after mock or *Pst* DC3000 inoculation, using RNA-seq (Fig. 6). First, we searched for the DEGs in both RipL-1 and RipL-2 lines compared to WT in mock and infected conditions. In total, 432 DEGs were commonly found in RipL-1 and RipL-2 lines; 390 and 42 genes with a higher and lower expression than in WT, respectively (Fig. 6A). The gene ontology analysis revealed an over-representation of genes involved in photosynthetic processes, water homeostasis and the responses to abiotic stresses such as cold and light intensity among the up-regulated genes in RipL lines (Fig. 6B). Molecular functions related to gene expression regulation such as mRNA binding and DNA-binding transcription factor activity were also over-represented among genes more expressed in both RipL lines than in the WT (Fig. 6C). Among the 40 DEGs classified as "DNA-binding transcription factor", 7 of the 36 members of the DNA binding with One Finger (DOF) family of transcription factors were up-regulated in both RipL-expressing lines compared to WT (Supplementary Fig. S5) (Gupta et al., 2015; Lijavetzky et al., 2003). Additionally, we searched for the known flowering regulator genes reported in the FLOR-ID database (www.flor-id.org; Bouché et al., 2016) in our dataset (Supplementary Fig. S6). Only 14 out of over 300 genes involved in flowering are significantly differentially expressed in our experiment, presumably because we perform the analysis on mature short-day grown plants. The negative regulators CYCLING DOF FACTOR2 and VARIANT IN METHYLATION2 (Fornara et al., 2009; Woo et al., 2008) displayed a modest (~2-fold change) mRNA accumulation in both RipL-1 and RipL-2 lines compared to WT, which might correlate with the flowering delay.

To confirm the RNA-seq data, we selected 8 DEGs and

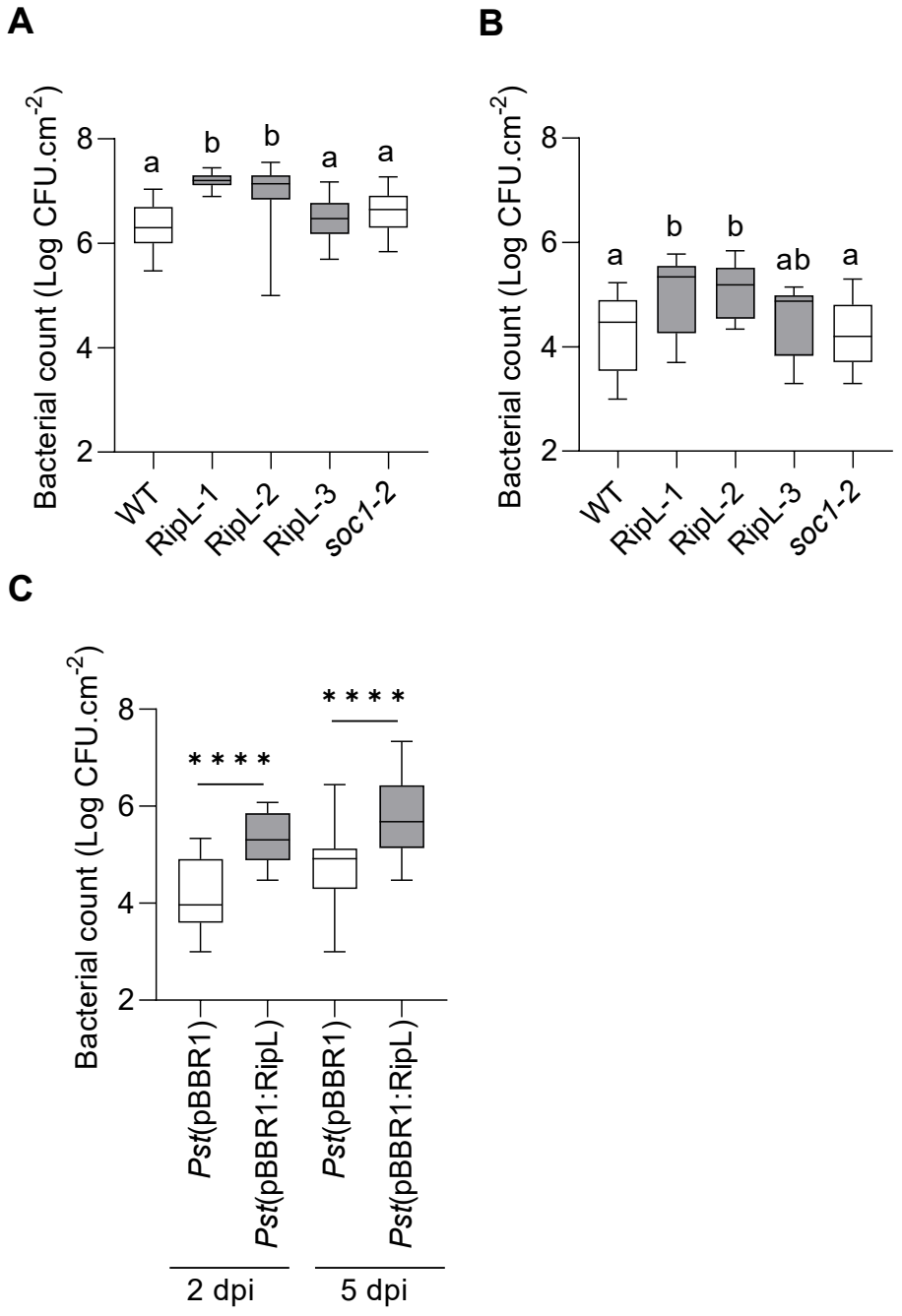

Fig. 4. Pathogen growth in RipL-expressing line and following RipL delivery from bacteria. Wild-type (WT) Col-0 and RipL-expressing lines were grown on soil in short day conditions for 5 weeks before infiltration with *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. (A) *Pst* DC3000 growth in WT and RipL-expressing lines. (B) *Pst* DC3000ΔAvrPtoΔAvrPtoB mutant growth in WT and RipL-expressing lines. (C) *Pst* DC3000(pBBR1) and *Pst* DC3000(pBBR1:RipL) growth in Col-0 plants. Bacterial enumeration was performed at 2 days or at 2 days and 5 days post-infiltration (dpi). Data are the number of colonies forming units (CFU) from three independent biological repeats shown in boxes, whiskers indicate the minimum and maximum values. Different letters indicate statistical significance of difference with WT as tested with ANOVA followed by Dunnett's test ($P < 0.05$). Asterisks indicate statistical difference with *Pst* DC3000(pBBR1) assessed by Student's *t*-test ($P < 0.0001$).

measured their expression using qRT-PCR in new batches of samples (Supplementary Fig. S7). *At3g21710*, *At4g17470*, *At4g31110*, and *At1g26450* expression was clearly up-regulated while *At4g08160*, *At5g48920*, *At2g05440*, and *At2g21890* expression was down-regulated in the two RipL-expressing lines compared to WT, in accordance with the respective transcript per million (TPM) values detected by RNA sequencing.

RipL expression dampens the overall amplitude of the transcriptional reprogramming during infection

We observed a large overlap of the genes regulated during *Pst* infection in RipL-expressing and WT lines (Fig. 7A). In accordance with previous studies, there was an over-representation of genes involved in defense response to biotic stress, regulation of signal transduction, hormone-mediated signaling or positive regulation of the RNA polymerase II in the DEGs up-regulated by *Pst* infection, while genes involved in development, photosynthesis or protein translation machinery were down-regulated (Supplementary Fig. S8) (Howard et al., 2013; Wang et al., 2008). The "hormone-mediated signaling" category included abscisic acid (ABA)-activated signaling, jasmonic acid (JA)-mediated signaling pathway, response to ethylene (ET) and systemic acquired resistance/

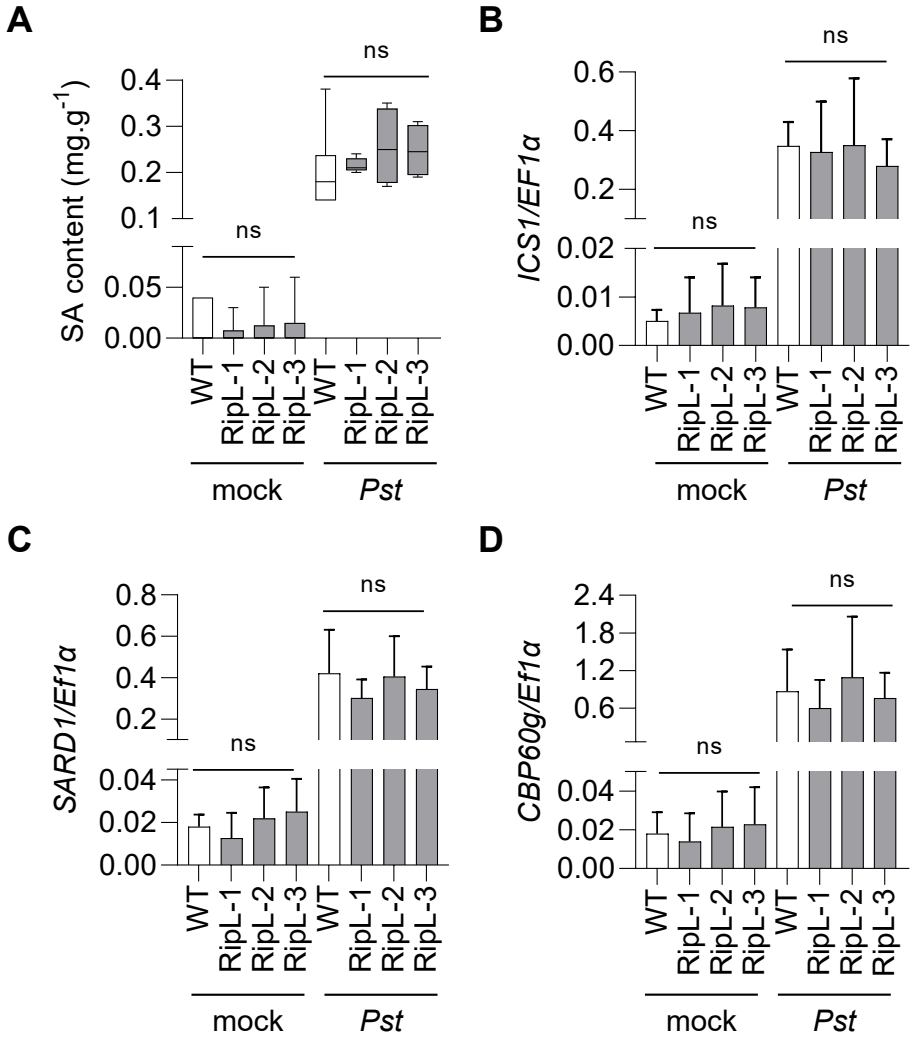

Fig. 5. Salicylic acid (SA) content and expression of SA regulator genes in RipL-expressing lines. Wild-type (WT) Col-0 and RipL-expressing lines were grown on soil in short day conditions for 5 weeks before infiltration with *Pst* DC3000 (*Pst*) or 10 mM $MgCl_2$ (mock). The samples were collected at 24 h post-inoculation and analyzed. (A) SA content measured in three biological repeats, shown as boxes, whiskers indicate minimum and maximum values. Expression of *ICS1* (B), *SARD1* (C), and *CBP60g* (D) relative to *EF1α*. The mean value of three biological repeats ± SD is presented. The statistical significance of difference with WT was tested with ANOVA followed by Dunnett's test.

SA-mediated pathway. These hormones are known regulators of plant defense responses during infection (Berens et al., 2017; Pieterse et al., 2012), supporting the validity of our experimental conditions. However, the genes in this category are similarly regulated by *Pst* infection in WT and the RipL-expressing lines, suggesting together with our previous results that RipL expression does not significantly affect SA nor JA, ET or ABA signaling pathways.

Despite the similarity of the overall transcriptional response to infection in WT and the RipL-expressing lines, the analysis of the z-score mean for all the commonly *Pst*-regulated genes revealed a significant difference in the amplitude of the expression change in the RipL-expressing lines (Fig. 7B). The fold-change of up-regulated or down-regulated genes was lower in RipL lines compared to WT. This general difference in z-score mean could be readily observed when we restricted the z-score mean analysis for the genes classified in the "response to bacterium" group. These genes were up-regulated to a higher extent in the WT than in the two RipL-expressing lines (Fig. 7C). Together, these results suggested a global buffering of the transcriptional response induced by bacterial infection in presence of RipL, which could contribute to the enhanced susceptibility observed in the RipL-expressing lines.

DISCUSSION

Rps delivers a considerably higher number of effectors than *P. syringae* or *Xanthomonas* spp. in the plant cell (Coll and Valls, 2013; Mukaihara et al., 2010). The role of these effectors in pathogenicity remains to be determined (Landry et al., 2020). Despite broad variation in the effector repertoire, certain effectors are conserved across strains of *Rps* and in the sister species *R. solanacearum* and *R. syzygii*, suggesting that they are required to establish and maintain the bacteria proliferation in the plant. RipL coding sequence is found in isolates from the three species, *R. solanacearum*, *Rps*, and *R. syzygii*, with a high degree of conservation (from 98.3% to 59.6% AA identity), suggesting a potential contribution to *Ralstonia* pathogenicity in diverse plant hosts. In this report, we investigated the role of the *Rps* effector RipL in plant immunity and development using a heterologous expression system. Our results indicate that RipL expression enhanced susceptibility

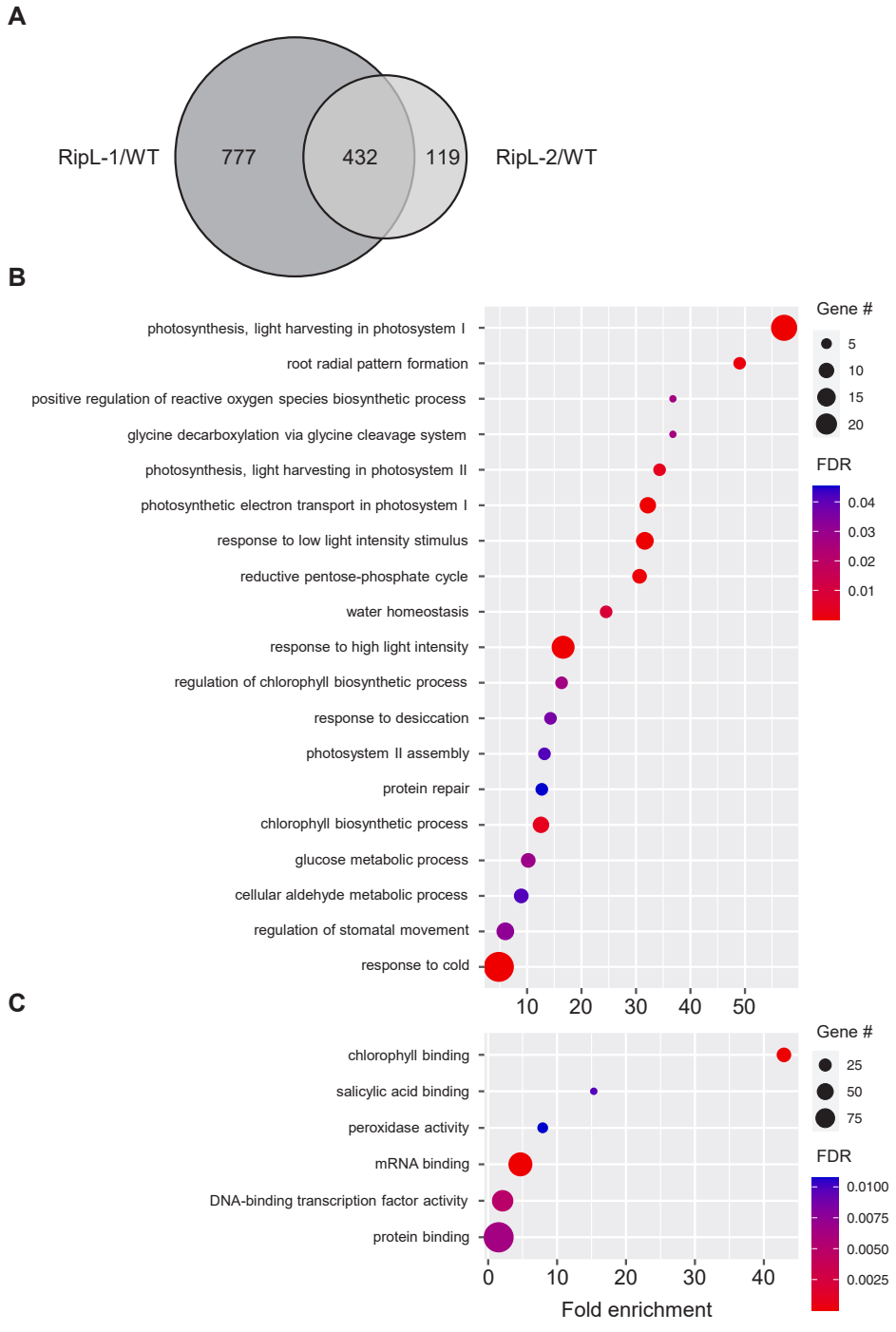

Fig. 6. Differentially expressed genes in RipL-expressing lines. Wild-type (WT) Col-0 and RipL-expressing lines were grown on soil in short day conditions for 5 weeks before mock- or *Pst* DC3000 infiltration. The samples were collected at 24 h post-inoculation. (A) Venn diagram representation of the number of differentially expressed genes (DEGs) identified in RipL-1 and RipL-2 lines compared to WT in both mock and *Pst* conditions. Over-representation of biological process (B) and molecular function (C) GO categories among the 390 DEGs up-regulated in both RipL-expressing lines compared to WT. FDR, false discovery rate.

to bacterial pathogens and delayed the flowering time in Arabidopsis.

Considering the substantial number of effectors encoded in the *Rps* genome, it is likely that some act redundantly. Therefore, the functional characterization of *Rps* effectors can benefit from expression of individual effectors rather study of single effector deletion. Several studies using heterologous expression of individual effectors have revealed their contribution to virulence. For example, Arabidopsis transgenic lines expressing the endoplasmic reticulum-associated RipN or the nuclear-localized RipAB are more susceptible to *Pst* DC3000 (Qi et al., 2022; Sun et al., 2019). In the case of RipAB, the hypersusceptibility of Arabidopsis expressing lines is in accordance with a loss of virulence reported for *ripAB* mutant strains in potato and in tomato (Qi et al., 2022; Zheng et al., 2019). Although our approach does not directly reflect the natural delivery of RipL during *Rps* root infection, the enhanced susceptibility of the RipL-expressing lines to

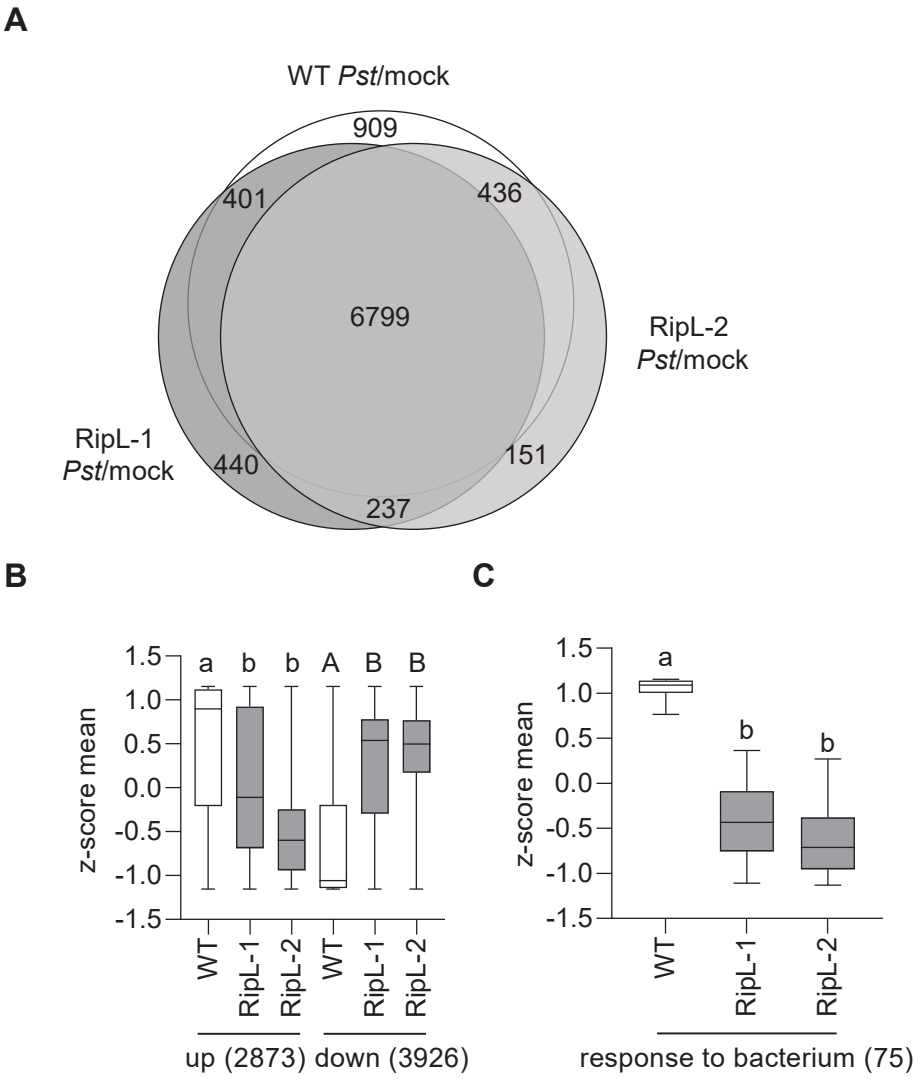

Fig. 7. Transcriptional response to pathogen infection in RipL-expressing lines. Wild-type (WT) Col-0 and RipL-expressing lines were grown on soil in short day conditions for 5 weeks before mock- or *Pst* DC3000 infiltration. The samples were collected at 24 h post-inoculation. (A) Venn diagram representation of the number of differentially expressed genes (DEGs) identified *Pst*-infected plants compared to mock in WT, RipL-1 and RipL-2 lines. (B) z-score mean of 6799 DEGs common to all the *Pst*-infected samples. (C) z-score mean of 75 DEGs annotated in the "response to bacterium" GO category. Different letters indicate statistical significance of difference with WT as tested with ANOVA followed by Dunnett's test ($P < 0.001$).

Pst infection and the higher titer of *Pst* DC3000(pBBR1:RipL) in Arabidopsis collectively indicate a notable contribution of RipL to *Rps* virulence in host plants.

Constitutive expression of pathogen effector *in planta* can lead to significant changes in the morphology or development of plants. For instance, Arabidopsis lines expressing constitutively the *Hyalenoperonospora arabidopsidis* RxLR effector HaRxL106 display longer hypocotyls and elongated leaves similar to the shade avoidance response (Wirthmueller et al., 2018). Plants constitutively expressing the phytoplasma effector SAP54 produce indeterminate leaf-like flowers and displayed enhanced susceptibility to the phytoplasma insect vector (MacLean et al., 2014). The *Meloidogyne incognita* candidate effector Mi8D05 promoted shoot development and accelerated flowering when constitutively expressed in Arabidopsis, although this effect did not directly correlate with improved colonization of the roots by the nematode (Xue et al., 2013). The smaller rosette size observed in RipL-expressing lines could be indicative of autoimmunity, usually accompanied by enhanced SA levels and reduced *Pst* susceptibility (Li et al., 2021). Here, we did not observe a significant correlation between the plant size and the accumulation of SA. The rosette size does not systematically correlate with heightened defense responses, as illustrated by the recently characterized RipAB-expressing Arabidopsis lines that display smaller rosette than WT plants and are more susceptible to *Pst* infection (Qi et al., 2022).

RipL-expressing plants exhibited delayed flowering, similar to *soc1-2* mutant (Lee et al., 2000). Several flowering time mutants have defects in immunity, but *soc1-2* plants do not display enhanced susceptibility to *Pst* DC3000 infection (March-Díaz et al., 2007; Wilson et al., 2017). Increased expression of SA biosynthetic genes correlates with early flowering (March-Díaz et al., 2008). As the total SA accumulation is not affected in RipL-expressing plants, the delayed flowering is likely the consequence of the reduced *FT* expression, rather than a collateral effect of impaired SA-dependent immunity. Several members of the DOF transcription factor family were found upregulated in the RipL-expressing lines. Interestingly, CYCLING DOF FACTOR (CDF) 1, 2, 3 and 5 redundantly

repressed *CONSTANS*, a positive regulator of *FT* expression (Fornara et al., 2009). CDF2 also affects the transcription and the processing of miRNA156 and miRNA172 that are important regulators of *FT* expression (Cho et al., 2012; Kim et al., 2012; Sun et al., 2015). Although further investigation is required, the altered expression of DOF transcription factors in RipL-expressing lines might be linked with the reduced expression of *FT*.

As SA is a positive regulator of both immunity and flowering, we initially hypothesized that the delayed flowering and the enhanced susceptibility in RipL-expressing lines could both stem from low accumulation of SA. SA is a major defense-associated hormone and the SA signaling pathway is targeted by effectors from multiple pathogens (Bauters et al., 2021). In Arabidopsis, SA biosynthesis results from two pathways initiated respectively by the isochorismate synthase (ICS) or the phenylalanine ammonia-lyase (PAL) (Lefevere et al., 2020). The ICS pathway is affected by the chloroplastic *Pst* effector HopI1 that remodels thylakoid structures and lowers plant SA contents (Jelenska et al., 2007). The *Verticillium dahlia* effector VdSCP41 directly targets the SA-signaling regulators SARD1 and CPB60g to impair SA biosynthesis (Qin et al., 2018). *SARD1* and *CPB60g* expression is controlled by TGA transcription factors, which are targeted by the *Rps* effector RipAB (Qi et al., 2022). *Rps* also possesses an internal system to degrade SA and prevent its toxicity (Lowe-Power et al., 2018). *Rps* can deploy other effectors such as RipAL involved in suppression of SA-signaling pathways through the promotion of jasmonate, which can antagonize SA-dependent signaling (Nakano and Mukaihara, 2018). In the RipL-expressing lines, total SA accumulation, SARD1/CBP60g-dependent signaling and the expression of SA-, JA-, ET-, and ABA-mediated signaling genes during *Pst* infection appeared unaffected. This indicates that RipL could promote plant susceptibility independent of SA or other hormones involved in defense responses.

RipL expression in Arabidopsis promotes susceptibility to bacterial infection without measurable modifications of the pattern-triggered immunity signaling nor of hormonal responses. Rather, the amplitude of the transcriptional response to infection appeared dampened in presence of RipL. Together with the smaller stature and the delayed flowering, this suggests that RipL-expressing plants might be more globally impaired in terms of energy allocation. We previously reported the association of RipL protein with lipid bodies in *N. benthamiana* leaves (Jeon et al., 2020). If RipL has a particular affinity for lipids, it might affect the availability of the FT protein and/or interfere more globally with energy storage (Yang and Benning, 2018). Phospholipids can regulate flowering time regulation through specific recruitment of the FT protein at the membranes, on phosphatidylcholine in shoot meristems or on phosphatidylglycerol in the companion cells of phloem tissues (Nakamura et al., 2014; Susila et al., 2021). On the other hand, roles of lipid bodies in plant defense responses recently emerged from the identification of proteins associated with this storage compartment in plant cells (Fernández-Santos et al., 2020). Lipid bodies accumulate in response to *Pst* infection in Arabidopsis leaves and are associated with important regulators of defense such as PHYTOALEXIN-DEFICIENT 3, an enzyme involved in the production of antimicrobial camalexin (Fernández-Santos et al., 2020; Nafisi et al. 2007). Immunolocalization of the RipL-3xFLAG protein in the RipL-expressing lines would clarify RipL interaction with lipid bodies and the possible interference of RipL with lipid storage/release from this compartment, which could explain both the reduced amplitude of transcriptional changes during *Pst* infection and the delayed flowering.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS

W.K. and C.S. conceived the experiments. W.K., H.J., and C.S. performed and analyzed the experiments. H.L. and K.H.S. contributed to the construction of the transgenic lines. W.K. and C.S. wrote the manuscript. All the authors approved the manuscript before submission.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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