Silencing of *AtRAP*, a target gene of a bacteria-induced small RNA, triggers antibacterial defense responses through activation of LSU2 and down-regulation of *GLK1*

Huan Wang[1],[*], **Jang-Kyun Seo**[2],[*], **Shang Gao**[1], **Xinping Cui**[3], and **Hailing Jin**[1]

[1]Department of Plant Pathology & Microbiology, Institute for Integrative Genome Biology, University of California, Riverside, CA 92521-0122, USA

[2]Graduate School of International Agricultural Technology, Seoul National University, Gangwon-do 25354, Korea

[3]Department of Statistics, University of California, Riverside, CA 92521-0122, USA

Summary

- Plants fine-tune their sophisticated immunity systems in response to pathogen infections. We previously showed that AtlsiRNA-1, a bacteria-induced plant endogenous small interfering RNA, silences the *AtRAP* gene, which encodes a putative RNA binding protein.

- In this study, we demonstrate that AtRAP functions as a negative regulator in plant immunity by characterizing molecular and biological responses of the knockout mutant and overexpression lines of *AtRAP* upon bacterial infection.

- AtRAP is localized in chloroplasts and physically interacts with Low Sulfur Upregulated 2 (LSU2), which positively regulates plant defense. Our results suggest that AtRAP negatively regulates defense responses by suppressing LSU2 through physical interaction. We also detected downregulation of the transcription factor *GOLDEN2-LIKE 1* (*GLK1*) in *atrap-1* using microarray analysis. The *glk1 glk2* double mutant showed enhanced resistance to *Pseudomonas syringae* pv. *tomato*, which is consistent with a previous study showing enhanced resistance of a *glk1 glk2* double mutant to *Hyaloperonospora arabidopsidis*.

- Taken together, our data suggest that silencing of *AtRAP* by AtlsiRNA-1 upon bacterial infection triggers defense responses through regulation of LSU2 and GLK1.

Author for correspondence: Hailing Jin, Tel: +1 9518277995, hailingj@ucr.edu.
[*]These authors contributed equally to this work.

Introduction

Plants have evolved complex immune systems to defend against pathogens (Dodds & Rathjen, 2010; Schwessinger & Ronald, 2012; Dangl *et al.*, 2013). In particular, pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) provides the first line of defense to protect the plants from infection of the vast majority of potential pathogens. In addition, plants have evolved a second line of defense to perceive pathogen effector proteins that suppress PTI. This second layer of protection is mediated by specific components, such as resistance (R) proteins that are involved in the recognition of effectors to activate effector-triggered immunity (ETI) (Chisholm *et al.*, 2006; Jones & Dangl, 2006; Dodds & Rathjen, 2010; Schreiber *et al.*, 2011; Dangl *et al.*, 2013). These plant defense responses are orchestrated by a complex transcriptional reprogramming of host cells that regulates activation of various defense-related genes, accumulation of plant hormones and production of reactive oxygen species (ROS) (Grant & Jones, 2009). To benefit plants, the pathways involved in defense responses need to be inactivated under normal growth conditions, but should be activated quickly upon pathogen attack. Thus, activation of the plant defense responses is controlled by complex interconnected signaling networks regulating various plant functions (Feys & Parker, 2000; Katagiri, 2004; Pieterse *et al.*, 2009).

Increasing evidence shows that small RNAs (sRNAs) have pivotal roles in regulating the complex defense signaling network (Ruiz-Ferrer & Voinnet, 2009; Katiyar-Agarwal & Jin, 2010; Seo *et al.*, 2013). sRNAs are short, noncoding RNA molecules that guide transcriptional and post-transcriptional silencing of gene expression (Baulcombe, 2004). Regulatory sRNAs are divided into two classes: microRNAs (miRNAs) and small interfering RNAs (siRNAs). Various miRNAs and siRNAs, as well as some sRNA pathway components are specifically induced by infections of different pathogens, and regulate host PTI and/or ETI pathways, as well as pathogen virulence (Ruiz-Ferrer & Voinnet, 2009; Zhang *et al.*, 2011; Weiberg *et al.*, 2013, 2014; Niu *et al.*, 2016). We previously identified a novel class of long siRNAs (lsiRNAs), which were induced by pathogen infection (Katiyar-Agarwal *et al.*, 2006). One of the identified lsiRNAs, AtlsiRNA-1, targets the *Arabidopsis thaliana protein containing a RNA-binding domain abundant in Apicomplexans* (*AtRAP*) gene (Katiyar-Agarwal *et al.*, 2006; Kleinknecht *et al.*, 2014). Because the knockout mutant of *AtRAP* (*atrap-1*) showed increased resistance to *Pseudomonas syringae* pv. *tomato* (*Pst*) infection, it was suggested that AtRAP negatively regulates plant defense responses (Katiyar-Agarwal *et al.*, 2007).

In this study, we further investigated the molecular mechanism underlying the function of AtRAP in plant disease resistance. We show that representative defense-related genes and immune responses were activated in the *atrap-1* mutant. We demonstrate that AtRAP protein directly interacts with Low Sulfur Upregulated 2 (LSU2) protein, which positively regulates plant defense. Microarray analyses revealed a set of genes that were differentially expressed

in the *atrap-1* mutant as compared with the wild type, including the transcription factor GOLDEN2-LIKE 1 (GLK1), another negative regulator of plant defense.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana L. Heynh. plants were grown in a controlled growth room at 23°C, with a 12 h : 12 h, light : dark photoperiod. The *Arabidopsis* knockout mutants *atrap-1* (CS_844807), *lsu2* (SALK_031648) and *glk1 glk2* (*Atglk1.1*; *Atglk2.1*) were obtained from the Arabidopsis Biological Resource Center (ABRC). The double mutant *atrap lsu2* was generated by crossing the single mutants *atrap-1* and *lsu2*. The double mutant was selected by PCR of the genomic DNA for the homologous T-DNA insertion and confirmed by reverse transcription polymerase chain reaction (RT-PCR) to show no or very low expression of *AtRAP* and *LSU2*, respectively, in the double mutant.

Pathogen inoculation

Pathogen inoculations were performed as previously described (Katiyar-Agarwal *et al.*, 2007). In brief, for RNA, protein and hypersensitive response (HR) analysis, plants were infiltrated with *Pst* DC3000 carrying an empty vector (EV) (pVSP61) or effector gene *avrRpt2* at a concentration of 2×10^7 colony-forming units (CFU) ml $^{-1}$. For bacterial growth assays, plants were infiltrated with *Pst* (EV) or *Pst* (*avrRpt2*) at a concentration of 2×10^5 CFU ml $^{-1}$. At least six leaf disks were collected at each time point for 0 and 3 d post-inoculation (dpi) by a cock borer. The 0 dpi samples were immediately collected after inoculation. Bacterial titers were measured by grinding, diluting, plating, culturing and counting colonies. Student's *t*-test (two samples) and ANOVA test (when comparing more than two samples) were used for significance difference calculation between Col-0 WT and mutant plants, or Col-0 WT and overexpression lines.

RNA extraction, quantitative real-time PCR and Northern blot analysis

Total RNA extraction was performed using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and mRNA extraction was carried out using the Oligotex mRNA Mini Kit (Qiagen).

To check the expression levels of *PR1* (AT2G14610), *PR2* (AT3G57260), *RBOHd* (AT5G47910), *RBOHf* (AT1G64060) and *PDF1.2* (AT5g44420), cDNA was synthesized from 500 ng of mRNA using Superscript III (Invitrogen). The resulting cDNA was subjected to quantitative real-time PCR using an iCycler iQ5 real-time PCR detection system (Bio-Rad) with specific primers. The sequences of specific primers are given in Supporting Information Table S1.

Northern blot analysis was performed as previously described with minor modifications (Seo *et al.*, 2009). Briefly, 10 μg of total RNA was fractionated by electrophoresis and transferred to positively charged nylon membranes (Amersham). Hybridization was performed using ULTRAhyb-Oligo hybridization buffer (Ambion) and ^{32}P end-labeled DNA oligonucleotide

probes specific for each target gene, according to the manufacturer's protocol. The sequences of probes are given in Table S2.

DAB staining

3,3′-Diaminobenzidine (DAB) staining was performed to detect ROS as described previously (ThordalChristensen *et al.*, 1997). Briefly, leaves were stained with 1 mg ml $^{-1}$ DAB (Sigma) by vacuum infiltration, and destained with 90% ethanol. The destained samples were mounted in 50% glycerol and observed under a light microscope.

Plasmid DNA constructs and generation of transgenic *Arabidopsis* plants

The full-length *AtRAP* coding sequence (CDS) was cloned into a p35SGATFH destination vector with a C-terminal Flag tag. The construct was transformed into *atrap-1* by *Agrobacterium tumefaciens* strain GV3101. The full-length *AtRAP* CDS was cloned into pEarlyGate101 with a yellow fluorescent protein (YFP) tag. The full-length *LSU2* CDS was cloned into pEarlyGate102 or pEarlyGate202, with a cyan fluorescent protein (CFP) or Flag tag, respectively. The vectors were transformed into *A. tumefaciens* strain GV3101 and used for transient expression of recombinant proteins.

Confocal microscopy

Subcellular localization of AtRAP-YFP and LSU2-CFP was observed by confocal microscopy using a Leica SP2/SP5 laser-scanning confocal microscope (Leica, Wetzlar, Germany), equipped with a specific laser/filter combination to detect CFP (excitation at 458 nm), YFP (excitation at 514 nm) and red fluorescent protein (RFP) (excitation at 594 nm).

Yeast two-hybrid screening

Yeast two-hybrid screening was performed using a cDNA library (generously provided by T. Eulgem) of pooled RNAs from 2-wk-old *Arabidopsis* Col-0 WT seedlings constructed using the HybriZAP-2.1 library construction system (Stratagene, la Jolla, CA, USA). The *Arabidopsis* library cDNAs (1.3×10^6) were screened by transformation into the AH109 yeast strain (Clontech, Palo Alto, CA, USA) expressing AtRAP-DNA-binding domain (BD) bait fusion protein generated by the GAL4 system (Stratagene,), as described by the manufacturer. Plasmids were isolated from the positive clones and transformed into *Escherichia coli* DH5α to amplify for sequencing. The plasmids from the positive clones were retransformed into the yeast transformant expressing AtRAP-BD to verify the interaction. The interactions between SV40 large T antigen (84–708) (pTD1-1) and either murine p53(72–390) (pVA3-1) or human lamin C(66–230) (pLAM5′-1) served as positive and negative controls, respectively.

Co-immunoprecipitation assay

Total protein extracts were prepared from the *Nicotiana benthamiana* leaves infiltrated with *A. tumefaciens* containing the AtRAP-YFP and/or LSU2-FLAG constructs. At 3 dpi, infiltrated leaves were homogenized in three volumes of protein extraction buffer (20mM Tris–HCl at pH 7.5, 300mM NaCl, 5 mM MgCl$_2$, 5mM dithiothreitol, 0.5% Triton X-100, proteinase inhibitor cocktail; Sigma). Cell debris was removed by centrifugation at 18 000 *g*

for 20 min at 4°C. The resulting supernatants were incubated with anti-YFP antibody-conjugated agarose beads (Roche) for 8 h at 4°C. The immunocomplexes were then precipitated by centrifugation for 1 min at 8200 *g*, and washed three times in 1 ml phosphate-buffered saline (PBS: 0.1M NaCl, 90mM sodium phosphate, pH 7.0). The resulting samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblot analysis using anti-YFP (Roche) and anti-FLAG (Sigma) antibodies.

Microarray analysis

For Affymetrix GeneChip array analysis, we normalized expression profiles with the Robust multiarray analysis (RMA) method (Irizarry *et al.*, 2003). A list of genes with statistically significant changes in expression between the genotypes was generated by the significance analysis of microarrays (SAM) method in which multiple testing was taken into account through *q*-value (Siggenes, R package v.1.50.0). We used a threshold *q*-value of 0.05 to select the genes whose expression levels are statistically significantly changed.

Accession number

The microarray data of this paper are deposited at NCBI (GSE98376).

Results

Knocking out *AtRAP* gene activates defense-related genes and increases ROS accumulation

We previously demonstrated that the *AtRAP* knockout mutant is more resistant to both virulent *Pst* carrying an empty vector (EV) and avirulent *Pst* (*avrRpt2*) than to the Col-0 wild-type (WT) plants (Katiyar-Agarwal *et al.*, 2007), suggesting that AtRAP has a negative role in defense against bacteria. Infection of *Arabidopsis* with *Pst* triggers transcriptional reprogramming of a variety of defense-related genes (Dong *et al.*, 1991; Panstruga *et al.*, 2009). To determine whether expression of the defense-related genes was altered in the *atrap* mutant, the leaf samples infiltrated with *Pst* (EV) or *Pst* (*avrRpt2*) were collected at different time points, and total RNAs were extracted and subjected to quantitative real-time RT-PCR. We first investigated the induction levels of two representative antimicrobial transcripts: *PATHOGENESIS-RELATED PROTEIN 1* (*PR1*, AT2G14610) and *PR2* (AT3G57260). *PR1* and *PR2* were induced to a higher level in the *atrap* mutant than in Col-0 WT plants at 10 h post-inoculation (hpi) with both *Pst* (EV) and *Pst* (*avrRpt2*) strains (Fig. 1a,b). *PR1* and *PR2* transcript levels were also slightly higher in the *atrap* mutant at 0 hpi than in the Col-0 WT plants (Fig. 1a,b), suggesting that the *atrap* mutant has an elevated basal defense level. Since *PR1* and *PR2* are two salicylic acid (SA)-dependent marker genes and the SA pathway is antagonistic to the jasmonic acid (JA) pathway (Kunkel & Brooks, 2002; Anderson *et al.*, 2004), we next examined the expression level of a JA-responsive marker gene: *PDF1.2*. As shown in Fig. S1(a), the expression of *PDF1.2* transcripts was significantly suppressed in the *atrap* mutant compared with Col-0 WT plants before and after infection.

Pst (*avrRpt2*) induces the HR in Col-0 WT plants that carry the cognate resistance gene *RPS2* (Kunkel *et al.*, 1993; Mackey *et al.*, 2003). When we examined the HR in the *atrap*

mutant, we observed an earlier and more severe induction of cell death (half leaf inoculation) than in the Col-0 WT plants (Fig. S1b). During the HR progression, the production of ROS is known to trigger programmed cell death and subsequent defense responses (Torres *et al.*, 2006). It has been demonstrated that plant *NADPH oxidase genes*, including *RBOHd* (AT5G47910) and *RBOHf* (AT1G64060), play crucial roles in regulating the generation of ROS in plant defense (Torres *et al.*, 2002; Miller *et al.*, 2009; Vellosillo *et al.*, 2010). We found that *RBOHf* was induced to a higher level in the *atrap* mutant than in the Col-0 WT in response to *Pst* (EV) and *Pst* (*avrRpt2*) infection, while no significant alteration of *RBOHd* mRNA expression was observed in the *atrap* mutant (Fig. 1c,d). This suggests that *RBOHf* may play a more important role than *RBOHd* in regulating ROS production and HR cell death in the *atrap* mutant upon pathogen attack.

Since we observed increased induction of *RBOHf* in the *atrap* mutant upon infection with *Pst* (*avrRpt2*) (Fig. 1d), we examined whether there is an increase in ROS accumulation in the *atrap* mutant. To detect ROS, DAB staining was performed on leaves of Col-0 WT and the *atrap* mutant plants 6 h post inoculation (hpi) with MgCl$_2$, *Pst* (EV) or *Pst* (*avrRpt2*). DAB staining was visualized as red–brown precipitates. We detected more intense staining in the *atrap* mutant infiltrated with *Pst* (*avrRpt2*) than in the Col-0 WT (Fig. 1e). Our results indicate that *atrap* more sensitively reacts to *Pst* to induce ROS production.

Overexpression of AtRAP results in reduced resistance to *Pst*

The *atrap* mutant is smaller and displays a virescent phenotype (Katiyar-Agarwal *et al.*, 2007). To determine whether the elevated defense responses observed earlier in the *atrap* mutant were due to the secondary effect from its developmental defect, or whether AtRAP is directly involved in plant defense responses, we generated overexpression lines of AtRAP in the *atrap* mutant background. The expression of AtRAP fused with a FLAG tag at the C-terminus was driven by the constitutive 35S promoter. The overexpression lines fully complemented the retarded growth and the virescent phenotype of the *atrap* mutant, and did not show any distinguishable phenotypes when compared with Col-0 WT plants (Fig. 2a). A transgenic line with a high expression level of AtRAP (#34) and a line with a medium expression level of AtRAP (#21) (Fig. 2b) were chosen and subjected to pathogen growth analysis. These overexpression lines exhibited enhanced disease susceptibility to both virulent *Pst* (EV) and avirulent *Pst* (*avrRpt2*) in proportion to the expression levels of AtRAP-FLAG (Fig. 2c,d). Our results suggest that AtRAP directly and negatively regulates plant defense responses. Taken together with our previous study (Katiyar-Agarwal *et al.*, 2006), the downregulation of AtRAP by AtlsiRNA-1-mediated silencing could be a regulatory mechanism to promptly elevate disease resistance in plants upon bacterial infection.

AtRAP interacts with LSU2, a positive regulator of plant resistance

To understand how AtRAP regulates plant immunity, we attempted to identify interacting partner proteins of AtRAP using yeast two-hybrid screening. We screened a total of 1.3×10^6 independent yeast transformants using AtRAP as bait and identified several positive candidates. Further analyses revealed that one of the positive candidates encodes LSU2 (AT5G24660) (Fig. 3a), which is known to be highly expressed under sulfur deprivation

(13.6-fold upregulation) according to the G$_{ENEVESTIGATOR}$ database (Zimmermann *et al.*, 2004).

To confirm the interaction between AtRAP and LSU2, we performed a co-immunoprecipitation assay (Co-IP). AtRAP and LSU2, which were respectively tagged with YFP and FLAG, were coexpressed in *N. benthamiana* leaves and the results shown in Fig. 3(b) confirmed that LSU2 is an interacting partner of AtRAP.

A recent study demonstrated that AtRAP is localized in chloroplast in *N. benthamiana* using a transient expression assay (Kleinknecht *et al.*, 2014). Because LSU2 interacts with AtRAP, we hypothesized that LSU2 is at least partially localized in the chloroplasts and interacts with AtRAP. The advanced protein subcellular localization prediction tool WoLF PSORT also predicted LSU2 to be present in the chloroplast/plastid (http://www.genscript.com/wolf-psort.html). To test our hypothesis, AtRAP tagged with YFP (AtRAP-YFP) at its C-terminal end and LSU2 with a C-terminal CFP (LSU2-CFP) were coexpressed in *N. benthamiana*. As expected, LSU2-CFP co-localized with AtRAP-YFP in the chloroplast (Fig. 3c,d). We then generated transgenic plants using the same construct to express the AtRAP-YFP in the *atrap* mutant background. These transgenic lines complemented the retarded growth and the virescent phenotype of *atrap* (Fig. S2), and showed expression of AtRAP-YFP in the chloroplasts (Fig. 3e). This result further confirmed our finding.

Next, to assess the biological function of LSU2, we examined the loss-of-function mutant *lsu2* in disease resistance to *Pst*. The *lsu2* mutant was developmentally and phenotypically indistinguishable from Col-0 WT plants. Pathogen growth assays revealed that the *lsu2* mutant expresses an enhanced disease susceptibility to both *Pst* (EV) and *Pst* (*avrRpt2*) compared with the Col-0 WT plants (Fig. 4a,b), indicating that LSU2 is a positive regulator of plant defense. Furthermore, the *LSU2* transcript level was slightly upregulated upon *Pst* (*avrRpt2*) infection in Col-0 WT (Fig. 4c), whereas the *atrap* mutant exhibited increased expression of *LSU2* transcripts, and significant upregulation of *LSU2* transcripts upon *Pst* infection (Fig. 4c). To confirm whether expression levels of AtRAP really affect the level of *LSU2* transcripts, we performed Northern blot analysis to examine *LSU2* RNA abundance in the AtRAP overexpression line using Col-0 WT and *atrap-1* as controls. As expected, the *LSU2* transcript level was downregulated in the AtRAP-overexpressed line (Fig. S3), suggesting that AtRAP negatively regulates *LSU2* transcript level.

Since we found that LSU2 interacts with AtRAP (Fig. 3), we examined whether bacterial infection affects the interaction between LSU2 and AtRAP. Because the antibodies specific for LSU2 and AtRAP are not yet available, experiments were performed by transiently expressing LSU2-Flag and AtRAP-YFP in *N. benthamiana* as shown in the Co-IP assay (Fig. 3b). At 2 d after agroinfiltration of AtRAP-YFP and LSU2-Flag, *Pst* (*avrRpt2*) was inoculated into the same leaves. After 1 d, total proteins were extracted from the infiltrated leaves and subjected to Western blot analysis using anti-YFP or anti-Flag antibodies. As expected, expression of AtRAP in *N. benthamiana* did not change significantly upon *Pst* (*avrRpt2*) infection (Fig. 5a) because the 3′-untranslated region which contains the lsiRNA target site is not present in the expression construct. However, a higher accumulation of LSU2-Flag was observed in the leaves inoculated with *Pst* (*avrRpt2*) than in the leaves

inoculated with inoculation buffer (control) (Fig. 5a), which is consistent with the RNA accumulation pattern of LUS2 after infection. To perform Co-IP, AtRAP-YFP and LSU2-Flag were coexpressed in *N. benthamiana* leaves by agroinfiltration. Two days later, *Pst* (*avrRpt2*) was inoculated into the *N. benthamiana* leaves. Total proteins were extracted from the inoculated leaves at 1 dpi and subjected to Co-IP using anti-YFP antibody-conjugated agarose beads. Western blot analyses using anti-YFP and anti-Flag antibodies were performed to examine the co-immunoprecipitated products. Interestingly, the amount of LSU2 which co-immunoprecipitated with AtRAP was significantly decreased in the leaf samples inoculated with *Pst* (*avrRpt2*) (Fig. 5b), although the entire accumulation level of LSU2 was significantly higher than that of the blank control. This result suggested that the physical interaction between AtRAP and LSU2 can be disrupted upon bacterial infection and that this results in release of LSU2, which positively regulates host defense. Conversely, this result suggests that AtRAP plays a regulatory role in suppressing defense responses by inactivating LSU2 through physical interaction.

To validate the antagonistic relationship between AtRAP and LSU2 *in vivo*, we generated the double mutant of *atrap lsu2*. The double mutant still displayed the virescent phenotype as with the *atrap* single mutant (Fig. S4a,b). We then performed pathogen growth assays on *atrap lsu2*. The double mutant became less resistant than the single *atrap* mutant, but less susceptible than the single *lsu2* mutant when challenged with both virulent and avirulent strains (Fig. 5c,d), indicating that AtRAP antagonistically regulates LSU2 by directing interacting with it.

Microarray analysis reveals altered gene expression in the *atrap* mutant

To identify genes that are regulated by *AtRAP*, we performed transcriptome analysis on the *atrap* mutant using Affymetrix ATH1 microarrays. Three biological replicates were analyzed using mRNAs extracted from rosette leaves of 3-wk-old Col-0 WT and the *atrap* mutant plants. The microarray results revealed that a total of 28 genes were expressed with significant differences between the *atrap* mutant and the Col-0 WT (i.e. 21 genes were downregulated and seven genes were upregulated in the *atrap* mutant; Tables S3, S4). Among them, the transcription factor *GLK1* was downregulated in the *atrap* mutant (Table S3). GLK1 is a member of the GARP superfamily (Waters *et al.*, 2008, 2009), and is closely related to another GARP family gene *GLK2*. GLK1 and GLK2 are differentially regulated, showing differential tissue expression patterns (Fitter *et al.*, 2002). Interestingly, the double knockout mutant plant (*glk1 glk2*) shows a pale green leaf phenotype, somewhat similar to that of the *atrap* mutant (Fitter *et al.*, 2002; Waters *et al.*, 2009). Thus, we examined the functional significance of the GLK transcription factors in disease resistance to *Pst*. We first performed Northern blot analysis to confirm the downregulation of the *GLK1* transcript level in the *atrap* mutant observed in the microarray analysis. To determine whether GLK1 is directly regulated by AtRAP or its downregulation in the *atrap* mutant is due to the retrograde signaling from the damaged plastids in the mutant, we examined the expression level of *GLK1* in the AtRAP overexpression lines where there is no obvious plastid damage. We found that GLK1 expression was elevated in the AtRAP overexpression line, suggesting that AtRAP is likely to positively regulate GLK1 directly (Fig. S5), although we cannot rule out the possibility of retrograde signaling regulation after AtRAP expression change.

We also infiltrated Col-0 WT and the *atrap* mutant with either *Pst* (EV) or *Pst* (*avrRpt2*) to examine whether the expression level of *GLK1* transcripts is altered by *Pst* infection. At 1 dpi, total RNAs were extracted from infected leaves and used for Northern blot analysis. Consistent with the microarray analysis, the Northern blot result showed that the *GLK1* transcript level was down-regulated in the *atrap* mutant (Fig. 6b). Interestingly, the *GLK1* transcript level was upregulated upon infection with *Pst* (EV) or *Pst* (*avrRpt2*) in both Col-0 WT and the *atrap* mutant. To assess the biological functions of the GLK1 and GLK2 transcription factors in disease resistance to *Pst*, we performed a pathogen growth assay using the double knockout mutant *glk1 glk2*, because GLK1 and GLK2 are functionally redundant. The analyses revealed that the *glk1 glk2* mutant exhibits enhanced disease resistance to *Pst* (*avrRpt2*) when compared with Col-0 WT (Fig. 6c,d), suggesting that GLKs are negative regulators of plant defense against *Pst* (*avrRpt2*). Consistent results were obtained from three independent experiments.

Since AtRAP has been shown to localize in the chloroplast (Fig. 3), it is noteworthy that, of 28 genes, eight have been annotated to be located in the chloroplast (TAIR; www.arabidopsis.org) (Tables S3, S4): ABC1 (a protein kinase superfamily protein; At5g05200), a dehydrin family protein (At1g54410), an aspartate-glutamate racemase family protein (At1g15410), FDH (an NAD-dependent formate dehydrogenase; At5g14780), MRL1 (a pentatricopeptide repeat protein; At4g34830), an RNA-binding (RRM motif) family protein (At1g70200), an FAD/NAD(P)-binding oxidoreductase family protein (At1g57770) and RNA polymerase beta′ subunit-2 (Atcg00170). These genes have been shown to be intimately involved in chloroplast development and/or metabolic processes (Lange & Ghassemian, 2003; Dal Bosco *et al.*, 2004; Friso *et al.*, 2004; Ytterberg *et al.*, 2006; Johnson *et al.*, 2010; Steiner *et al.*, 2011). Since the *atrap* mutant shows a virescent phenotype, further experiments may reveal how these genes are involved in contributing to its biological phenotype.

Discussion

Through loss- and gain-of-function studies using the knockout mutant and overexpression lines of AtRAP, respectively, we showed that AtRAP functions as a negative regulator of plant innate immunity because alteration of the AtRAP expression affects both compatible and incompatible interactions between *Arabidopsis* and *Pst* bacterial strains. Analyses of transcriptional levels of representative defense-related marker genes suggest that AtRAP is directly involved in suppression of plant immunity under normal growth conditions because the levels of *PR1* and *PR2* were constitutively upregulated in the *atrap* mutant (Fig. 1a,b). In addition, the *atrap* mutant induced more rapid and strong HR cell death upon pathogen infection than seen in the Col-0 WT (Fig. S1b). This seems to be associated with the constitutive accumulation, and sensitive production, of ROS in the *atrap* mutant (Fig. 1e), because ROS play a key role in both triggering HR and inducing defense-related genes (Torres *et al.*, 2006). Both *RBOHd* and *RBOHf* are known to be involved in generating ROS in plant defense (Torres *et al.*, 2002; Miller *et al.*, 2009; Vellosillo *et al.*, 2010). Our transcriptional analyses suggested that *RBOHf*, but not *RBOHd*, is responsible, at least in part, for sensitive production of ROS in the *atrap* mutant upon *Pst* infection (Fig. 1c,d). It has been shown that *RBOHf* is more important for the activation of HR cell death, although

RBOHd contributes to the generation of total detected ROS in *Arabidopsis* (Torres *et al.*, 2002). Transgenic plants that overexpress AtRAP, which have no obvious developmental defects, showed a more susceptible phenotype, further supporting that AtRAP is directly involved in regulating plant immunity.

Using a yeast two-hybrid screening with AtRAP as bait, we identified the AtRAP interacting protein, LSU2. A strong interaction between AtRAP and LSU2 was confirmed by Co-IP (Fig. 3b). LSU2 is known to be strongly expressed under sulfur deprivation (Zimmermann *et al.*, 2004). A recent study showed that LSU2 is highly induced by a combination of light and plastid signaling, suggesting that LSU2 is involved in the optimization of chloroplast function (Ruckle *et al.*, 2012). Chloroplast localization of LSU2 and its association with AtRAP may be associated with the function of LSU2 in chloroplast biogenesis. In this study, analysis of the *lsu2* knockout mutant revealed that LSU2 is involved in disease resistance to both virulent and avirulent strains of *Pst* (EV) and *Pst* (*avrRpt2*), respectively (Fig. 4). While little information is available on the biological function of LSU2 in disease resistance, Mukhtar *et al.* (2011) have shown that LSU2 physically interacts with multiple effector proteins of *Pst*, and with *Hyaloperonospora arabidopsidis* (*Hpa*), and is required for full immune function during *Hpa* infection. Consistent with our results, their preliminary observations suggested that LSU2 is also required for *Pst* bacterial resistance mediated by the RPS2-dependent disease resistance pathway (Mukhtar *et al.*, 2011). It is not clear how LSU2 mediates plant immunity against *Pst* (EV) and *Pst* (*avrRpt2*). Our observation that LSU2 was upregulated upon *Pst* infection at both the RNA and the protein levels (Figs 4c, 5a, S3) supports the role of LSU2 as a positive regulatory component in the surveillance system of plant defense. In addition, upon bacterial infection, the physical interaction between LSU2 and AtRAP was disrupted (Fig. 5b), indicating that an increase of the released LSU2 has a positive effect on plant defense. In this regard, it is likely that the activity of LSU2 as an immune activator may be suppressed by a physical interaction with AtRAP under normal growth conditions because these two proteins have antagonistic effects on plant immunity. This model was also supported by the results from functional characterization of the *atrap lsu* double mutant.

GLK1 and GLK2 are involved in chloroplast development and maintenance, and coordinate the expression of the photosynthetic apparatus (Waters *et al.*, 2008, 2009). In the *atrap* mutant, the *GLK1* transcript level was significantly downregulated (Figs 6b, S5), whereas in the AtRAP overexpression lines, *GLK1* was highly induced (Fig. S5), suggesting that *GLK1* expression is regulated by the AtRAP protein. Furthermore, our microarray results showed that expression levels of some nuclear genes involved in chloroplast development and/or metabolic processes were also significantly decreased in the *atrap* mutant (Table S3). Although it is unknown if GLK1 is a transcriptional activator that directly regulates these genes, the downregulation of those nuclear genes seems to be highly correlated with the biological phenotype of the *atrap* mutant, which is similar to the *glk1 glk2* mutant. Interestingly, the overexpression of GLK1 resulted in significant down-regulation of PR1, but upregulation of PR10, suggesting that GLK1 may negatively modulate the defense signaling pathway (Qiu *et al.*, 2007; Savitch *et al.*, 2007). Our results are consistent with a study by Murmu *et al.* (2014), which indicates that GLK1 negatively regulates plant defense responses to the biotrophic oomycete pathogen *H. arabidopsidis* by activating a JA-

dependent pathway. They and others also showed that GLK1 positively regulates plant defense responses to the necrotrophic fungal pathogens *Botrytis cinerea* and *Fusarium graminearum* (Savitch *et al.*, 2007; Schreiber *et al.*, 2011; Murmu *et al.*, 2014). Thus, GLK1 is a negative regulator of plant immune responses against biotrophs, and a positive regulator of plant defense against necrotrophs.

Chloroplasts are important organelles for plant immunity. Retrograde signaling from chloroplasts regulates expression of various nuclear genes involved in the defense response (Nott *et al.*, 2006). A recent study showed that expression of nuclear encoded chloroplast-targeted genes (NECGs) was significantly altered upon *Pst* infection (Zabala *et al.*, 2015). Thus, some pathogen effectors, such as Hop1J, Hop U1 and HopR1 suppress plant immunity by targeting the chloroplast genes (Fu *et al.*, 2007; Jelenska *et al.*, 2007; Caplan *et al.*, 2008; Zabala *et al.*, 2015). The phenotypic characteristics of the *atrap* mutant, which are virescent and with retarded growth, further support the important role of AtRAP in the regulation of chloroplast functions under normal growth conditions. Accumulating evidence suggests a strong correlation between chloroplasts and the modulation of innate immunity (Wildermuth *et al.*, 2001; Torres *et al.*, 2006; Vlot *et al.*, 2009). The discovery of photosynthesis inhibition by *Pst* infection suggests that comprehensive photosynthesis is essential for the plant defense system (Zabala *et al.*, 2015). The chloroplast is not only a major source of ROS production, but also of antioxidants required to maintain ROS homeostasis (Mittler *et al.*, 2004, 2011). The absence of functional AtRAP in chloroplasts might result in an imbalance of ROS homeostasis in the *atrap* mutant. Our results show that the *atrap* mutant exhibited sensitive induction of HR cell death and ROS accumulation upon pathogen inoculation (Figs 1, S1b). In addition, a recent study demonstrated that AtRAP functions in the maturation of *16S* rRNA (Kleinknecht *et al.*, 2014). Therefore, it is likely that AtRAP plays an essential role in the balancing and coupling of plant immunity and chloroplast functions.

A hypothetical working model for the AtRAP proteinmediated regulation in plant antibacterial immune responses is presented in Fig. S6. Under normal growth conditions in the absence of pathogen infection, the AtRAP protein directly interacts with LSU2 and suppresses LSU2-associated plant defense responses. At the same time, AtRAP is also required for the constitutive expression of GLK1, which is a transcription factor regulating the expression of various genes involved in chloroplast development and stress responses (Fig. S5a). Upon *Pst* infection, AtlsiRNA-1 is highly induced and silences its target gene *AtRAP*. Silencing of *AtRAP* results in the release and functional activation of LSU2, which initiates plant immune responses, as well as modulates chloroplast activities. Silencing of *AtRAP* also alters expression of the GLK1 transcription factor, resulting in the reprogramming of associated gene expression. Eventually, the AtlsiRNA1-induced silencing of *AtRAP* promptly activates defense responses upon pathogen attack. Further characterization of the biological functions of AtRAP and its associated proteins in plant defense and chloroplast function will expand our understanding of how plants fine-tune their signaling regulatory pathways to balance growth and defense.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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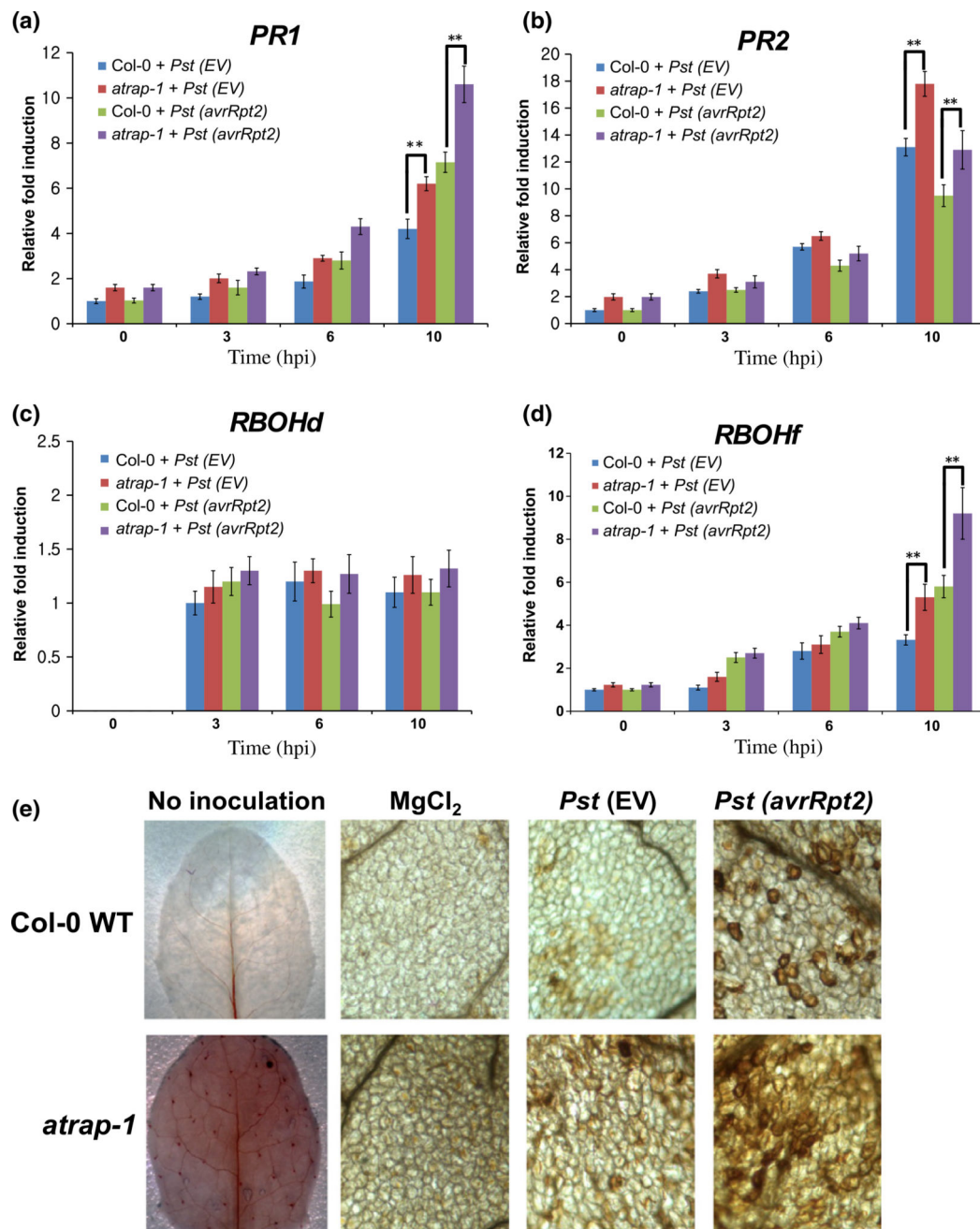
Fig. 1.
Effect of AtRAP knockout mutation on defense responses. Five-week-old *Arabidopsis* Col-0 wild-type (WT) and the *atrap* mutant plants were infiltrated with *Pseudomonas syringae* pv. *tomato* (*Pst*) (EV) or *Pst* (*avrRpt2*) at a concentration of 2×10^7 colony-forming units (CFU) ml^{-1}. Total RNAs were extracted from the infiltrated leaves at the times indicated on the horizontal axes. Relative accumulation levels of (a) *PR1*, (b) *PR2*, (c) *RBOHd* and (d) *RBOHf* transcripts were quantified by quantitative real-time RT-PCR. Error bars represent the ±SEM from three independent experiments. **, $P < 0.01$ (determined by Student's *t*-test). (e) The *atrap-1* mutant exhibits increased ROS accumulation. Five-week-old

Arabidopsis Col-0 WT and the *atrap* mutant plants were infiltrated with $MgCl_2$, *Pst* (EV), or *Pst* (*avrRpt2*) at a concentration of 2×10^7 CFU ml^{-1}. DAB staining was performed to visualize reactive ROS accumulation at 6 hpi.
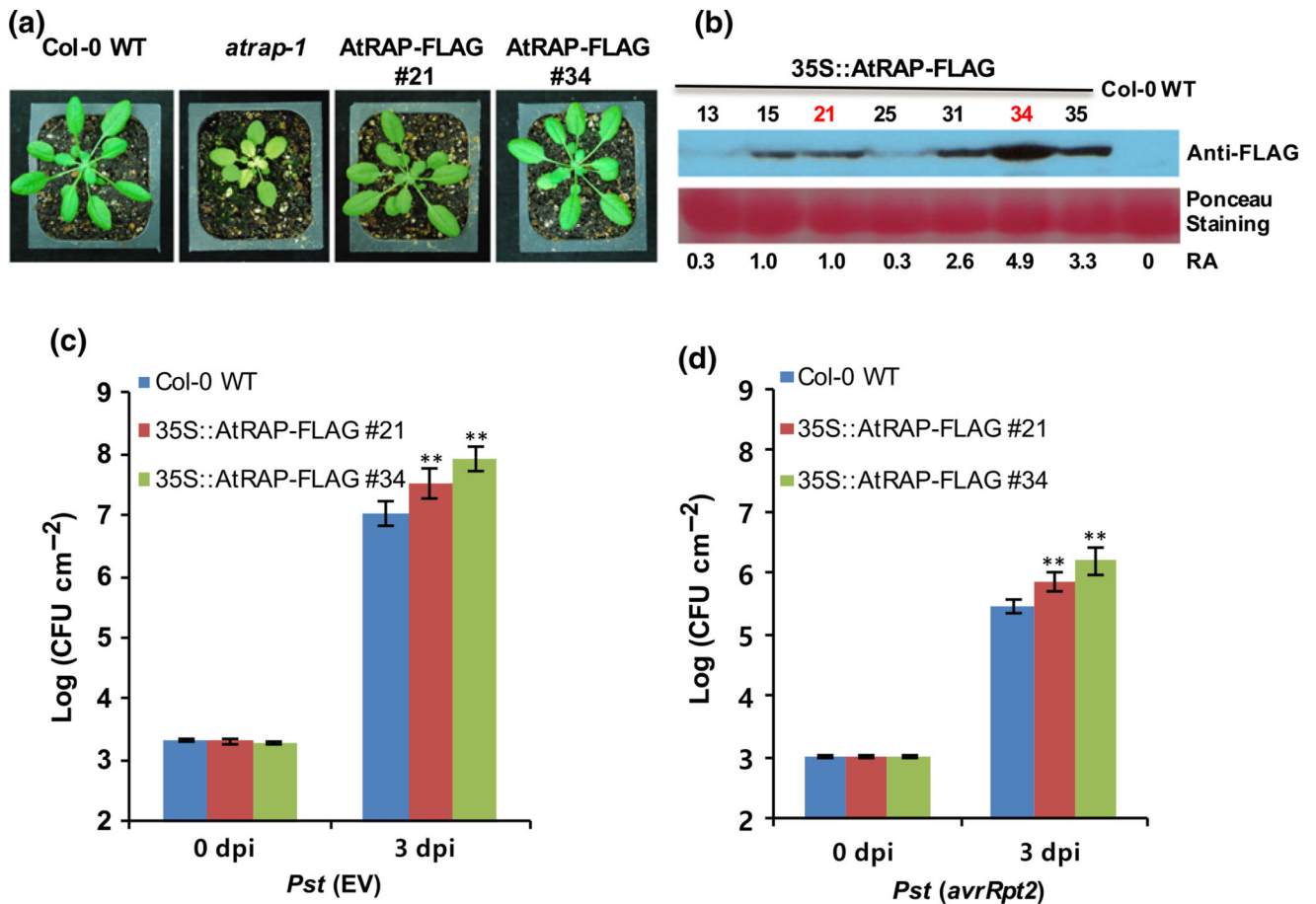
Fig. 2.
Effects of AtRAP overexpression on disease resistance to *Pseudomonas syringae* pv. *tomato* (*Pst*) in *Arabidopsis*. (a) Phenotypes of the AtRAP knockout mutant and overexpression plants. (b) Immuno-detection of AtRAP-FLAG with anti-FLAG antibody in extracts from seven independent AtRAP overexpression lines. (c, d) Growth of *Pst* (EV) and *Pst* (*avrRpt2*) in two independent AtRAP overexpression lines at 0 and 3 dpi. *Arabidopsis* plants were infiltrated with *Pst* (EV) or *Pst* (*avrRpt2*) at a concentration of 2×10^5 CFU ml^{-1}. At least six leaf disks were collected at 0 and 3 dpi by a cock borer. The 0 dpi samples were immediately collected after inoculation. Bacterial titers were measured by grinding, plating, culturing and counting colonies. An ANOVA test was used for significance difference calculation between Col-0 wild-type (WT) and overexpression lines. Error bars represent ±SD of more than six replicates. Similar results were obtained from three independent experiments. **, $P < 0.01$ (determined by Student's *t*-test).
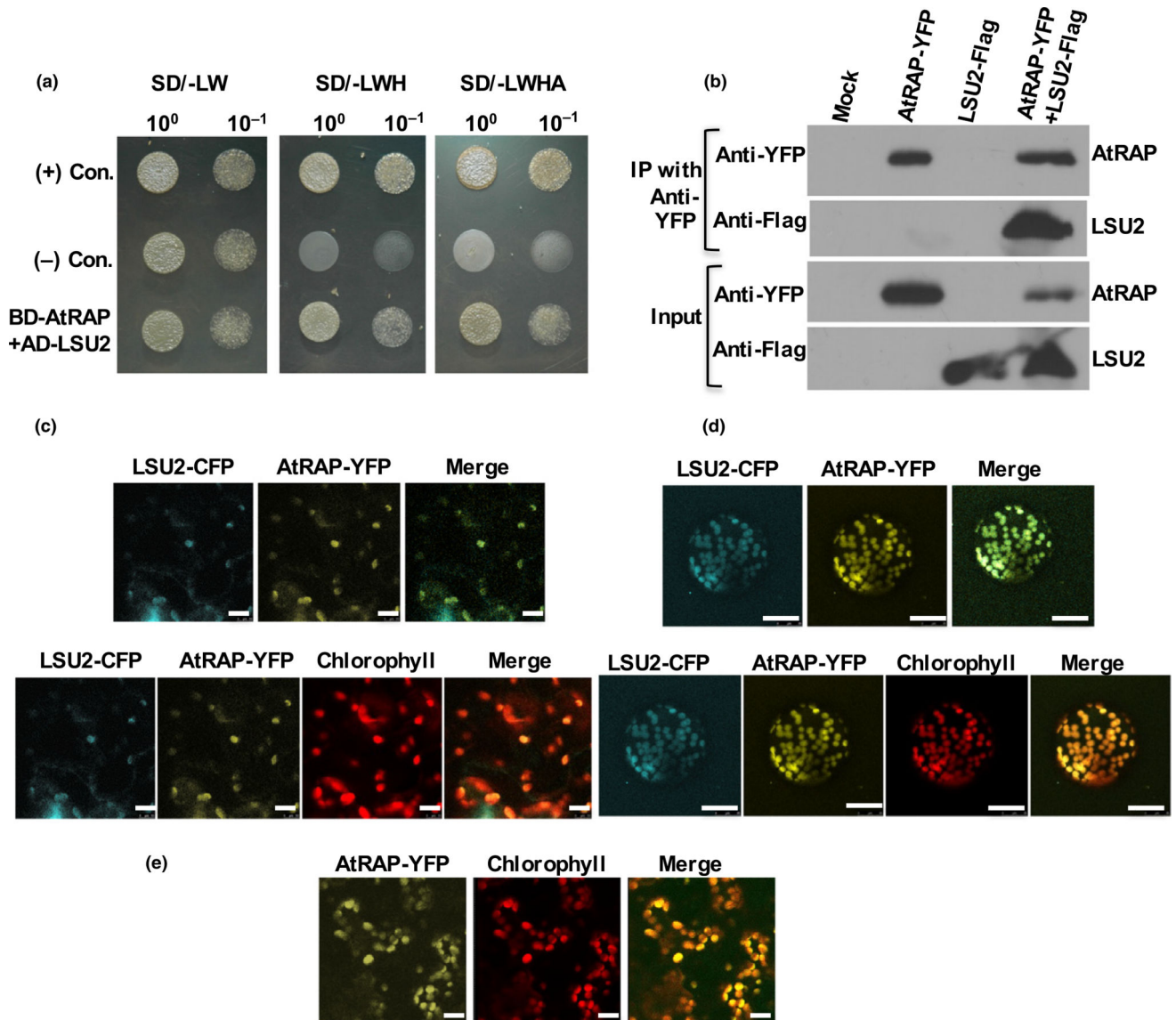
Fig. 3.
AtRAP interacts with LSU2. (a) Interaction between AtRAP and LSU2 in yeast two-hybrid assay. Full-length LSU2 was fused downstream of GAL4-AD in pACT2. Full-length AtRAP was fused downstream of GAL4-BD of pAS2-1. Yeast cells co-transformed with pACT2 and pAS2-1 fusion derivatives were selected on Synthetic Defined (SD) lacking leucine, tryptophan and histidine (SD/-LWH) and SD laking leucine, tryptophan, histidine and adenine (SD/-LWHA) agar media. (b) Co-immunoprecipitation assay in *Nicotiana benthamiana* leaves. AtRAP and LSU2, tagged with YFP and FLAG, respectively, were coexpressed in *N. benthamiana* leaves by agro-infiltration and immunoprecipitated with anti-YFP antibody-conjugated agarose beads. Expression of AtRAP-YFP and LSU2-FLAG in total protein extracts was confirmed by Western blotting using anti-YFP and anti-FLAG antibodies, respectively. Total protein extract from leaves infiltrated with the infiltration buffer was used as a negative control (mock). (c, d) AtRAP is co-localized with LSU2 in chloroplasts. AtRAP and LSU2, which were respectively tagged with YFP and CFP, were

coexpressed in *N. benthamiana* leaves by agro-infiltration. The fluorescence signals of YFP and CFP are shown in the yellow and cyan channels, respectively. Both the (c) infiltrated leaves and (d) protoplasts of *N. benthamiana* were subjected to confocal microscopy. Chloroplasts emit red fluorescence. (e) AtRAP-YFP localizes in chloroplasts from transgenic plants expressing AtRAP-YFP under the 35S promoter. The fluorescence signal of YFP is shown in the yellow channel. Chloroplasts emit red fluorescence. Bars: (c, e) 10 μm; (d) 25 μm.
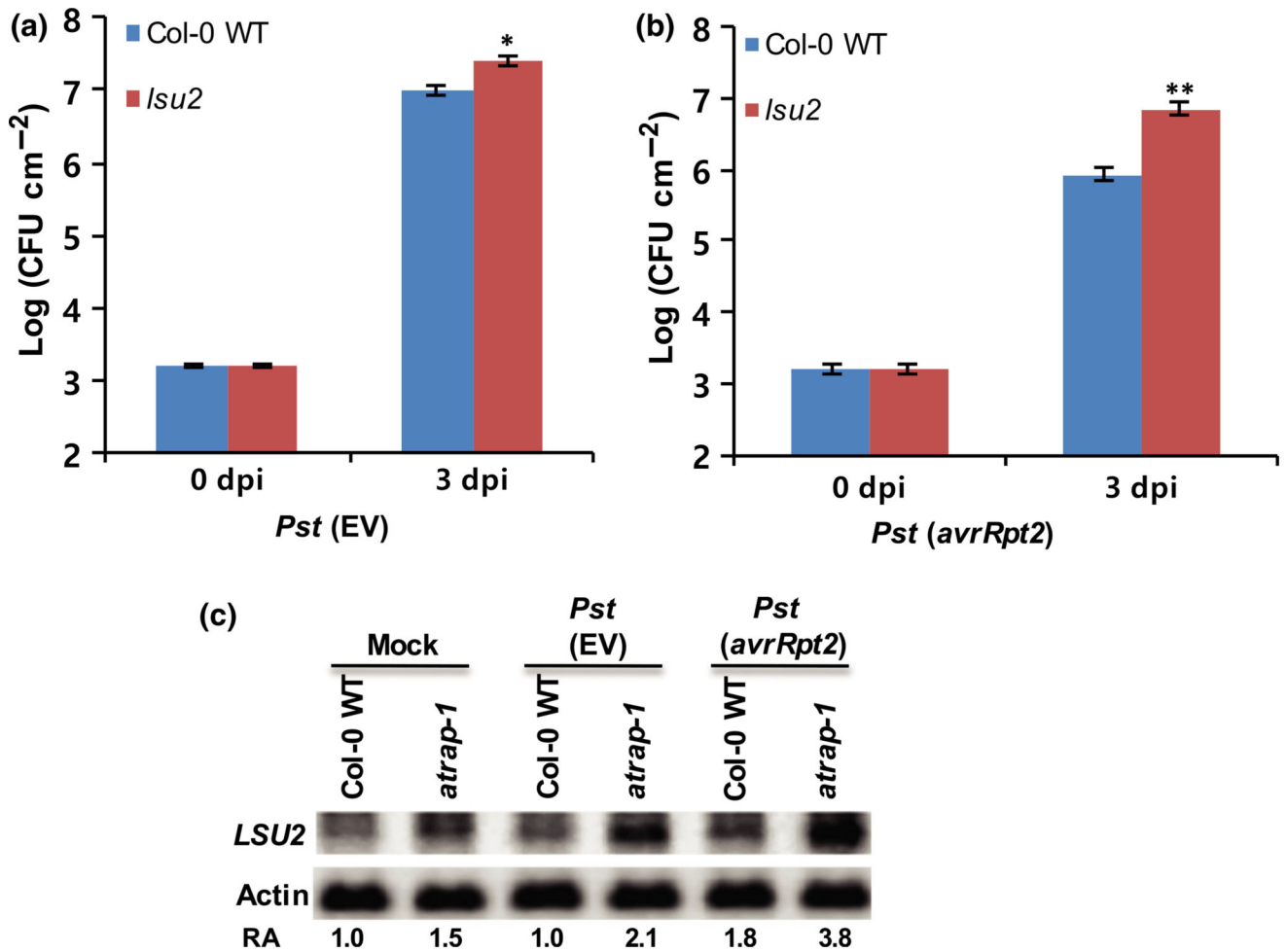
Fig. 4.
LSU2 is a positive regulator of plant defense against *Pseudomonas syringae* pv. *tomato* (*Pst*) in *Arabidopsis*. (a, b) Growth of *Pst* (EV) and *Pst* (*avrRpt2*) in the *lsu2* mutant plants at 0 and 3 dpi. Error bars represent ±SD of more than six replicates. Similar results were obtained from three independent experiments. **, $P < 0.01$; *, $P < 0.05$ (determined by Student's *t*-test). (c) Detection of *LSU2* mRNA in the Col-0 wild-type (WT) and the *atrap* mutant plants. Five-week-old *Arabidopsis* Col-0 WT and the *atrap* mutant plants were infiltrated with *Pst* (EV) or *Pst* (*avrRpt2*) at a concentration of 2×10^7 CFU ml^{-1}. Total RNAs were extracted from the infiltrated leaves at 8 hpi and subjected to Northern blot analysis.
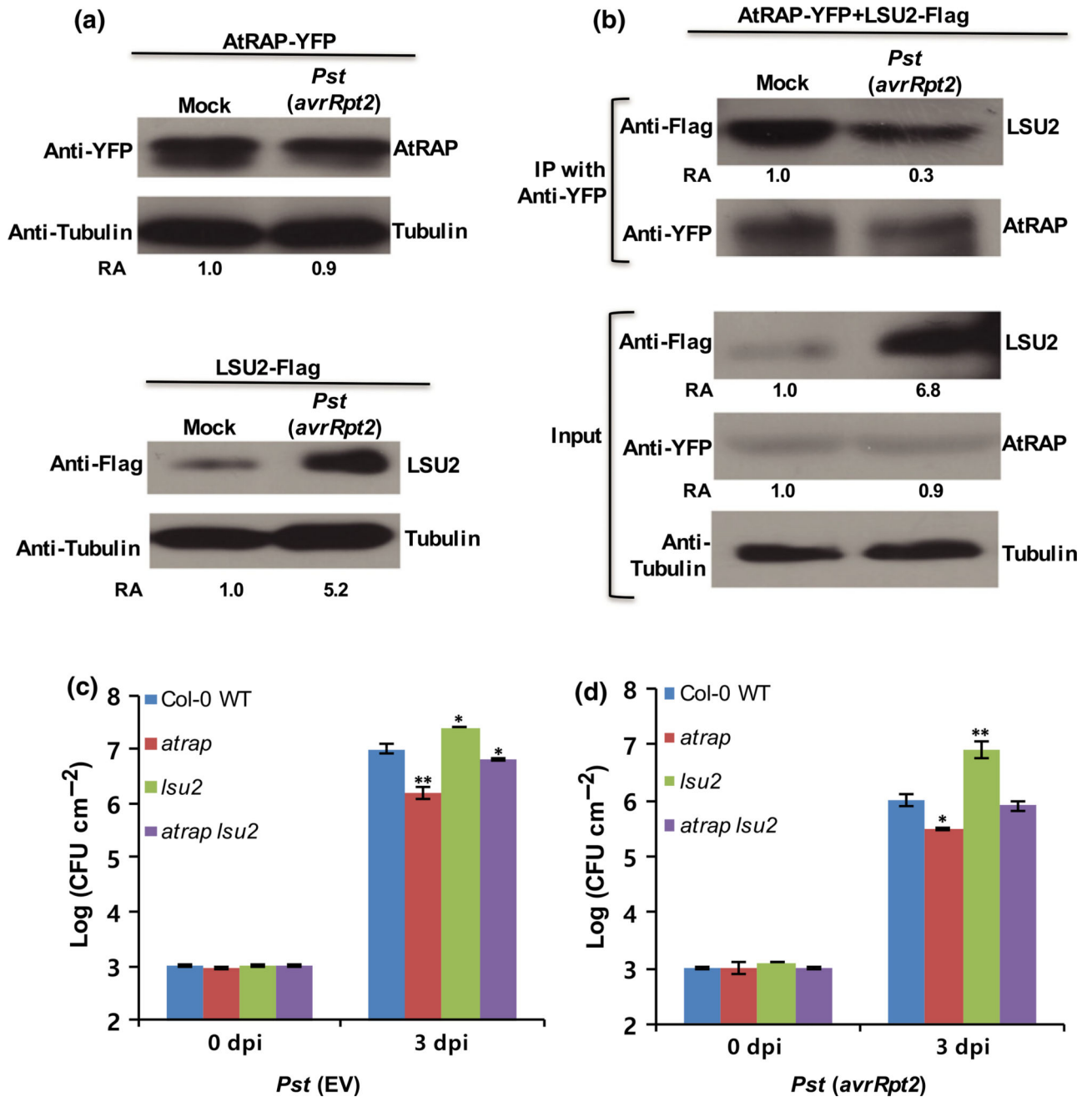
Fig. 5.
Effects of bacterial infection on the interaction between AtRAP and LSU2. (a) AtRAP-YFP and LSU2-Flag were separately expressed in *Nicotiana benthamiana* leaves by agro-infiltration. After 2 d, *Pseudomonas syringae* pv. *tomato* (*Pst*) (*avrRpt2*) was inoculated into the leaves infiltrated with AtRAP-YFP or LSU2-Flag. After 1 d, total proteins were extracted from the infiltrated leaves and subjected to Western blot analysis using anti-YFP or anti-Flag antibodies. (b) Co-immunoprecipitation of AtRAP with LSU2. AtRAP-YFP and LSU2-Flag were coexpressed in *N. benthamiana* leaves by agro-infiltration. After 2 d, *Pst* (*avrRpt2*) was inoculated into the leaves. After 1 d, total proteins were extracted from the

infiltrated leaves and subjected to co-immunoprecipitation using anti-YFP antibody-conjugated agarose beads. Western blot analysis using anti-YFP or anti-Flag antibodies was performed to analyze the resulting co-immunoprecipitated products. (c, d) Growth of *Pst* (EV) and *Pst* (*avrRpt2*) in the *atrap lsu2* mutant plants at 0 and 3 dpi. The *atrap lsu2* double mutant is less resistant than the *atrap* single mutant, and is less susceptible than the *lsu2* single mutant to bacterial infection. Error bars represent ±SD of more than six replicates. Similar results were obtained from three independent experiments. **, $P < 0.01$; *, $P < 0.05$ (determined by Student's *t*-test).
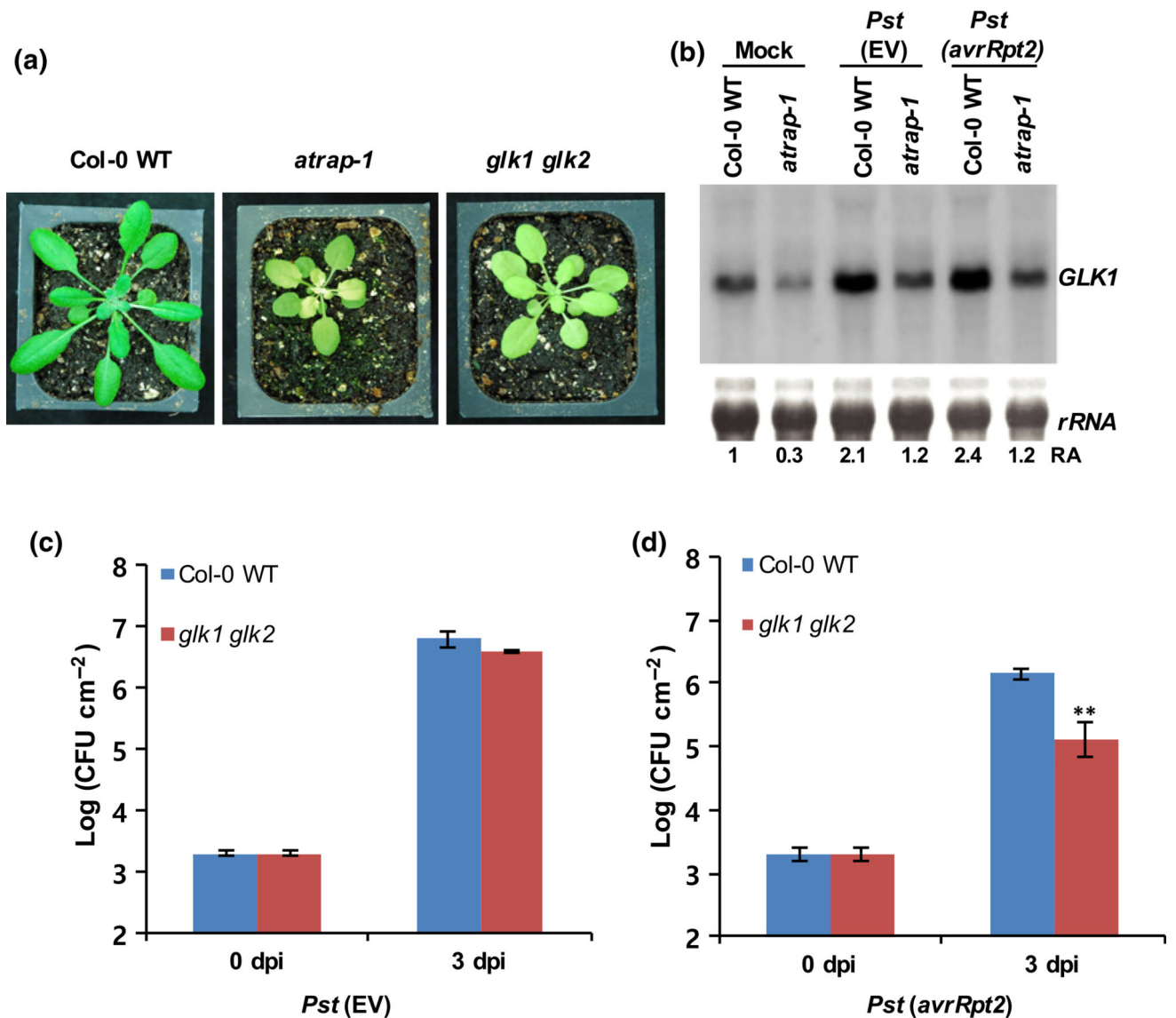
Fig. 6.
GLK1 negatively regulates defense responses in *Arabidopsis*. (a) Phenotypes of the *atrap-1* mutant and the *glk1 glk2* mutant plants. (b) Detection of *GLK1* mRNA in the Col-0 wild-type (WT) and the *atrap* mutant plants. Five-week-old *Arabidopsis* Col-0 WT and the *atrap* mutant plants were infiltrated with *Pst* (EV) or *Pst* (*avrRpt2*) at a concentration of 2×10^7 CFU ml^{-1}. Total RNAs were extracted from the infiltrated leaves at 8 hpi after inoculation and subjected to Northern blot analysis. (c, d) Growth of *Pst* (EV) and *Pst* (*avrRpt2*) in the *glk 1gkl2* mutant plants at 0 and 3 dpi. Error bars represent ±SD of more than six replicates. Similar results were obtained from three independent experiments. **, $P < 0.01$ (determined by Student's *t*-test).