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Characterization of AtRAP Function in Plant Immunity and in RNA Transportation

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Characterization of AtRAP Function in Plant Immunity and in RNA Transportation

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by

Huan Wang

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ABSTRACT OF THE DISSERTATION

Characterization of AtRAP Function in Plant Immunity and in RNA Transportation

by

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Chloroplasts are essential semi-autonomous organelles that regulate energy production, metabolites synthesis, signal transduction, and stress response in plants and algae. They are responsible for photosynthesis, a process that converts carbon dioxide and water to sugars and oxygen, using light to support nearly all organisms on Earth. Despite their functional importance and their ability to perform transcription and translation within themselves, the chloroplast genome size is very small. Consequently, proper function of the chloroplasts largely relies on anterograde signaling from the nucleus. The mechanisms that regulate the import of nucleus-encoded proteins into chloroplasts have been extensively studied. However, much less is known about nucleus-encoded RNAs imported into chloroplasts, and it has been unclear whether chloroplast RNAs are modified for their proper function. In addition to their function in energy production, chloroplasts are also significant in plant defense. Retrograde signaling of the chloroplast can reprogram numerous nucleus-encoded genes which are involved in plant immunity. Chloroplasts are capable of modulating levels of defense-related molecules by controlling photosynthesis.

This project had its beginnings in a study in which our lab discovered the target of a bacterial-induced small RNA: *Arabidopsis thaliana* protein containing an RNA-binding domain abundant in Apicomplexans (AtRAP). Two separate approaches were taken to further study the function of AtRAP:

1) AtRAP was characterized as the target protein of a siRNA induced by bacteria *Pseudomonas syringae* pv. *tomato* (*Pst*) (*avrRpt2*). We show that AtRAP acts as a negative regulator of plant defense by using loss-of-function and gain-of-function analysis. AtRAP functions through direct interaction with Low Sulfur Upregulated 2 (LSU2), a positive regulator of plant defense. AtRAP also regulates transcription factor GOLDEN2-LIKE 1 (GLK1) that is involved in plant biotic and abiotic stress responses. Thus, this approach aims to study the functional mechanism of AtRAP in plant immunity.

2) AtRAP was characterized as a chloroplast-localized RNA-binding protein. By characterizing AtRAP-associated RNAs, we discovered a group of nucleus-encoded RNAs, which are translocated into chloroplasts. Further structural analysis suggests that many of these AtRAP-bound chloroplast-localized nuclear RNAs are small nucleolar RNAs (snoRNAs). These nucleus-encoded snoRNAs are imported into chloroplasts, where they methylate chloroplast-encoded rRNAs and mRNAs. Thus, this approach aims to study the translocation and function of nucleus-encoded RNAs, mainly snoRNAs, inside chloroplasts.

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CHAPTER 1

General Introduction

Chloroplast Functions

Eukaryotic cells rely on intracellular organelles for cell integrity and survival. Chloroplasts, which are semi-autonomous organelles, originated from cyanobacteria approximately 1 to 1.5 billion years ago and have evolved to support global ecosystems (Waters and Langdale, 2009). Chloroplasts are responsible for photosynthesis, a process that comprises of light-dependent and light-independent reactions. During light-dependent reactions, which occur on the thylakoid membrane, light activates the electron transport chain, in which electrons flow from photosystem I (PSI) to photosystem II (PSII). ATP and oxygen are generated in this process. During light-independent reactions, which occur on chloroplast stroma, CO_2 is converted into glucose, which is transported to the cytosol or stored as starch using the chemical energy produced during light-dependent reactions. In essence, the chloroplast is a crucial organelle which can convert solar energy into chemical energy while simultaneously maintaining environmental oxygen levels that are vital for nearly all organisms on Earth (Eberhard et al., 2008).

Besides photosynthesis, chloroplasts are also crucial for fatty acid synthesis, nitrate assimilation, and amino-acid synthesis (Jarvis and Lopez-Juez, 2013). Fatty acid synthesis is very restricted in plastids. Many intermediates which are purified from plastids can support fatty acid synthesis. For example, acetate is capable of entering the stroma of chloroplasts and participating fatty acid *de novo* synthesis by acting as a primary carbon source (Lopez-Juez and Pyke, 2005; Preiss et al., 1993). Although the beginning steps of nitrate assimilation is in the cytosol, the remaining steps all occur in plastids, which include green tissue (chloroplasts) as well as non-green tissue (Neuhaus and Emes, 2000).

Chloroplasts also play essential roles in plant defense. Photosynthesis can be either increased by plants to synthesize defense-related molecules and energy, by pathogens to synthesize carbon compounds to feed pathogens; or suppressed by plants to limit carbon availability for pathogens to defense over pathogen infection (Chen et al., 2010; Eom et al., 2015; Swarbrick et al., 2006). Chloroplast retrograde signaling, which refers to the singling from chloroplast to nucleus, regulates lots of nucleus-encoded genes which are involved in plant immunity (Nott et al., 2006). The nucleus-encoded chloroplast-targeted genes (NECGs) were significantly reprogrammed during bacterial *Pseudomonas syringae* infection in *Arabidopsis*. The effectors from bacterial disrupted chloroplast photosystem II by inhibiting chloroplast CO_2 assimilation (Zabala et al., 2015). Chloroplasts are involved to synthesize a series plant immunity mediators, such as reactive oxygen species (ROS), salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA) (Nambara and Marion-Poll, 2005; Stael et al., 2015; Torres et al., 2006; Wasternack and Hause, 2013; Wildermuth et al., 2001).

Chloroplast Genome

Despite its importance in the cell, the chloroplast has a genome that is small in size and limited in function. Chloroplast genomes range from 120 to 160 Kbp. The chloroplast genome in higher plants is remarkably conserved, and large inverted repeats are present as a physical peculiarity. Chloroplast genomes usually contain 120 to 135 genes and are organized in operons. The *Arabidopsis thaliana* genome size is 154 Kbp and includes 130 genes. There are 31 genes which are in charge of transcription or translation: 26 encode ribosomal proteins (*rps2-rps19*; *rpl2-rpl36*), 4 encode RNA polymerase subunits (*rpoA*, *B*, *C1*, *C2*), and 1 encodes a putative intron splicing protein (*matK*). There are 45 genes which encode functional RNAs: 37 are tRNAs (*trnA-trnV*) and 8 are ribosomal RNAs (*rrn4.3-rrn23*). There are 35 genes which encode components of complexes involved in photosynthesis: 15 encode photosystem II or photosystem II assembly proteins (*psbA-psbI*; *ycf9*), 7 encode photosystem I or photosystem I assembly proteins (*psaA-psaJ*; *ycf3*; *ycf4*), 5 encode cytochrome b_6/f or cytochrome b_6/f assembly proteins (*petA-petG*; *ORF31*), 7 encode subunits of the plastid ATP synthase complex (*atpA-atpI*), and 1 encodes rubisco large subunit (*rbcL*). The remaining 19 genes are involved in NADH complex, proteolysis, and lipid biosynthesis (Sugiura, 1992).

Protein Transport Pathways into Chloroplasts

Chloroplasts are believed to have arisen from an endosymbiotic event involving ancestors of cyanobacteria. After endosymbiosis, most of the bacterial genomic circular DNA and associated genes were lost or transferred to the nucleus to benefit the endosymbiosis. Then, in order to import the host-encoded bacterial protein back in, they established two complexes in the envelope which are called Translocon at the Outer envelope membrane of Chloroplasts (TOC) and Translocon at the Inner envelope membrane of Chloroplasts (TIC).

Due to its limited size, the chloroplast genome synthesizes less than 100 proteins. Compared to that, the chloroplast requires approximately 3000 proteins for its structure and function. Therefore, anterograde import and signaling from the nucleus are essential to the chloroplast. More than 95% of chloroplast proteins are encoded in the nucleus, synthesized in the cytosol, and transported to the chloroplast. These chloroplast-localized proteins usually contain N-terminal signaling peptides that can be recognized by the Toc-Tic system, which directs proteins to chloroplasts and their final destinations inside of chloroplasts. When protein precursors are imported into chloroplasts by Toc-Tic components, the N-terminal signaling peptide is cleaved by stroma processing peptidase (SPP). The Toc-Tic system can be divided into two parts: the elements which were originated from prokaryotic endosymbionts are Toc75, Tic20-22, Tic32, Tic55, and Tic62; the components which were originated from the eukaryotic host are Toc34, Toc64, Toc159, Tic40, and Tic 110. These elements were discovered mainly from *Pisum sativum* (pea). The core proteins of the Toc complex are Toc75, a β-barrel structured transporting channel protein, and Toc159 and Toc 34, two GTPases acting as receptor proteins. The core proteins of the Tic complex are Tic20, Tic22, Tic40, and Tic110. Tic20 is predicted to span the inner envelope membrane four times and has an exposed N-termini and C-termini. Tic22 is a scaffold between the Toc and Tic complexes and is located on the outer face of the chloroplast envelope inner membrane. Tic40 is in charge of the Tic complex chaperone activity. Tic110 contains a helix-repeat domain which is in rod shape into stroma (Kessler and Schnell, 2009; Kikuchi et al., 2013; Li and Chiu, 2010; Schleiff and Becker, 2011; Shi and Theg, 2013).

Non-Canonical Protein Transport Pathways into Chloroplasts

Although the Toc-Tic pathway is the main machinery for protein import to the chloroplast, several non-canonical transport pathways have been discovered. Both the N-terminal peptide and Toc-Tic mechanism are not essential for the localization of Quinone Oxidoreductase Homologue (ceQORH), a chloroplast envelope protein (Miras et al., 2007). The import of ceQORH requires a high concentration of ATP and proteinaceous receptor components which are chloroplast surface-exposed outer envelope proteins. Tic32 also lacks a chloroplast transit peptide and can travel into the chloroplast without Toc-Tic elements in low ATP conditions (Jarvis, 2008; Miras et al., 2007; Nada and Soll, 2004).

RNA Transport into Organelles (Mammalian Mitochondria)

Compared with the well-studied protein trafficking pathway from the nucleus to the chloroplast, very little is known about RNA trafficking into chloroplasts. The prominent example of anterograde RNA import is found in the mammalian mitochondrial system which is mediated by Polynucleotide Phosphorylase (PNPase), an exoribonuclease and poly-A polymerase localized in the mammalian mitochondrial intermembrane space. Reduction of PNPase results in impaired RNA processing and accumulated polycistronic transcripts in mitochondria. PNPase trans-locates nucleus-encoded RNase P, 5S rRNA, and

MRP RNA into mitochondria. After incubation of mitochondria with these nucleus-encoded radioisotope-labeled RNAs, nuclease was applied to remove any membrane attached RNAs. The RNAs within the mitochondria were extracted and fractionated by electrophoresis. The labeled nucleus-encoded RNAs were present in WT yet significantly reduced in *the pnpase* mutant. PNPase RNA processing and RNA import are separate activities (Wang et al., 2010). PNPase recognizes a stem-loop RNA structure originating from RNase P RNA that is described as a mediator of PNPase-dependent RNA import. Myoclonic epilepsy with ragged red fibers (MERRF) and mitochondrial encephalomyopath, lactic acidosis, and strokelike episodes (MELAS) are human diseases caused by mutations of mitochondrial $tRNA^{Lys}$ and $tRNA^{Leu}$, respectively. Fusing correct WT tRNAs of $tRNA^{Lys}$ / $tRNA^{Leu}$ with a stem-loop sequence resulted in import into mitochondrial and partially rescued the mitochondrial defects. The successful import includes a stem-loop sequence for tRNA transporting into mitochondria; a 3' UTR sequence from human *MRPS12*, which encodes mitochondrial ribosomal protein S12, for tRNA transporting from nucleus to mitochondrial outer membrane; an extended paired aminoacyl stem for reduction of tRNA stem-loop sequence processing in the nucleus. (Wang et al., 2012).

Several nucleus-encoded tRNAs have also been discovered in mammalian mitochondria. RNA Import Complex (RIC) was isolated from the mitochondrial inner membrane of *Leishmania tropica*, a species of trypanosome. Correct cytosolic $tRNA^{Lys}$ could be imported in the MERRF mitochondria to rescue its respiratory defects with the help of RIC from *in vitro* assay. The accumulation of cytosolic $tRNA^{Lys}$ in mitochondria correlated well with the accumulation of RIC incubated amount. The cytosolic tRNA^{Lys} in mitochondrial was dramatically reduced when RIC was changed to RIC-D, which disarmed the RIC binding site to tRNA^{Lys}. There were several other cytosolic tRNAs (tRNA^{Gln}, tRNA^{Phe}, tRNA^{Leu}) which can travel into MERRF mitochondria treated with RIC (Mahata et al., 2006). Additionally, nucleus-encoded tRNA^{Gln} could be detected in both rat mitochondria and human mitochondria *in vivo*. *In vitro*, the augmentation of imported tRNA^{Gln} correlated well with augmentation of incubated tRNA^{Gln} and ATP concentration. Valinomycin and nigericin were applied to reduce mitochondrial membrane potential and the pH, while the imported tRNA^{Gln} was unaffected. Carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), which is an oxidative phosphorylation inhibitor, was used to reduce the mitochondrial electrochemical potential, while the imported tRNA^{Gln} was unaffected. The mitochondrial membrane potential, pH, and electrochemical potential are essential for protein trafficking into mitochondria. Thus, tRNA^{Gln} is imported into mitochondria by a different mechanism compared with protein import (Rubio et al., 2008).

RNA Transport into Organelles (Plant Mitochondria)

In plants, the transportation of RNA into mitochondria has also been noted. Potato transgenic plants expressing a bean nuclear tRNA^{Gln} vector had bean nuclear tRNA^{Gln} in both the cytosol and mitochondria. When a 4 bp sequence was inserted into the anticodon loop of bean tRNA^{Gln} bean and transformed into potato, it was detected in mitochondria as well. This suggests that foreign RNA may be engineered into mitochondria by the tRNA^{Gln} import system (Small et al., 1992). Another study discovered that cytosolic tRNA$^{Gly}_{(UCC)}$

was present in potato mitochondria. The cytosolic $tRNA^{Gly}_{(CCC)}$ was also discovered in potato mitochondria, although at a lower amount than $tRNA^{Gly}_{(UCC)}$. However, the cytosolic $tRNA^{Gly}_{(GCC)}$ was not detected in potato mitochondria. This suggests that the $tRNA^{Gly}$ travels into mitochondria through a unique selective mechanism (Brubacher-Kauffmann et al., 1999). A similar result was found in tobacco. Arabidopsis nucleus-encoded $tRNA^{Val}$ was transformed into tobacco cells. The Arabidopsis nucleus-encoded cytosol-localized $tRNA^{Met}$ and mitochondria-encoded $tRNA^{Ser}$ were transformed into tobacco cells as negative and positive controls, respectively. The $tRNA^{Val}$ could be detected in tobacco mitochondria. When $tRNA^{Val}$ anticodon was mutated to be similar with cytosol-localized $tRNA^{Met}$, the mutated $tRNA^{Val}$ could not be detected in mitochondria. When the $tRNA^{Val}$ D domain was mutated to be similar with cytosol-localized $tRNA^{Met}$, the mutated $tRNA^{Val}$ could not be detected in mitochondria. Thus, both the tRNA anticodon and D-domain are required for important into mitochondria (Delage et al., 2003). The nucleus-encoded mitochondria-localized $tRNA^{Ala}$ in potato was found to interact with mitochondria voltage-dependent anion channel (VDAC) *in vitro*. The imported $tRNA^{Ala}$ could be abolished by adding antibody of VDAC protein (anti-VDAC) or ruthenium red (RuR) which can close VDACs. This indicates that VDAC is required for tRNA import into mitochondria in plants. Additionally, The imported $tRNA^{Ala}$ ould be abolished by adding antibodies to the translocase of the outer mitochondrial membrane 20 (TOM20) or TOM40. However, the $tRNA^{Ala}$ import assay was not affected by adding peptide pF1b, which can inhibit various protein import into mitochondria. Thus, tRNA import into mitochondria require protein import components (TOM20/TOM40), but the import mechanism is distinct from protein import (Salinas et al., 2006).

RNA Transport into Organelles (Plant Chloroplast)

RNA anterograde trafficking is significantly less well studied in chloroplasts. The relevant studies have been in viroid-infected plants. Viroids from the *Avsunviroidae* family contain major functional noncoding RNAs (ncRNAs) that have been reported to be taken up by the chloroplast (Ding, 2009; Flores et al., 2005). Eggplant latent viroid (ELVd), which was previously discovered to travel into the chloroplast, was engineered as a 5' UTR region fused with a GFP mRNA sequence and transiently expressed in tobacco. Results showed that the GFP protein was expressed in chloroplasts. This trafficking process is proved to be a nuclear-dependent step (Gomez and Pallas, 2010, 2012). *Epifagus virginiana*, which is a root holoparasitic plant, possesses small chloroplasts lacking thylakoids. 13 of the 30 tRNAs are missing in the *E. virginiana* chloroplast genome. However, the chloroplast genes are still transcribed, suggesting the possibility that necessary tRNAs are imported into chloroplasts (Bungard, 2004). So far, the only reported plant nucleus-encoded RNA detected in the chloroplast is the *eukaryotic translation initiation factor 4E* (*eIF4E)*. However, the transport mechanism and the function of the *eIF4E* inside the chloroplast are still unclear, the eIF4E-GFP protein was not expressed in chloroplasts (Nicolai et al., 2007). Additionally, there was nuclear contamination in isolated chloroplast RNA. The nucleus-encoded *28S rRNA* and *18S rRNA* both were detected from ethidium bromide-stained gel of chloroplast RNA. The nucleus-encoded 28S *rRNA* was detected by Northern blot analysis.

SnoRNAs and SnoRNA Trafficking

Small nucleolar RNAs (SnoRNAs) are one of the most ancient non-coding RNAs with diverse functions and a range of sizes (Kiss, 2002). They are essential for RNA modification (Abel et al., 2014; Bratkovic and Rogelj, 2014; Makarova et al., 2013). SnoRNAs are divided into two classes based on their conserved nucleotides and secondary structures: 1) box C/D snoRNAs, which direct 2'-*O*-ribose methylation, and 2) box H/ACA snoRNAs, which direct pseudouridylation.

SnoRNA trafficking is poorly understood even in the nucleus itself. By definition, the localization of snoRNAs is in nucleolus, where the ribosomes are synthesized and assembled. The box C/D motif is sufficient for nucleolus localization (Lange et al., 1998; Narayanan et al., 1999; Samarsky et al., 1998). In vertebrates, the box C/D motif and box H/ACA motifs are also sufficient to direct RNA into Cajal bodies (Samarsky et al., 1998). The Cajal body is a nuclear compartment close to nucleoli and contains snoRNA and small nucleolar ribonucleoprotein (snoRNP) (Gall, 2000; Lange et al., 1998; Narayanan et al., 1999). snoRNAs localize with their associated proteins; however, the trafficking mechanisms are not clear. In mammalian systems, box C/D snoRNAs form snoRNP particles, which are involved in a complex intranuclear trafficking process (Kiss et al., 2006; Verheggen et al., 2001). However, snoRNA trafficking outside nucleus has never been reported.

AtRAP Protein Background

Previously, our lab discovered a class of long small interfering RNAs (lsiRNAs) which are 30–40-nt in length and are induced by bacterial infection or specific developmental conditions. AtlsiRNA-1 is one of the lsiRNAs induced by bacterial *Pseudomonas syringae* carrying effector *avrRpt2*. AtlsiRNA-1 targets the *Arabidopsis thaliana protein containing an RNA-binding domain abundant in Apicomplexans* (*AtRAP*) gene (Katiyar-Agarwal et al., 2007). AtRAP processes a predicted 78 amino acid (AA) chloroplast-transit signaling peptide (1-78 AA) (Emanuelsson et al., 1999), a putative RNA-binding domain abundant in Apicomplexans (RAP) (607–665 AA) (Lee and Hong, 2004), and four Octotricopeptide Repeats (OPRs) (333–370 AA, 372–409 AA, 409–446 AA, and 476–513 AA) (Kleinknecht et al., 2014).

The N-terminal chloroplast-transit peptide is essential for nucleus-encoded protein import into chloroplast (Bruce, 2000; Lee et al., 2008). By dissecting the transit sequence, Becker, et al. discovered that the transit peptide is composed of multiple different domains which are involved in interacting with imported components. The N-terminal transit peptide of the preprotein small subunit of Rubisco (pSSU) interacts with Toc-Tic component Toc159 (Becker et al., 2004). A biochemical study found that the chloroplast transit peptide forms α-helical structures which may associate with the chloroplast envelope membrane (Wienk et al., 1999; Wienk et al., 2000). Other studies, which took a bioinformatics approach, found that chloroplast transit peptides lack acidic amino acids and are rich in hydroxylated amino acids (Bhushan et al., 2006; Zhang and Glaser, 2002).

The RAP (RNA-binding domain abundant in Apicomplexans) domain is named due to the overrepresentation in Apicomplexans (*Toxoplasma gondii* and *Toxoplasma annulata*) and the inferred RNA-binding ability. There are 15 RAP-domain containing proteins in *T. gondii* and 9 RAP-domain containing proteins in *T. annulata*. The RAP domain comprises of blocks of charged and aromatic residues and is predicted to form α-helical and β-strand structures. It is highly conserved from green algae to land plants, such as *Arabidopsis thaliana* and *Oryza sativa*, as well as from Apicomplexans to flies and mammals, such as *Plasmodium falciparum*, *Drosophila melanogaster*, *Mus musculus*, and *Homo sapiens* (Lee and Hong, 2004). Raa3, which is a nucleus-encoded and chloroplast-localized protein in green algae *Chlamydomonas reinhardtii* and contains a RAP-domain at its C-terminus, is required for *trans*-splicing of *psaA* disparate transcripts. The *psaA* encodes a protein of photosystem I and needs *tscA* (a small RNA in chloroplast) to splice its introns. Raa3 specifically binds to *tscA* RNA to participate in a ribonucleoprotein complex to *trans*-splice *psaA* disparate transcripts. The trans-splicing function is Raa3 C-terminal dependent. The C-terminus includes a RAP domain and a pyridoxamine 5'-phosphate oxidase (PDX) sequence (Rivier et al., 2001). PDX is not responsible for RNA binding, the PDX-like sequence is not vital for *trans*-splicing. Thus, it suggests that the binding site of Raa3 is the RAP domain (Lee and Hong, 2004). Another RAP domain-containing protein is Fas-activated serine/threonine phosphoprotein (FAST) which is located on the mitochondrial outer membrane in *H. sapiens*. FAST promotes the inclusion of exon IIIb of fibroblast growth factor receptor 2 (*FGFR2*) mRNA. *FGFR2* is well known to be alternatively spliced to include one of the exons (exon IIIb or IIIc). FAST targets a

U-rich intronic sequence (IAS1) which is adjacent to the 5' exon IIIb splicing site, so that FAST can regulate FGFR2 transcripts splicing (Simarro et al., 2007).

It is postulated that OPRs, which are composed of tandem repeated amino acids units, can form α-helical RNA-binding domains. OPRs have been discovered in green algae to land plants: such as *Chlamydomonas reinhardtii*, *Arabidopsis thaliana*, *Nicotiana tabacum*, *Oryza sativa*, *Zea mays*, and *Physcomitrella patens* (Eberhard et al., 2011; Kleinknecht et al., 2014; Rahire et al., 2012). Almost all the characterized OPR-containing proteins function in RNA processing, RNA stabilization, and translation. TDA1, which belongs to chloroplast T factors in *C. reinhardtii*, is encoded by the nucleus and functions in chloroplast *atpA* translation. *atpA* encodes an ATP synthase α subunit, and the T factors are a set of nucleus-encoded proteins which are required for chloroplast mRNA translation. TDA1 is characterized to have 8 OPRs at the C-terminus (Eberhard et al., 2011). Tab1 is another T factor and contains 10 OPRs. These OPRs participate in the association of Tab1 with the 5'UTR region of *psaB* mRNA. PsaB is important in PSI assembly since it serves as an anchor protein. The Chloroplast transgene of the 5'UTR *psaB* mRNA was fused to a reporter gene. The reporter gene was expressed in WT but not in the *tab1* mutant. This suggests that Tab1 specifically affects the translation initiation of *psaB* mRNA (Rahire et al., 2012; Stampacchia et al., 1997). Since OPR is represented more in unicellular organisms and bacteria and is rare in land plants, and the RAP domain is highly conserved in green algae and land plants, we will mainly characterize RAP domain in the dissertation.

Based on the introduction above, our study of AtRAP is separated into two parts: the first approach uncovers the function of AtRAP in plant immunity as a small RNA target protein. The second approach uncovers the fundamental function of AtRAP as a putative chloroplast-localized RNA-binding protein.

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CHAPTER 2

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*

Abstract

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 demonstrated 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 down-regulation 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* (*Pst*), which is consistent with a previous study showing enhanced resistance of *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.

Key words: AtRAP, LSU2, GLK1, *glk1 glk2*, Plant defense

Introduction

Plants have evolved complex immune systems to defend against pathogens (Dangl et al., 2013; Dodds and Rathjen, 2010; Schwessinger and Ronald, 2012). 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; Dangl et al., 2013; Dodds and Rathjen, 2010; Jones and Dangl, 2006; Schreiber et al., 2011). 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 and 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 and 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 (Katiyar-Agarwal and Jin, 2010; Ruiz-Ferrer and Voinnet, 2009; Seo et al., 2013). sRNAs are short, non-coding RNA molecules that guide transcriptional and posttranscriptional 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 small RNA pathway components are specifically induced by infections of different pathogens, and regulate host PTI and/or ETI pathways, as well as pathogen virulence (Niu et al., 2016; Ruiz-Ferrer and Voinnet, 2009; Weiberg et al., 2014; Weiberg et al., 2013; Zhang et al., 2011). 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 to the wild type, including the transcription factor GOLDEN2-LIKE 1 (GLK1), another negative regulator of plant defense.

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 hours post infection (hpi) with both *Pst* (EV) and *Pst* (*avrRpt2*) strains (Fig. 2.1). The *PR1* and *PR2* transcript levels were also slightly higher in the *atrap* mutant at 0 hpi than in the Col-0 WT plants (Fig. 2.1a, 2.1b), suggesting that the *atrap* mutant has elevated basal defense level. Since *PR1* and *PR2* are two SA-dependent marker genes and SA pathway is antagonistic to JA pathway (Anderson et al., 2004; Kunkel and Brooks, 2002), we next examined the expression level of JA-responsive marker gene: *PDF1.2*. As shown in supporting information Fig.2.2a, the expression of *PDF1.2* transcripts was significantly suppressed in the *atrap* mutant compared to Col-0 WT plants before and after infection.

Pst (*avrRpt2*) induces the hypersensitive response (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. 2.2b). During the HR progression, the production of reactive oxygen species (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 (Miller et al., 2009; Torres et al., 2002; 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. 2.1c, 2.1d). This suggests that *RBOHf* may play a more important role than *RBOHd* in regulating the 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. 2.1d), we sought to examine whether there is an increase of the

ROS accumulation in the *atrap* mutant. To detect ROS level, DAB staining was performed on leaves of Col-0 WT and the *atrap* mutant plants 6 hours 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. 2.1e). 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 virescent phenotype (Katiyar-Agarwal et al., 2007). To determine whether the elevated defense responses observed above in the *atrap* mutant is due to the secondary effect from its developmental defect, or 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 to Col-0 WT plants (Fig. 2.3a). A transgenic line with a high expression level of AtRAP (#34) and a line with a medium expression level of AtRAP (#21) (Fig. 2.3b) 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. 2.3c, 2.3d). Our results suggest that AtRAP directly and negatively regulates plant defense responses. Taken together with our previous study (Katiyar-Agarwal et al., 2006), the down-regulation 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 which is 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. 2.4a), which is known to be highly expressed under sulfur deprivation (13.6-fold up-regulation) according to the GENEVESTIGATOR 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 co-expressed in *N. benthamiana* leaves and the results shown in Fig. 2.4b confirmed that LSU2 is an interacting partner of AtRAP.

A recent study demonstrated that AtRAP is localized in chloroplast in an *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. 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 and LSU2 with a C-terminal CFP (LSU2-CFP) were co-expressed in *N. benthamiana*. As expected, LSU2-CFP co-localized with AtRAP-YFP in the chloroplast (Fig. 2.4c, 2.4d). 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. 2.5), and showed expression of AtRAP-YFP in the chloroplasts (Fig. 2.4e). 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*) than the Col-0 WT plants (Fig. 2.6a, 2.6b), 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. 2.6c), whereas the *atrap* mutant exhibited increased expression of *LSU2* transcripts, and significant upregulation of *LSU2* transcripts upon *Pst* infection (Fig. 2.6c). To confirm whether expression levels of AtRAP really affect the level of *LSU2* transcripts, we performed Northern blot analysis to examine the *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. 2.7), suggesting that AtRAP negatively regulates *LSU2* transcript level.

Since we found that LSU2 interacts with AtRAP (Fig. 2.4), we sought to examine if bacterial infection affects the interaction between LSU2 and AtRAP. Because the antibodies specific for LSU2 and AtRAP are not available yet, experiments were performed by transiently expressing LSU2-Flag and AtRAP-YFP in *N. benthamiana* as shown in the Co-IP assay (Fig. 2.4b). At 2 days after agroinfiltration of AtRAP-YFP and LSU2-Flag, *Pst* (*avrRpt2*) was inoculated into the same leaves. After one day, total proteins were extracted from the infiltrated leaves and subjected to Western blot analysis using anti-YFP or anti-Flag antibodies. As expected, the expression of AtRAP in *N. benthamiana* did not change significantly upon *Pst* (*avrRpt2*) infection (Fig. 2.8a) because the 3'-UTR 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. 2.8a), which is consistent with the RNA accumulation pattern of LUS2 after infection. To perform Co-IP, AtRAP-YFP and LSU2-Flag were co-expressed 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. 2.8b), although the entire accumulation level of LSU2 is 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 releasing 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 virescent phenotype as the *atrap* single mutant (Fig. 2.9a, 2.9b). We then performed pathogen growth assays on *atrap lsu2*. The double mutant became less resistance than the single *atrap* mutant, but less susceptible than the single *lsu2* mutant when challenged with both virulent and avirulent strains (Fig. 2.8c, 2.8d), supporting that AtRAP antagonistically regulate 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-week-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 down-regulated and 7 genes were up-regulated in the *atrap* mutant; Table 2.1 and 2.2). Among them, the transcription factor *GOLDEN2-LIKE 1* (*GLK1*) was down-regulated in the *atrap* mutant (Table 2.1). GLK1 is a member of the GARP superfamily (Waters et al., 2008; Waters et al., 2009), and is closed 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 (Fig. 2.10a), somewhat similar to that of the *atrap* mutant (Fitter et al., 2002; Waters et al., 2009). Thus, we sought to examine the functional significance of the GLK transcription factors in disease resistance to *Pst*. We first performed Northern blot analysis to confirm the down-regulation 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 down-regulation 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 is elevated in the AtRAP overexpression line, suggesting that AtRAP is likely to positively regulates GLK1 directly (Fig. 2.11), 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 result showed that the *GLK1* transcript level was down-regulated in the *atrap* mutant (Fig. 2.10b). Interestingly, the *GLK1* transcript level was up-regulated 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 to Col-0 WT (Fig. 2.10c, 2.10d), 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. 2.4), it is noteworthy that, out of 28 genes, 8 genes have been annotated to be located in the chloroplast (TAIR; www.arabidopsis.org) (Table 2.1 and 2.2): 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), a RNA-binding (RRM motif) family protein (At1g70200), a 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 (Dal Bosco et al., 2004; Friso et al., 2004; Johnson et al., 2010; Lange and Ghassemian, 2003; Steiner et al., 2011; Ytterberg et al., 2006). 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 up-regulated in the *atrap* mutant (Fig. 2.1a, 2.1b). In addition, the *atrap* mutant induced more rapid and strong HR cell death upon pathogen infection than seen in the Col-0 WT (Fig. 2.2b). This seems to be associated with the constitutive accumulation, and sensitive production, of ROS in the *atrap* mutant (Fig. 2.1e), because ROS plays 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 (Miller et al., 2009; Torres et al., 2002; 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. 2.1c, 2.1d). 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 overexpressing AtRAP, which has no obvious developmental defects, showed more susceptible phenotype, further supporting that AtRAP is directly involved in regulating plant immunity.

Using a yeast two-hybrid screening with AtRAP as a bait, we identified the AtRAP interacting protein, LSU2. A strong interaction between AtRAP and LSU2 was confirmed by Co-IP (Fig. 2.4b). 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. 2.6). While little information is available on the biological function of LSU2 in disease resistance, Mukhtar and collaborators (Mukhtar et al., 2011) have shown that LSU2 physically interacts with multiple effector proteins of *Pst*, and *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 still not clear how LSU2 mediates plant immunity against *Pst* (EV) and *Pst* (*avrRpt2*). Our observation that LSU2 was up-regulated upon *Pst* infection at both the RNA and protein levels (Fig. 2.6c, 2.7, 2.8a) 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. 2.8b), 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; Waters et al., 2009). In the *atrap* mutant, the *GLK1* transcript level was significantly down-regulated (Fig. 2.10b, 2.11), whereas in the AtRAP overexpression lines, *GLK1* was highly induced (Fig. 2.11), 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 2.1). although it is unknown if GLK1 is a transcriptional activator that directly regulates these genes, the down-regulation 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 up-regulation of PR10, suggesting that GLK1 may negatively modulating the defense signaling pathway (Qiu et al., 2007; Savitch et al., 2007). Our results are consistent with a previous study by Murmu et al., which indicates that GLK1 negatively regulates plant defense responses to biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis* by activating jasmonic acid (JA)-dependent pathway (Murmu et al., 2014). Murmu et al. as well as others also showed that GLK1 positively regulates plant defense responses to necrotrophic fungal pathogen *Botrytis Cinerea* and *Fusarium graminearum* (Murmu et al., 2014; Savitch et al., 2007; Schreiber et al., 2011). 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 defense response (Nott et al., 2006). A recent study showed that expression of nuclear encoded chloroplast-targeted genes (NECGs) were significantly altered upon *Pst* infection (Zabala et al., 2015). Thus, some pathogen effectors, such as Hop1J and Hop U1, HopR1 suppress plant immunity by targeting the chloroplast genes (Caplan et al., 2008; Fu et al., 2007; Jelenska et al., 2007; Zabala et al., 2015). The phenotypic characteristics of the *atrap* mutant, which are virescent and 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 (Torres et al., 2006; Vlot et al., 2009; Wildermuth et al., 2001). The discovery of photosynthesis inhibition by *Pst* infection suggests that comprehensive photosynthesis is essential for plant defense system (Zabala et al., 2015). The chloroplast is not only a major source of ROS production, but also antioxidants required to maintain ROS homeostasis (Mittler et al., 2004; Mittler et al., 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 (Fig. 2.1, Fig. 2.2b). 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 protein-mediated regulation in plant antibacterial immune responses is presented in Fig. 2.12. Under the normal growth condition 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. 2.11). 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 alter the 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.

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 light/12h 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 HR analysis, plants were infiltrated with *Pst* DC3000 carrying EV (pVSP61) or effector gene *avrRpt2* at a concentration of 2×10^7 colony-forming units (cfu)/mL. For bacterial growth assays, plants were infiltrated with *Pst* (EV) or *Pst* (*avrRpt2*) at a concentration of 2×10^5 cfu/ml. At least six leave discs were collected at each time point for 0 and 3 days 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, USA) according to the manufacturer's instructions, and mRNA extraction was carried out using the Oligotex mRNA Mini Kit (Qiagen, USA).

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, USA). The resulting cDNA was subjected to quantitative real-time PCR using an iCycler iQ5 real-time PCR detection system (Bio-Rad, USA) with specific primers. The sequences of specific primers are in Table 2.3.

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, UK). Hybridization was performed using ULTRAhyb®-Oligo hybridization buffer (Ambion, USA) and ^{32}P end-labeled DNA oligonucleotide probes specific for each target gene, according to the manufacturer's protocol. The sequences of probes are in Table 2.4.

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 3,3′-diaminobenzidine (DAB; Sigma, USA) 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 YFP tag. The full length *LSU2* CDS was cloned into pEarlyGate102 or pEarlyGate202, with a 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, Germany), equipped with a specific laser/filter combination to detect CFP (excitation at 458 nm), YFP (excitation at 514 nm) and RFP (excitation at 594 nm).

Yeast two-hybrid screening

Yeast two-hybrid screening was performed using a cDNA library (generously provided by Eulgem, T.) of pooled RNAs from 2-week-old *Arabidopsis* Col-0 WT seedlings constructed using the HybriZAP-2.1 library construction system (Stratagene, USA). The *Arabidopsis* library cDNAs (1.3 × 106) were screened by transformation into the AH109 yeast strain (Clontech, USA) expressing AtRAP-DNA-binding domain (BD) bait fusion protein generated by the GAL4 system (Stratagene, USA), as described by the manufacturer's instructions. 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 (20 mM Tris–HCl at pH 7.5, 300 mM NaCl, 5 mM MgCl2, 5 mM dithiothreitol, 0.5% Triton X-100, proteinase inhibitor cocktail [Sigma, USA]). 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, USA) 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 of PBS (0.1 M NaCl, 90 mM sodium phosphate, pH 7.0). The resulting samples were analyzed by SDS-PAGE, followed by immunoblot analysis using anti-YFP (Roche, USA) and anti-FLAG (Sigma, USA) antibodies.

Microarray analysis

For Affymetrix GeneChip array analysis, we normalized expression profiles with the RMA method (Irizarry et al., 2003). A list of genes with statistically significant changes in expression between the genotypes was generated by the SAM method in which multiple testing was taken into account through q-value (Siggenes, R package version 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 is deposited into NCBI (GSE98376).

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Author Contribution

H.J. conceived the idea and designed the project. H.W., J.-K.S. and S.G. performed the experiments. H.W., J.-K.S., and H.J. analyzed the data and wrote the manuscript. X.C. analyzed microarray data. All authors have read and approved the manuscript.

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Figures and Tables

Figure 2.1 Effect of AtRAP knockout mutation on defense responses.
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. Total RNAs were extracted from the infiltrated leaves at the times indicated on the horizontal axes. Relative accumulation levels of *PR1* (a), *PR2* (b), *RBOHd* (c), and *RBOHf* (d) 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 MgCl2, *Pst* (EV), or *Pst* (*avrRpt2*) at a concentration of 2×10^7 cfu/mL. DAB staining was performed to visualize ROS accumulation at 6 hours post inoculation (hpi).

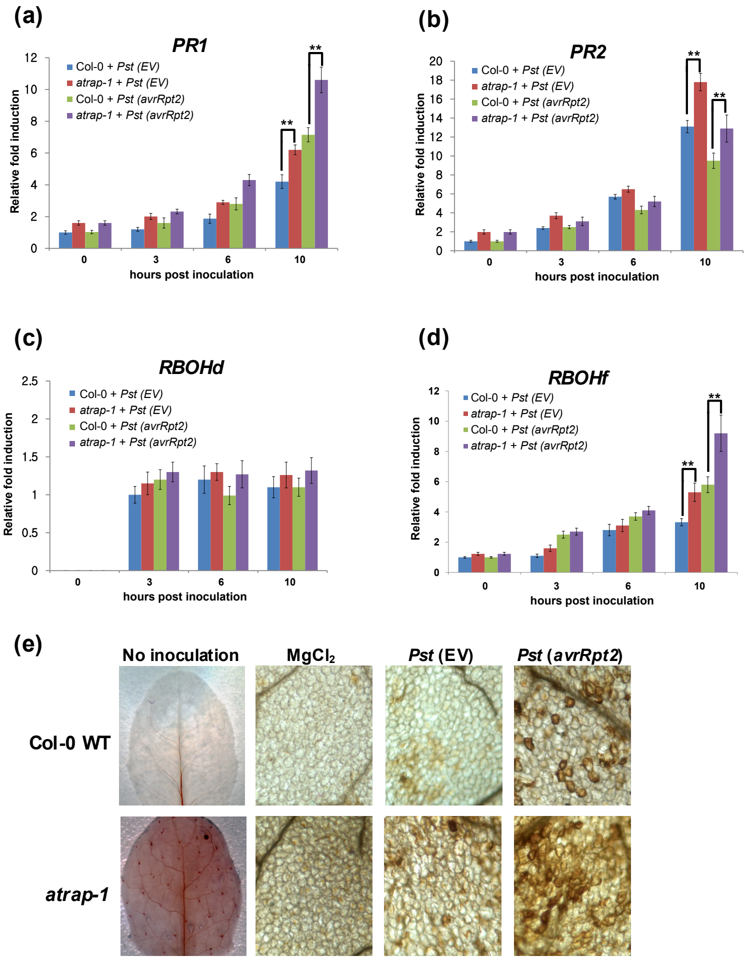
(a)
PR1
Relative fold induction
12
10
8
6
4
2
0
Col-0 + Pst (EV)
atrap-1 + Pst (EV)
Col-0 + Pst (avrRpt2)
atrap-1 + Pst (avrRpt2)
**
**
0
3
6
10
hours post inoculation
(b)
PR2
Relative fold induction
20
18
16
14
12
10
8
6
4
2
0
Col-0 + Pst (EV)
atrap-1 + Pst (EV)
Col-0 + Pst (avrRpt2)
atrap-1 + Pst (avrRpt2)
**
**
0
3
6
10
hours post inoculation
(c)
RBOHd
Relative fold induction
2.5
2
1.5
1
0.5
0
Col-0 + Pst (EV)
atrap-1 + Pst (EV)
Col-0 + Pst (avrRpt2)
atrap-1 + Pst (avrRpt2)
0
3
6
10
hours post inoculation
(d)
RBOHf
Relative fold induction
12
10
8
6
4
2
0
Col-0 + Pst (EV)
atrap-1 + Pst (EV)
Col-0 + Pst (avrRpt2)
atrap-1 + Pst (avrRpt2)
**
**
0
3
6
10
hours post inoculation
(e)
No inoculation
MgCl₂
Pst (EV)
Pst (avrRpt2)
Col-0 WT
atrap-1

Figure 2.2 AtRAP negatively regulates plant defense to *Pst*.
(a) Five-wees-old *Arabidopsis* Col-0 WT and the *atrap* mutant plants were infiltrated with *Pst* (EV) or *Pst (avrRpt2)* at a concentration of 2 x 10^7 cfu/mL. Total RNAs were extracted from the infiltrated leaves at the times indicated on the horizontal axes. Relative accumulation levels of *PDF1.2* transcripts were quantified by quantitative real-time PCR. Error bars represent the SEM from three independent experiments. ** $p < 0.01$ (determined by Student's t test). (b) HR cell death phenotypes in the leaves infiltrated with *Pst (avrRpt2)* at a concentration of 2 x 10^7 cfu/mL at 8 hpi. The red arrowheads indicate collapse of infiltrated leaf areas caused by HR cell death.

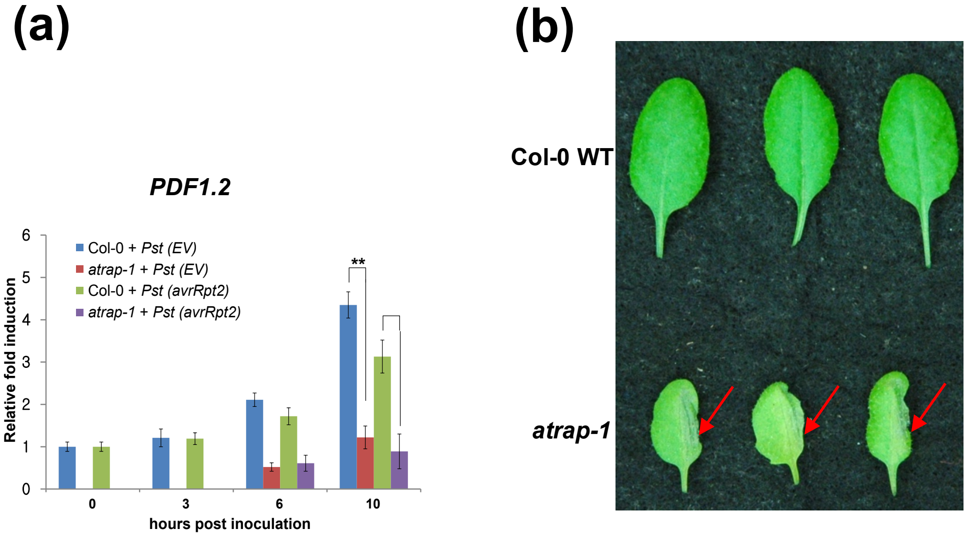

Figure 2.3 Effects of AtRAP overexpression on disease resistance to *Pst* in *Arabidopsis*. (a) Phenotypes of the AtRAP knockout mutant and overexpression plants. (b) Immunodetection of AtRAP-FLAG with anti-FLAG antibody in extracts from seven independent AtRAP overexpression lines. (c,d) Growth of *Pst* (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. At least six leave discs were collected at 0 day post inoculation (dpi) 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. ANOVA-test was used for significance difference calculation between Col-0 WT and overexpression lines. Error bars represent standard deviation of more than six replicates. Similar results were obtained from three independent experiments. ** $p < 0.01$ (determined by Student's t test).

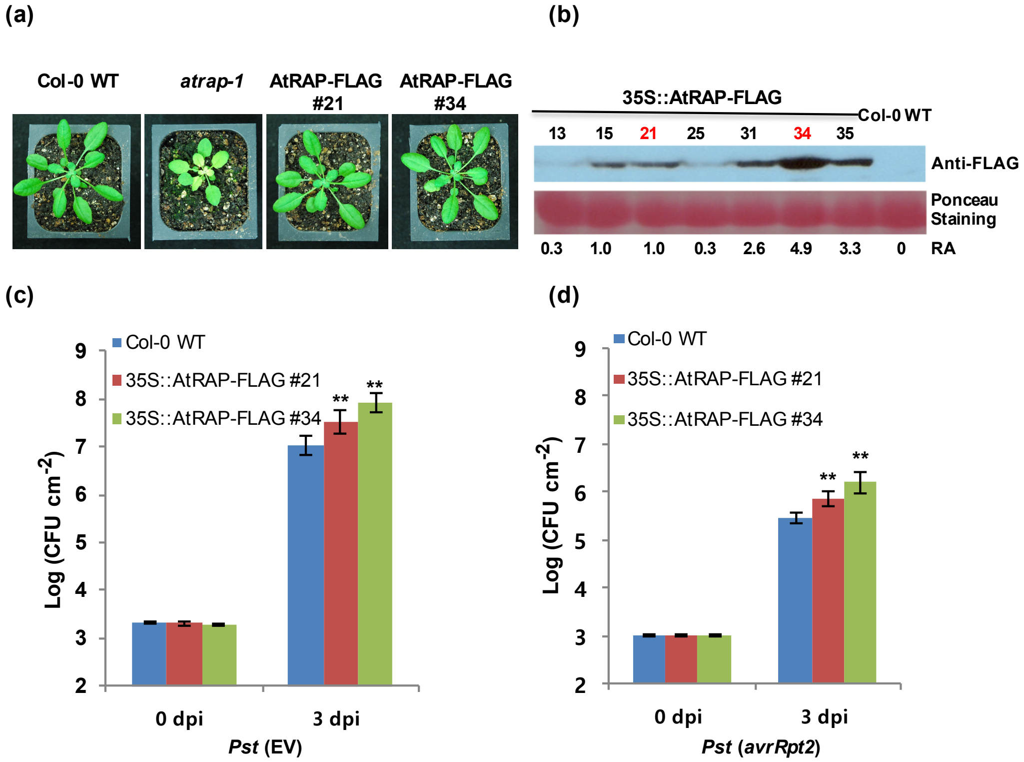
(a)
Col-0 WT
atrap-1
AtRAP-FLAG #21
AtRAP-FLAG #34
(b)
35S::AtRAP-FLAG
Col-0 WT
13
15
21
25
31
34
35
Anti-FLAG
Ponceau Staining
0.3
1.0
1.0
0.3
2.6
4.9
3.3
0
RA
(c)
Col-0 WT
35S::AtRAP-FLAG #21
35S::AtRAP-FLAG #34
Log (CFU cm^{-2})
9
8
7
6
5
4
3
2
**
**
0 dpi
3 dpi
Pst (EV)
(d)
Col-0 WT
35S::AtRAP-FLAG #21
35S::AtRAP-FLAG #34
Log (CFU cm^{-2})
9
8
7
6
5
4
3
2
**
**
0 dpi
3 dpi
Pst (avrRpt2)

Figure 2.4 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 SD/-LWH and SD/-LWHA agar media. (b) Co-immunoprecipitation assay in *N. benthamiana* leaves. AtRAP and LSU2, tagged with YFP and FLAG, respectively, were co-expressed in *N. benthamiana* leaves by agro-infiltration and immuno-precipitated 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 co-expressed 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 infiltrated leaves (c) and protoplasts (d) of *N. benthamiana* were subjected to confocal microscopy. Chloroplasts emit red fluorescence. Bar = 10 μm and 25 μm separately. (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. Bar = 10 μm.

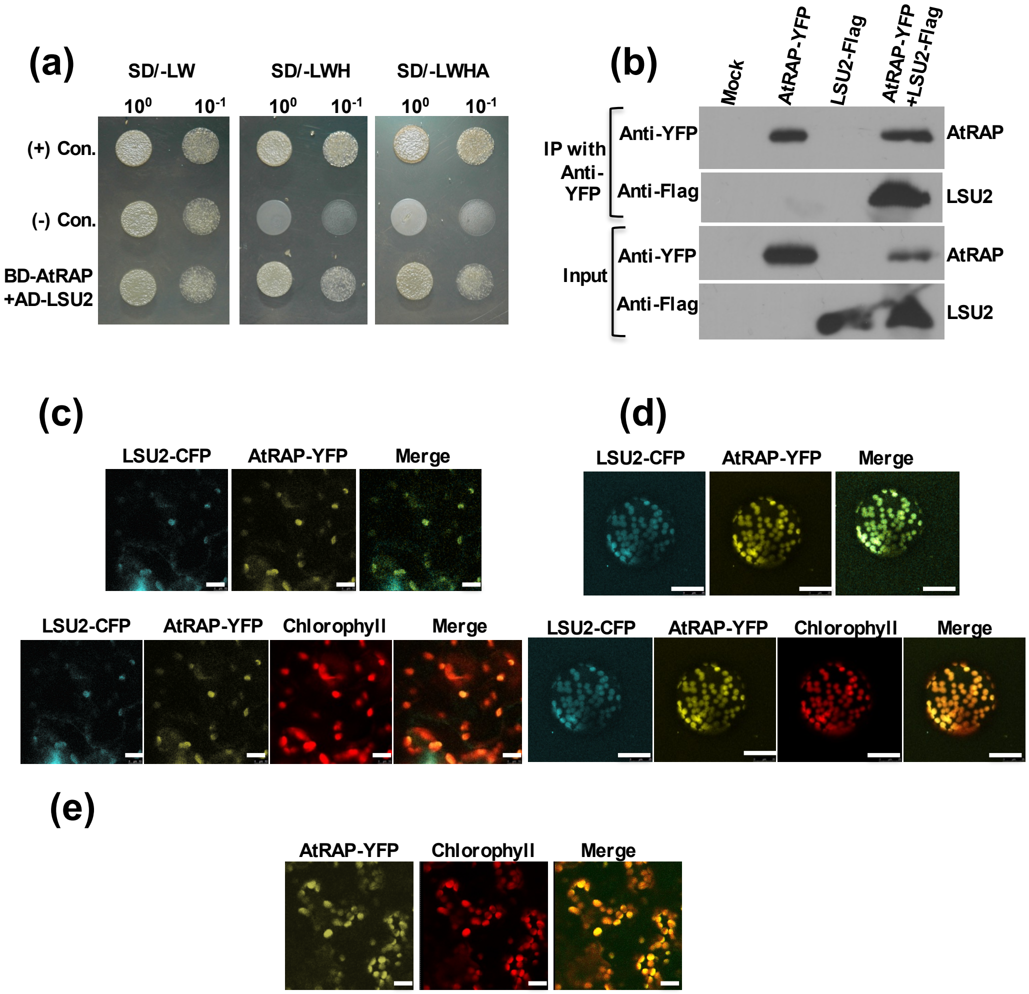
(a)
SD/-LW
SD/-LWH
SD/-LWHA
10⁰
10⁻¹
(+) Con.
(-) Con.
BD-AtRAP
+AD-LSU2
(b)
Mock
AtRAP-YFP
LSU2-Flag
AtRAP-YFP
+LSU2-Flag
IP with
Anti-
YFP
Anti-YFP
Anti-Flag
Input
Anti-YFP
Anti-Flag
AtRAP
LSU2
AtRAP
LSU2
(c)
LSU2-CFP
AtRAP-YFP
Merge
LSU2-CFP
AtRAP-YFP
Chlorophyll
Merge
(d)
LSU2-CFP
AtRAP-YFP
Merge
LSU2-CFP
AtRAP-YFP
Chlorophyll
Merge
(e)
AtRAP-YFP
Chlorophyll
Merge

Figure 2.5 Phenotype of AtRAP overexpressing plant.
35S::AtRAP-YFP was transformed into the *atrap-1* mutant background. The virescent phenotype and the growth retardation defect of *atrap-1* was complemented.

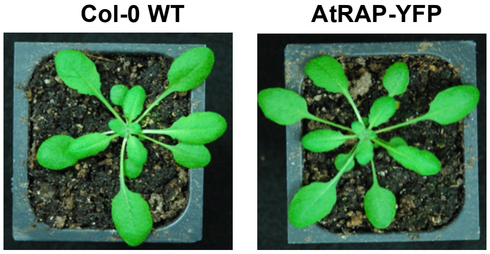

Figure 2.6 LSU2 is a positive regulator of plant defense against *Pst* in *Arabidopsis*. (a,b), Growth of *Pst* (EV), and *Pst* (*avrRpt2*) in the *lsu2* mutant plants at 0 and 3 dpi. Error bars represent standard deviation of more than six replicates. Similar results were obtained from three independent experiments. ** $p < 0.01$ (determined by Student's t test). * $p < 0.05$ (determined by Student's t test). (c) Detection of *LSU2* mRNA in the Col-0 WT and the *atrap* mutant plants. Five-weeks-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. Total RNAs were extracted from the infiltrated leaves at 8 hours post inoculation (hpi) after inoculation and subjected to Northern blot analysis.

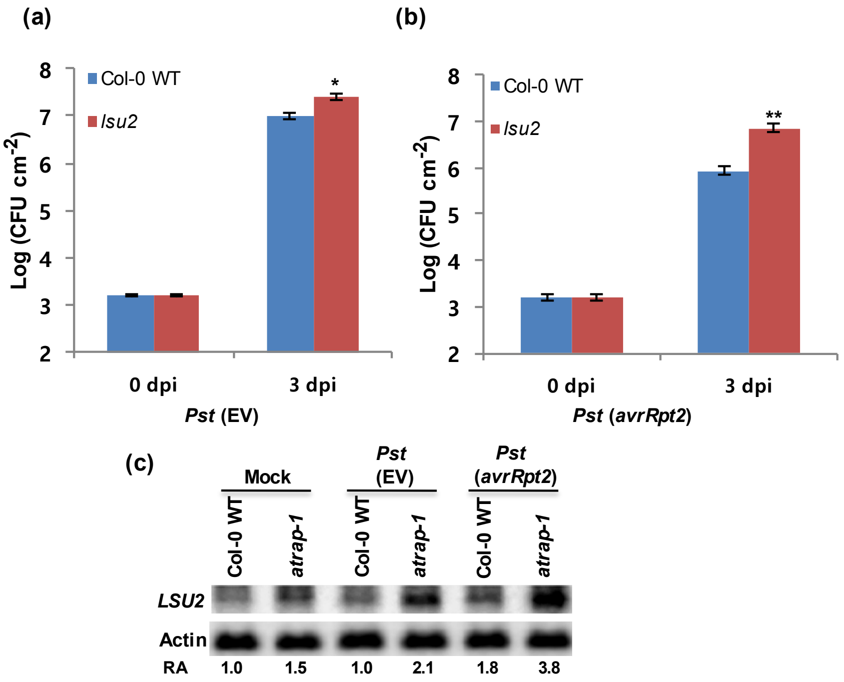
(a)
(b)
Log (CFU cm^{-2})
8
7
6
5
4
3
2
Col-0 WT
lsu2
*
0 dpi
3 dpi
Pst (EV)
Log (CFU cm^{-2})
8
7
6
5
4
3
2
Col-0 WT
lsu2
**
0 dpi
3 dpi
Pst (*avrRpt2*)
(c)
Mock
Pst (EV)
Pst (*avrRpt2*)
Col-0 WT
atrap-1
Col-0 WT
atrap-1
Col-0 WT
atrap-1
LSU2
Actin
RA
1.0
1.5
1.0
2.1
1.8
3.8

Figure 2.7 *LSU2* is negatively regulated by *AtRAP*.
Detection of *LSU2* mRNA in the Col-0 WT, the *atrap* mutant and the 35S::AtRAP plants. Five-week-old *Arabidopsis* Col-0 WT, the *atrap* mutant and the 35S::AtRAP plants were collected. Total RNAs were extracted and subjected to Northern blot analysis.

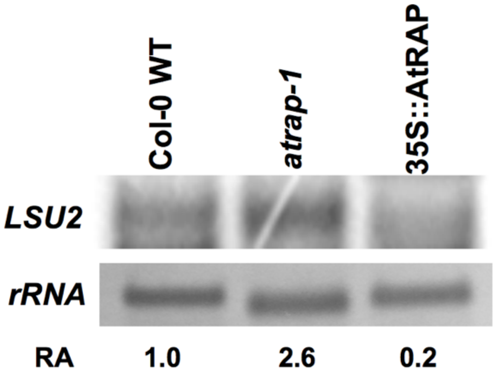

Figure 2.8 Effects of bacterial infection on the interaction between AtRAP and LSU2. (a) AtRAP-YFP and LSU2-Flag were separately expressed in *N. benthamiana* leaves by agro-infiltration. After 2 days, *Pst* (*avrRpt2*) was inoculated into the leaves infiltrated with AtRAP-YFP or LSU2-Flag. After one day, 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 co-expressed in *N. benthamiana* leaves by agro-infiltration. After 2 days, *Pst* (*avrRpt2*) was inoculated into the leaves. After one day, 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 resistance than the *atrap* single mutant, and is less susceptible than the *lsu2* single mutant to bacterial infection. Error bars represent standard deviation of more than six replicates. Similar results were obtained from three independent experiments. ** $p < 0.01$ (determined by Student's t test). * $p < 0.05$ (determined by Student's t test).

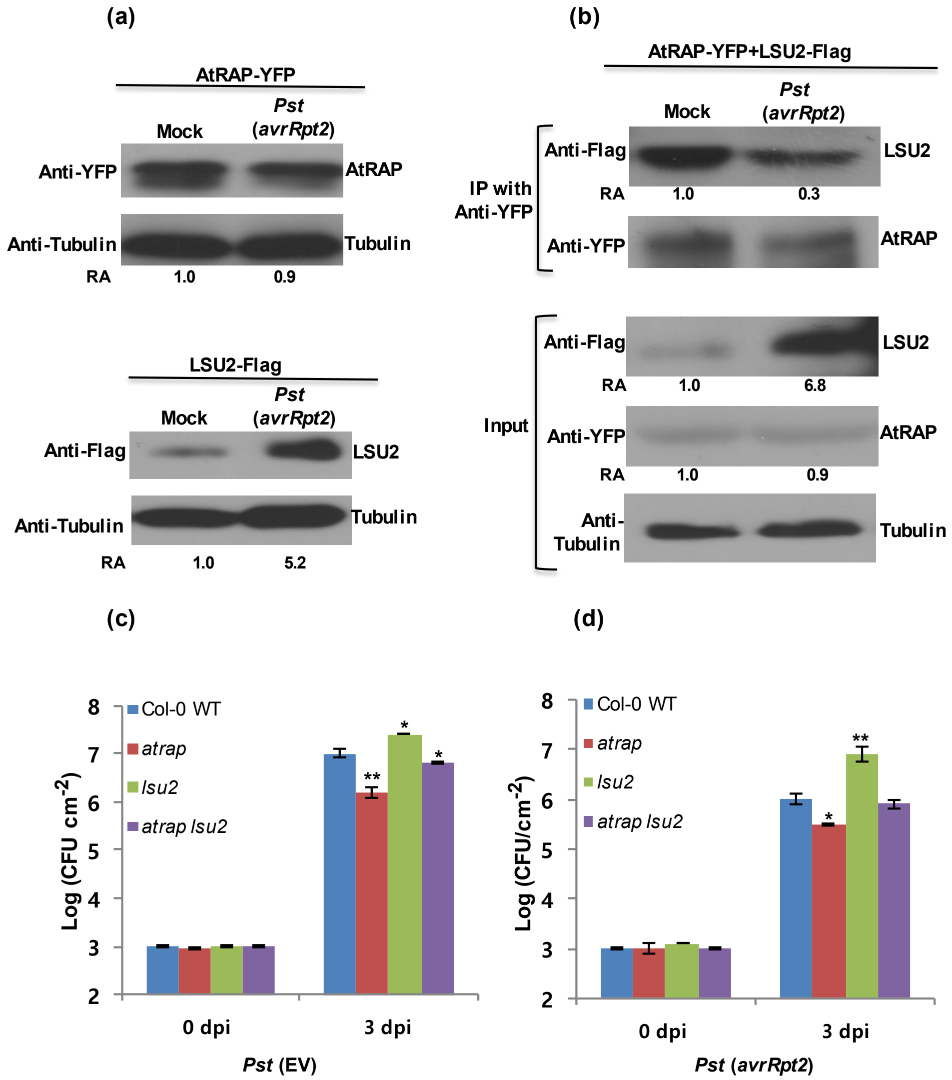
(a)
AtRAP-YFP
Mock
Pst (*avrRpt2*)
Anti-YFP
AtRAP
Anti-Tubulin
Tubulin
RA
1.0
0.9
LSU2-Flag
Mock
Pst (*avrRpt2*)
Anti-Flag
LSU2
Anti-Tubulin
Tubulin
RA
1.0
5.2
(b)
AtRAP-YFP+LSU2-Flag
Mock
Pst (*avrRpt2*)
IP with Anti-YFP
Anti-Flag
LSU2
RA
1.0
0.3
Anti-YFP
AtRAP
Input
Anti-Flag
LSU2
RA
1.0
6.8
Anti-YFP
AtRAP
RA
1.0
0.9
Anti-Tubulin
Tubulin
(c)
Log (CFU cm^{-2})
8
7
6
5
4
3
2
Col-0 WT
atrap
lsu2
atrap lsu2
**
*
*
0 dpi
3 dpi
Pst (EV)
(d)
Log (CFU/cm^{-2})
8
7
6
5
4
3
2
Col-0 WT
atrap
lsu2
atrap lsu2
*
**
0 dpi
3 dpi
Pst (*avrRpt2*)

Figure 2.9 Characterization of the *atrap lsu2* double mutant.
(a) Phenotype of the *atrap lsu2* double mutant. (b) Genotyping and RT-PCR result of the *atrap lsu2* double mutant. F: Genomic DNA forward primer across T-DNA insertion site; R: Genomic DNA reverse primer across T-DNA insertion site; T: T-DNA insertion primer; F': cDNA forward primer; R': cDNA reverse primer. The primers follow Katiyar-Agarwal *et al*., 2006 and Ruckle *et al*., 2012. The *lsu2* T-DNA insertion line (SALK_031648) is a knock-down line within 5' UTR insertion. The double mutant was selected by PCR of the genomic DNA for the homologous T-DNA insertion and confirmed by RT-PCR to show no or very low expression of *AtRAP* and *LSU2*, respectively, in the double mutant.

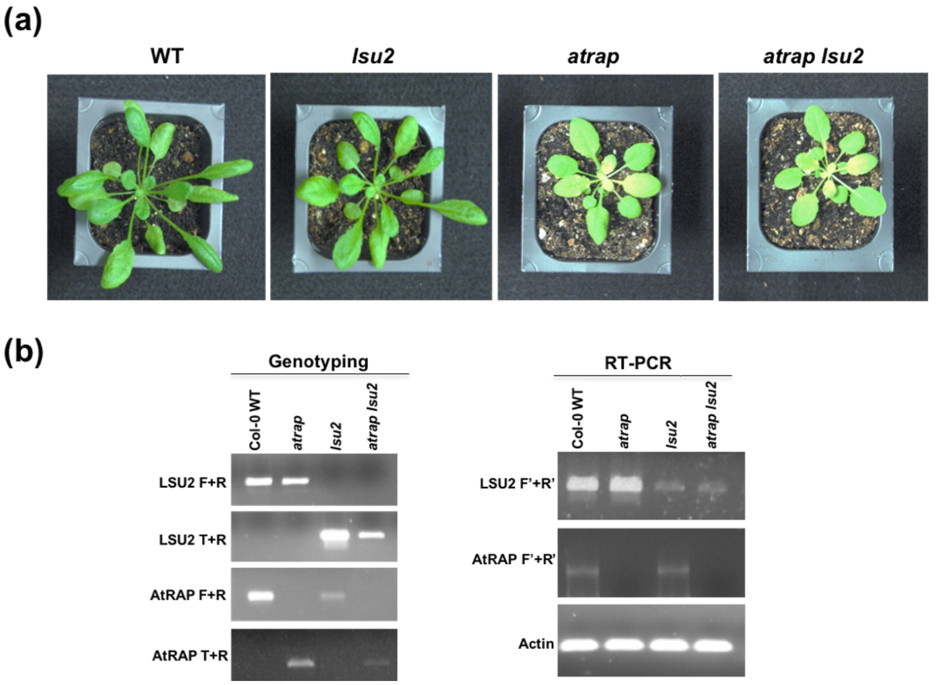
(a)
WT
lsu2
atrap
atrap lsu2
(b)
Genotyping
Col-0 WT
atrap
lsu2
atrap lsu2
LSU2 F+R
LSU2 T+R
AtRAP F+R
AtRAP T+R
RT-PCR
Col-0 WT
atrap
lsu2
atrap lsu2
LSU2 F'+R'
AtRAP F'+R'
Actin

Figure 2.10 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 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. Total RNAs were extracted from the infiltrated leaves at 8 hours post inoculation (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 standard deviation of more than six replicates. Similar results were obtained from three independent experiments. ** $p < 0.01$ (determined by Student's t test).

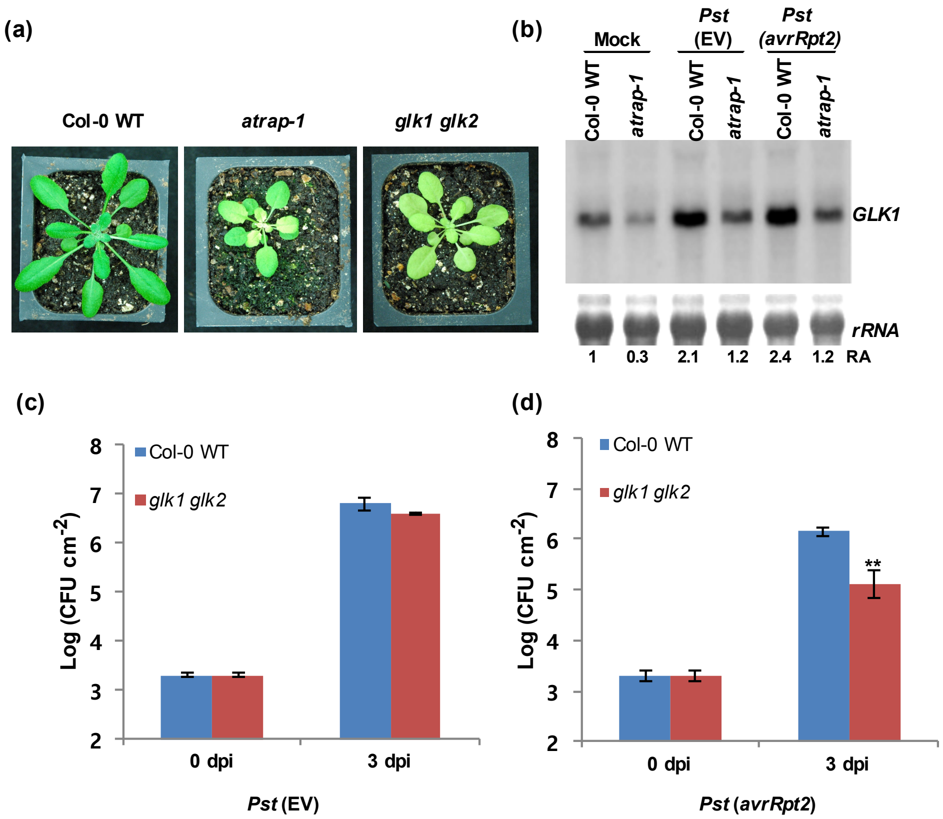
(a)
Col-0 WT
atrap-1
glk1 glk2
(b)
Mock
Pst (EV)
Pst (avrRpt2)
Col-0 WT
atrap-1
Col-0 WT
atrap-1
Col-0 WT
atrap-1
GLK1
rRNA
1
0.3
2.1
1.2
2.4
1.2
RA
(c)
Col-0 WT
glk1 glk2
Log (CFU cm^{-2})
8
7
6
5
4
3
2
0 dpi
3 dpi
Pst (EV)
(d)
Col-0 WT
glk1 glk2
Log (CFU cm^{-2})
8
7
6
5
4
3
2
**
0 dpi
3 dpi
Pst (avrRpt2)

Figure 2.11 *GLK1* positively regulated by *AtRAP*.
Detection of *GLK1* mRNA in the Col-0 WT, the *atrap* mutant and the 35S::AtRAP plants. Detection of *GLK1* mRNA in the Col-0 WT, the atrap mutant and the 35S::AtRAP plants. Five-weeks-old Arabidopsis Col-0 WT, the *atrap* mutant and the 35S::AtRAP plants were collected. Total RNAs were extracted and subjected to Northern blot analysis.

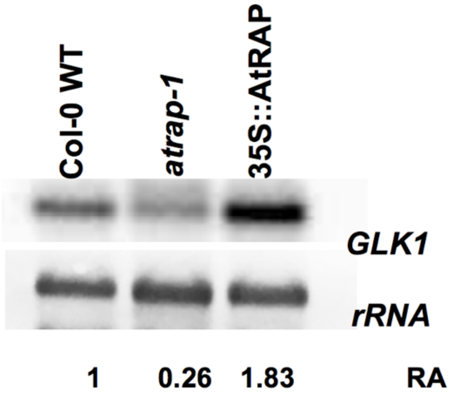

Figure 2.12 A hypothetical working model for the regulation of the AtRAP protein in plant antibacterial disease resistance.
Under the normal growth condition in the absence of pathogen infection, AtRAP suppresses the positive regulator of plant defense - LSU2 by directly interacting with the LSU2 protein. Upon bacterial infection, AtlsiRNA1 is highly induced and silences the *AtRAP* gene, which quickly activates defense responses by releasing LSU2. Expression of the transcription factor GLK1 is positively regulated by AtRAP protein and acts as a negative regulator of plant antibacterial defense responses.

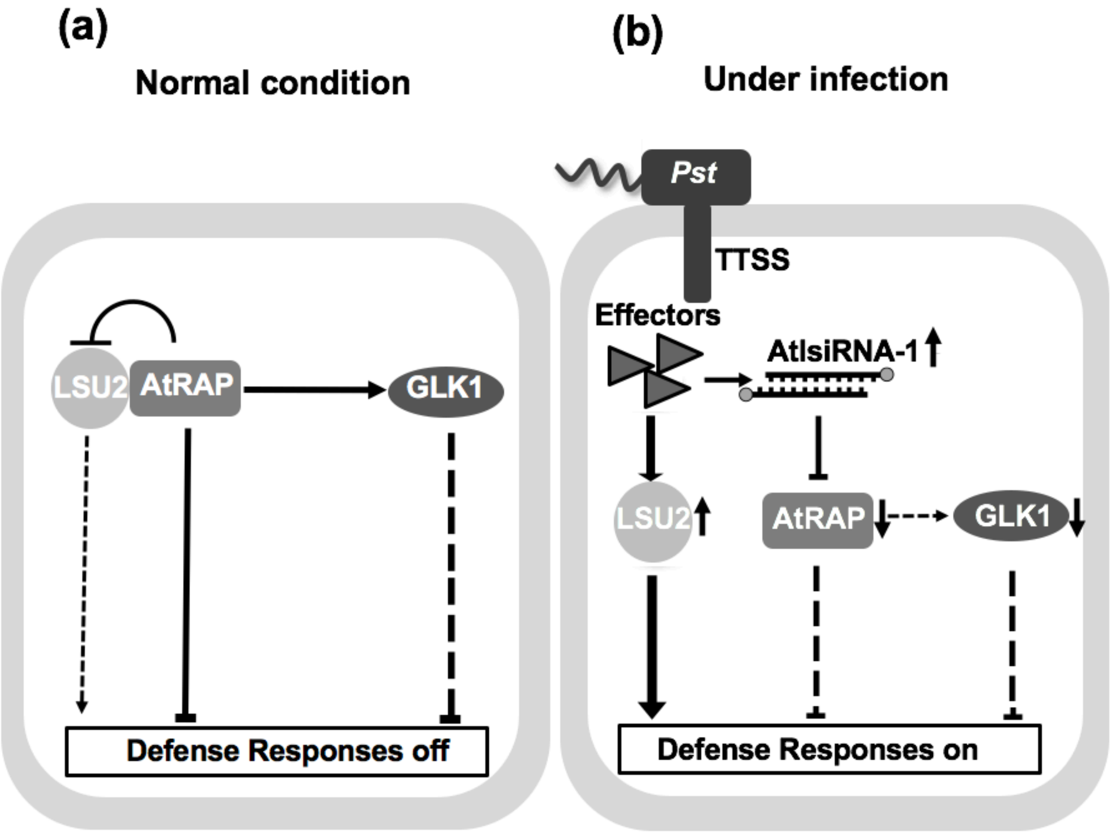
(a)
Normal condition
LSU2
AtRAP
GLK1
Defense Responses off
(b)
Under infection
Pst
TTSS
Effectors
AtlsiRNA-1
LSU2
AtRAP
GLK1
Defense Responses on

Table 2.1 Genes which are down-regulated in the *atrap* mutant are listed.

Affymetrix code	Relative level (WT/*atrap-1*)	AGI code	Gene description
254716_at	0.1347	AT4G13560	UNE15 (unfertilized embryo sac 15)
250824_at	0.1462	AT5G05200	Protein kinase superfamily protein
262357_at	0.3179	AT1G73040	jacalin lectin family protein
260869_at	0.3228	AT1G43800	acyl-(acyl-carrier-protein) desaturase
265948_at	0.3668	AT2G19590	ACC oxidase 1
260004_at	0.4208	AT1G67860	unknown protein
252612_at	0.4555	AT3G45160	unknown protein
254386_at	0.4634	AT4G21960	PRXR1 (Peroxidase superfamily protein)
263715_at	0.4643	AT2G20570	GLK1 (ARABIDOPSIS GOLDEN2-LIKE 1)
255626_at	0.4983	AT4G00780	TRAF-like family protein
245196_at	0.5058	AT1G67750	pectate lyase family protein
259001_at	0.5220	AT3G01960	unknown protein
259535_at	0.5271	AT1G12280	disease resistance protein (CC-NBS-LRR class)
262958_at	0.5329	AT1G54410	dehydrin family protein
264611_at	0.5543	AT1G04680	pectate lyase family protein

262582_at	0.5608	AT1G15410	aspartate-glutamate racemase family
245307_at	0.5688	AT4G16770	2-oxoglutarate and Fe(II)-dependent oxygenase superfamily protein
255578_at	0.5757	AT4G01450	nodulin MtN21 family protein
247685_at	0.5917	AT5G59680	leucine-rich repeat protein kinase
246595_at	0.5983	AT5G14780	Encodes a NAD-dependent formate dehydrogenase
261368_at	0.6011	AT1G53070	legume lectin family protein

Table 2.2 Genes which are up-regulated in the *atrap* mutant are listed.

Affimetrix code	**Relative level (WT/*atrap-1*)**	**AGI code**	**Gene description**
253208_at	1.5466	AT4G34830	MRL1 (Pentatricopeptide repeat protein)
264698_at	1.8240	AT1G70200	RNA-binding (RRM)-containing protein
251638_at	1.9411	AT3G57490	RPS2D (40S ribosomal protein S2)
254907_at	2.3464	AT4G11190	disease resistance-responsive family protein
246411_at	2.3464	AT1G57770	FAD/NAD(P)-binding oxidoreductase family protein
244997_at	2.6193	ATCG00170	RNA polymerase beta' subunit-2
249645_at	2.9723	AT5G36910	thionin 2.2

Table 2.3 Q-PCR primers which are used in this study are listed.

Primer ID	Primer sequence
PR1 (AT2G14610)-QRT-F	TTCCCTCGAAAGCTCAAGATA
PR1 (AT2G14610)-QRT-R	GGCACATCCGAGTCTCACTGA
PR2 (AT3G57260)-QRT-F	GGCTTGAAGTCAAGGTCTCA
PR2 (AT3G57260)-QRT-R	CCCTGGATCGTTATCAACAG
RBOHd (AT5G47910)-QRT-F	TGCTTTCAGATCAAGCATCA
RBOHd (AT5G47910)-QRT-R	TGAGCTTACGTGTCCAGTCA
RBOHf	ACACCGAGGCATACGTGTAA

(AT1G64060)- QRT-F	
RBOHf (AT1G64060)- QRT-R	ACATGGACCTTATCAACGCA
PDF1.2 (AT5G44420) QRT-F	TCATCATGGCTAAGTTTGCT
PDF1.2 (AT5G44420) QRT-R	CCTTCAAGGTTAATGCACTG

Table 2.4 DNA probes which are used in this study are listed.

Primer ID	**Primer sequence**
LSU2 (AT5G24660) Probe1	ATCTCTTTCTTCATCTCCTCCACAGCTTTCTCCATCTCT
LSU2 (AT5G24660) Probe2	AGCCGCCACCGTCACATAGTTTCCTCCTTTCCCCATTT
GLK1 (AT2G20570) Probe1	AAACGGCGGATTAGGCATGGCGGTAGAAGGCGGAGGTA
GLK1 (AT2G20570) Probe2	CGTCATAAGTCACCGTCTCCGCCGCAACATCGTCTCT

Chapter 3

Nucleus-encoded snoRNAs move into chloroplasts and direct methylation of chloroplast RNAs

Abstract

Chloroplasts are important organelles that capture solar energy and convert it to chemical energy in the form of sugars for plants and algae as well as oxygen that is vital for the whole biosphere (Eberhard et al., 2008; Waters and Langdale, 2009). They contain a small genome that is limited in size and function and require anterograde signaling from the nucleus for proper functioning (Sato et al., 1999; Sugiura, 1992). Although protein trafficking from the nucleus to chloroplasts has been well-studied (Keegstra, 1989; Kikuchi et al., 2013; Li and Chiu, 2010; Soll and Schleiff, 2004), it is largely unknown whether nuclear RNA, especially functional non-coding RNA, can travel into chloroplasts and what the relevant function of these RNAs would be. Here, we report that a group of nucleus-encoded small nucleolar RNAs (snoRNAs) can move into chloroplasts and direct methylation of chloroplast-encoded ribosomal RNA and photosynthesis-related mRNAs. AtRAP, which is a proved chloroplast-localized RNA-binding protein, is important in snoRNA trafficking and chloroplast RNA modification. Thus, we discovered these nucleus-encoded chloroplast-localized snoRNAs as a novel anterograde signaling into chloroplast to help chloroplast maintain proper function. What's more, we characterized

the first protein component-AtRAP in this anterograde signaling which facilitates these snoRNAs transport into chloroplasts.

Introduction

Chloroplasts are semi-autonomous organelles in plants and algae that drive photosynthesis, a process by which they use solar energy to convert carbon dioxide and water to sugars and oxygen to support nearly all organisms on Earth (Eberhard et al., 2008; Waters and Langdale, 2009). Chloroplasts are believed to have arisen from an endosymbiotic event involving ancestors of cyanobacteria. Following this endosymbiotic process, most of the cyanobacterial circular genomic DNA and its associated genes were lost or transferred to the nucleus (Timmis et al., 2004). Despite the functional importance of the chloroplast and its ability to perform transcription and translation within itself, its genome size is rather limited. Proper functioning of chloroplasts largely relies on anterograde import and signaling from the nucleus.

The mechanisms that regulate the import of nucleus-encoded proteins into chloroplasts have been extensively studied. In chloroplasts, about 95% of the proteins are encoded in nucleus, synthesized in cytosol, and finally imported into chloroplasts (Keegstra, 1989; Kikuchi et al., 2013; Li and Chiu, 2010; Soll and Schleiff, 2004). Compared to the well-studied protein transport pathways, very little is known about RNA trafficking into chloroplasts. Furthermore, although functional non-coding RNAs, such as small nuclear (snRNAs) and small nucleolar RNAs (snoRNAs), are crucial for post-transcriptional RNA splicing and RNA modification of mRNAs, tRNAs, and rRNAs (Brown et al., 2003; Kiss, 2001; Storz, 2002; Weil et al., 2016; Will and Luhrmann, 2001), they are not encoded in the meager genome of the chloroplast or reported to be present in chloroplasts (Sato et al., 1999).

In several previous studies, tRNAs were detected in mammalian and plant mitochondria (Brubacher-Kauffmann et al., 1999; Delage et al., 2003; Mahata et al., 2006; Rubio et al., 2008; Salinas et al., 2006; Small et al., 1992). A long-standing question is whether plant endogenous, especially non-coding RNAs, can travel into chloroplasts. *Epifagus virginiana*, which is a root holoparasitic plant, possesses vestigial chloroplasts which are size-reduced, lack thylakoids, and are missing 13 of the 30 tRNAs. However, the remaining genes in the chloroplasts are still expressed and functional, suggesting that necessary tRNAs may be imported into the chloroplasts (Bungard, 2004; Wolfe et al., 1992). Although the plant *eukaryotic translation initiation factor 4E* (*eIF4E)* nucleus-encoded mRNA has been detected in the chloroplast, the eIF4E protein has not. Thus, the transport mechanism and function of the *eIF4E* transcript inside the chloroplast are still unclear. Additionally, there was nuclear contamination in the isolated chloroplast RNA, which may confound the results (Nicolai et al., 2007). A prominent example of anterograde RNA import is found in the mammalian mitochondrial system. It is mediated by Polynucleotide Phosphorylase (PNPASE), an exoribonuclease and poly-A polymerase localized in the mammalian mitochondrial intermembrane space. It translocates nucleus-encoded *RNase P*, *5S rRNA*, and *MRP* RNA into mitochondria (Wang et al., 2010). PNPASE recognizes a stem-loop RNA structure that was described as a mediator of PNPASE-dependent RNA import (Wang et al., 2012). Thus, the critical open question is whether plant endogenous non-coding RNA can move into chloroplasts, and if so, what is the potential function of imported RNA in the chloroplasts.

Results

Our discovery was made during our previous study of an *Arabidopsis thaliana* protein containing a RNA-binding domain abundant in Apicomplexans (AtRAP). AtRAP possesses a predicted 78-amino acid chloroplast-transit signaling peptide (SP) (Emanuelsson et al., 1999), a putative RNA-binding domain abundant in Apicomplexans (RAP) (Kleinknecht et al., 2014; Lee and Hong, 2004) and four Octotricopeptide Repeats (OPRs) (Kleinknecht et al., 2014). In order to determine the function of the domains in AtRAP, We generated transgenic *atrap-1* mutant plants expressing vectors with deleted portions of AtRAP under the native AtRAP promoter. Compared to the AtRAP-Green Fluorescent Protein (GFP) transgenic control, plants with deleted signaling peptide (AtRAP-ΔSP-GFP) and deleted RAP domain (AtRAP-ΔRAP-GFP) could not rescue the mutant *atrap-1* virescent phenotype. This indicates that the predicted SP and RAP domains are essential for AtRAP function. (Fig. 3.1). The AtRAP-ΔSP and the AtRAP-ΔRAP transgenic plants show similar chlorophyll deficiencies as the *atrap-1* mutant (Fig. 3.2). AtRAP was proven to be localized in chloroplast by transient expressed AtRAP fused with GFP on C-terminus under 35S promoter-driven in *Nicotiana benthamiana* protoplasts (Kleinknecht et al., 2014). In agreement with those results, We confirmed that AtRAP is localized in the chloroplast using AtRAP-GFP transgenic plants (Fig. 3.1b, 3.1c). Moreover, the signaling peptide is essential for its localization. AtRAP-ΔSP-GFP could not be detected from chloroplasts extracted from AtRAP-ΔSP-GFP transgenic. Nuclear protein tubulin was used as control to indicate the purity of the chloroplast fraction proteins (Fig. 3.1b). Also, confocal microscopy showed that AtRAP-ΔSP-GFP was predominantly localized in the cytosol instead of the chloroplasts (Fig. 3.1c). The RAP domain is indispensable for AtRAP localization (Fig. 3.1b, 3.1c). Taken together, AtRAP is indeed a chloroplast-localized protein, and the signaling peptide is essential for AtRAP transport into the chloroplast.

It has been postulated that OPRs, which are composed of tandemly repeated amino acids units, can form α-helical RNA-binding domains (Eberhard et al., 2011; Rahire et al., 2012). OPRs are mostly found in unicellular organisms and bacteria and rare in land plants. OPRs have been found in several green algae and land plants, such as *Chlamydomonas reinhardtii*, *Arabidopsis thaliana*, *Nicotiana tabacum*, *Oryza sativa*, *Zea mays*, and *Physcomitrella patens* (Kleinknecht et al., 2014). The RAP domain, which comprises of blocks of charged aromatic residues and is predicted to form α-helical and β-strand structures, is a putative RNA-binding domain. It is highly conserved across many eukaryotes: green algae, land plants (*Arabidopsis thaliana* and *Oryza sativa*), Apicomplexans (*Plasmodium falciparum*), flies (*Drosophila melanogaster*), mammals (*Mus musculus*) and humans (Lee and Hong, 2004). Because the OPR domain is rare in land plants, and the RAP domain is represented more in eukaryotes, we decided to focus on the characterization of the RAP domain in AtRAP.

To characterize AtRAP's putative RNA-binding activity and RAP domain function, RNA-immunoprecipitation (RIP) was performed to pull down RNAs bound to AtRAP-GFP and AtRAP-ΔRAP-GFP proteins. Anti-GFP antibody-conjugated agarose beads were used to precipitate AtRAP-GFP and AtRAP-ΔRAP-GFP from transgenic AtRAP-GFP and AtRAP-ΔRAP-GFP plants, respectively (Fig. 3.1a). Potential RNAs associated with AtRAP-GFP or AtRAP-ΔRAP-GFP were then extracted and subjected to end labeling. A strong autoradiography signal for the AtRAP-GFP sample indicates that AtRAP is bound to a large amount of polynucleotides (DNA or RNA) (Fig. 3.4a). The RNase A treatment abolished the presence of polynucleotides, suggesting that the polynucleotides bound to AtRAP were all RNAs. The amount of RNAs bound to AtRAP-ΔRAP-GFP were considerably less than AtRAP-GFP, suggesting that the RAP domain is significant for AtRAP RNA binding activity (Fig. 3.4a). From these results, we concluded that AtRAP is a genuine RNA-binding protein, and the RAP domain is important for its RNA-binding activity.

Next, to investigate the identity and function of the AtRAP-bound RNAs, we did deep sequencing the RNAs pulled down with AtRAP. To our surprise, the majority of highly abundant RNAs bound to AtRAP were nucleus-encoded (Table 3.1). More interestingly, most of the nucleus-encoded RNAs are annotated as small nucleolar RNAs (snoRNAs) (Table 3.1). SnoRNAs are one of the most ancient non-coding RNAs. They are highly structured and are essential for RNA modification. SnoRNAs are divided into two classes based on their conserved motifs and secondary structures: box C/D snoRNAs, which direct 2'-*O*-ribose methylation, and box H/ACA snoRNAs, which direct pseudouridylation (Brown et al., 2003; Kiss, 2002; Tollervey, 1996). SnoRNA trafficking is poorly defined even in the nucleus itself. By definition, the localization of snoRNAs is in nucleolus, where the ribosomes are synthesized and assembled (Narayanan et al., 1999; Samarsky et al., 1998; Verheggen et al., 2001). SnoRNA trafficking outside of the nucleus has never been reported. Because AtRAP is a chloroplast-localized RNA-binding protein and anterograde signaling from the nucleus is required due to the the limited chloroplast, we speculated that these nucleus-encoded RNAs, specifically the snoRNAs, may have been transported into the chloroplast.

To test our hypothesis, the foremost and crucial issue we had to address was improving the chloroplast isolation method to remove all nuclear and cytoplasmic contamination and obtain ultra-pure chloroplasts. We modified the traditional isopycnic centrifugation method by using eight-layer miracloth filtering, two or more Percoll centrifugations, and very selective removal of the central layer of intact chloroplasts after centrifugation (Seigneurin-Berny et al., 2008). From microscope determination, most isolated chloroplasts were intact (Fig. 3.3a). The RNAs of chloroplasts were then extracted and detected by methylene blue staining. As seen in the stained blot, both nucleus-encoded *25S rRNA* and *18S rRNA* were eliminated in the chloroplast RNA (Fig. 3.3b). We then applied more sensitive northern blot analysis and chose *18S rRNA* which is one of the most abundant nuclear RNAs to detect chloroplast RNA quality. We did not detect *18S rRNA* by northern blot analysis (Fig. 3.3c). This suggests that there was no nuclear or cytoplasmic contamination in our isolated chloroplasts and that our improved method is selective enough to obtain ultra-pure chloroplasts. Since we were able to obtain chloroplasts with high integrity and purity, we were able to test whether nucleus-encoded snoRNAs are transported into chloroplasts and to determined whether AtRAP is involved in the trafficking process. Using this isolation method, pure chloroplasts were obtained from WT and *atrap-1* plants. Many highly abundant snoRNAs identified from AtRAP-IP RNA-seq were also detected in abundance from chloroplast RNA-seq from WT plants, but a low abundance was detected from *atrap-1* mutants (Table 3.2). Based on the deep-sequencing results, we hypothesized that these nucleus-encoded snoRNAs are potentially imported into chloroplasts and AtRAP may participate this in process.

Next, we performed Northern blot analysis to detect whether nucleus-encoded snoRNAs identified from chloroplast WT RNA-seq (Table 3.2) localize in isolated chloroplast fraction. SnoR Z37, which was highly expressed both in chloroplast WT RNA-seq and AtRAP-IP RNA-seq, SnoR 77Y, which was highly expressed in chloroplast WT RNA-seq, and SnoR 68, which was higly expressed in AtRAP-IP RNA-seq, were three candidates from table S2. All three could be detected in isolated chloroplast RNA (Fig. 3.4b, 3.4c, 3.4d). The snoRNA Northern blot analysis, *18S rRNA* Northern blot analysis, and methylene blue staining in each panel were all performed on the same blot to ensure the purity of the chloroplasts and quality of chloroplast RNA. Moreover, lower levels of *SnoR Z37*, *SnoR 68*, and *SnoR 77Y* were detected from the *atrap-1* chloroplast fraction compared with the Col-0 WT chloroplast fraction. Taken together, the results indicate that nucleus-encoded snoRNAs are indeed trafficked to the chloroplast, and AtRAP plays an important role in this trafficking process (Fig. 3.4b, 3.4c, 3.4d). Furthermore, because the snoRNAs were only reduced but not completely abolished in the *atrap-1* chloroplast fraction, it is very likely there are other components, besides AtRAP, which participate in snoRNA trafficking.

Next, we confirmed whether AtRAP functions in snoRNA trafficking into chloroplasts using the AtRAP-ΔSP-GFP transgenic plants, in which protein does not localize in the chloroplast, and the AtRAP-ΔRAP-GFP transgenic plants, in which the protein have lost most of its binding activity (Fig. 3.1b, 3.1c, 3.4a). Chloroplasts were isolated from these plants and RNA was extracted. Northern blot analysis revealed that chloroplast-localized nucleus-encoded snoRNAs levels were largely decreased in both transgenic lines compared to Col-0 WT chloroplasts, which was similar with *atrap-1* (Fig. 3.4b, 3.4c, 3.4d). It suggests that both AtRAP transportation into chloroplasts and AtRAP binding ability are essential for snoRNA trafficking. Therefore, we further characterized AtRAP as a significant component in snoRNA trafficking.

To rule out the possibility that the detected snoRNAs are adhering to the surface of the chloroplast outer membrane, we treated the isolated chloroplasts with Micrococcal nuclease (MNase), which degrades single and double stranded RNA and DNA and eliminates any nuclear and cytoplasmic RNA contamination attached to the outer membrane of chloroplasts. We were still able to detect the snoRNAs after treatments ranging a low amount (1 U) to a high amount (100 U) of MNase (Fig. 3.5a, 3.5b, 3.5c), indicating the snoRNAs are indeed inside the chloroplasts.

Next, we addressed what is the function of these snoRNAs that traffick into chloroplasts. The box C/D snoRNAs mainly direct 2'-*O*-ribose methylation, and box H/ACA snoRNAs mainly direct pseudouridylation. The three snoRNAs which we detected among chloroplast RNAs using Northern blot (SnoR Z37, SnoR 77Y, SnoR 68) (Fig. 3.4 and Fig. 3.5) are C/D box snoRNAs. Thus, we hypothesized that there may be snoRNA targets in chloroplasts, and our future direction include testing whether these chloroplast-localized nucleus-encoded snoRNAs functionally direct methylation of chloroplast RNAs. First, we searched for potential targets of these snoRNAs using "SnoScan," a program for predicting box C/D class snoRNAs and their corresponding targets. Here, we used the chloroplast genome as the snoRNA input target pool. Using the box C and D structure analysis, target sequence complementary base pairing comparison, the relative distance between the snoRNA features within snoRNAs, and other criteria, we could predict the snoRNA targets in chloroplasts. A target score of 12 or higher was the cut-off for acceptable target sequences. Table 3.3 showed predicted chloroplast targets of snoRNAs from chloroplast WT RNA-seq. Table S4 showed chloroplast target sequences of three box C/D snoRNAs: SnoR Z37, SnoR 77Y, and SnoR 68. To our surprise, some of these snoRNAs are predicted to target chloroplast protein-coding mRNAs, even though in the nucleus most snoRNAs modify rRNAs. Interestingly, several protein-coding mRNAs are photosystem II-related RNAs, which may explain why *atrap-1* shows the virescent phenotype (Table 3.3, Table 3.4).

To experimentally test the function of these snoRNAs in the methylation of the predicted targets, we performed primer extension experiments. We adapted and modified the method used to detect nuclear RNA modification in animal and yeast systems for the plant system. Reverse transcription of the three snoRNA targets: *23S rRNA*, *Photosynthetic electron transfer A* (*PETA*) mRNA, and the *Photosystem II reaction center protein D*

(PSBD) mRNA were done using a ^{32}P end-labeled primer with a low dNTP concentration (0.004 mM). Transcription was terminated at the methylation site, resulting in a smaller-sized band after electrophoresis, which could be detected by autoradiography. Reverse transcription proceeded to completion beyond the methylation sites, in high dNTP concentration conditions (1 mM) (Figure 3.6a). The results indicated that reverse transcription of *23S rRNA* (the putative target of chloroplast-localized *SnoR Z37*), *PETA* (the putative target of chloroplast-localized *SnoR 77Y*), and *PSBD* (the putative target of chloroplast-localized *SnoR 68*) all stopped at the methylation target site, resulting in a 30-nt, 30-nt, and 100-nt bands, respectively, in the WT RNA samples (Fig 3.6b, 3.6c, 3.6d). This suggests that chloroplast-localized snoRNAs do function in the methylation of chloroplast-encoded RNA. The methylation of *23S* rRNA, *PETA* mRNA, and *PSBD* mRNA was abolished, reduced, or slightly reduced, respectively, in the *atrap-1* RNA samples. Taken together, the nucleus-encoded snoRNAs do methylate chloroplast-encoded RNAs and AtRAP is important for the methylation function.

Discussion

While there is evidence from plant and mammalian studies showing RNA is imported into mitochondria, there is not clear evidence showing whether RNAs, especially non-coding RNAs can import into chloroplasts and what is the function of these potential imported RNAs. Here, we provide evidence that nucleus-encoded snoRNAs can travel into chloroplasts and methylate chloroplast RNAs. The modified chloroplast RNA could be essential for choroplat RNA metabolism (Stern et al., 2010). Additionally, we discovered a chloroplast-localized RNA binding protein-AtRAP which facilitates the snoRNA import process. Overall, our study sheds light on RNA trafficking between organelles and paves the way for us to engineer RNAs to be imported into chloroplast (Maliga, 2002).

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana plants were grown at 23 °C in a controlled growth room with a 12-light/12-dark photoperiod. The *Arabidopsis* knockout mutant *atrap-1* (CS_844807) was obtained from Arabidopsis Biological Resource Center (ABRC).

Generation of transgenic plants

The native promoter-driven transgenic plants were generated by fusion of the *AtRAP* promoter with *AtRAP* CDS, *AtRAP-ΔSP* CDS (Δ1-78AA), *AtRAP-ΔRAP* CDS (Δ607-665AA), or *AtRAP-ΔOPR* CDS (Δ354-415AA). The fused fragment was cloned into the *PMDC107* destination vector with a C-terminal GFP tag (Curtis and Grossniklaus, 2003). Mutant *atrap-1* plants were transformed using the floral dip method with *Agrobacterium tumefaciiens* strain GV3101 carrying the cloned vectors. The transgenic plants were selected on Murashige-Skoog medium supplemented with 20 μg/ml hygromycin B.

Confocal microscopy

The subcellular localization of AtRAP-GFP and AtRAP-ΔSP-GFP were observed using a Leica SP5 laser-scanning confocal microscope (Leica, Germany). The GFP excitation wavelength was 488 nm, and the RFP excitation wavelength was 594 nm.

RNA immunoprecipitation (RIP) of AtRAP-GFP and AtRAP-ΔRAP-GFP

Transgenic *atrap-1* plants with AtRAP-GFP and AtRAP-ΔRAP-GFP constructs driven by the native AtRAP promoter were collected and ground into fine powder in liquid nitrogen and homogenized with 5 ml/g of extraction buffer (20 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 300 mM NaCl, 5 mM Dithiothreitol, 0.5% Triton X-100, 1 tablet/50 ml Protein Inhibitor Cocktail [Roche]). After removing cell debris, GFP antibody (Roche) and Protein A Agarose (Roche) were added, and the solution was rotated for 8 hours at 4 °C. The immunocomplexes were then precipitated and washed before the RNA was extracted using TRIzol reagent (Invitrogen). The RNA samples were treated with Calf Intestine Alkaline Phosphatase (CIAP) (Invitrogen), then with or without RNase A (Thermo Fisher Scientific), and subsequently labeled with radioisotope ATP (γ-^{32}P) (Perkin Elmer) using T4 polynucleotide kinase (NEB) and purified on MicroSpin G-25 Columns (GE Healthcare). The polyacrylamide gels were then used for labeled RNA samples electrophoresis.

Chloroplast isolation

The traditional isopycnic centrifugation method for chloroplast isolation was modified to improve chloroplast quality (Seigneurin-Berny et al., 2008). 4-week-old *Arabidopsis* plants totaling 100 g of tissue were pre-cooled overnight at 4 °C to prevent the starch grains from damaging the chloroplasts during centrifugation. The *Arabidopsis* leaves were harvested and homogenized using a grinding medium (pH 8.4) (0.4 M sorbitol, 20 mM Tricine buffer (pH 8.4), 10 mM EDTA (pH 8.0), 10 mM $NaHCO_3$, 0.1% w/v BSA) (Seigneurin-Berny et al., 2008). The homogenate was filtered using 8-layer miracloth, collected, and separated using a Percoll gradient. The middle layer of intact chloroplasts was extracted and separated again using a Percoll gradient. The layer of intact chloroplasts layer was extracted, washed, and pelleted. RNA and protein was extracted from the chloroplasts.

Northern blot analysis

RNA samples were fractionated by electrophoresis in 1.5% formaldehyde agarose gel and transferred to Hybond N^+ membranes (GE Healthcare). Probes were made from full length snoRNA PCR products which were randomly labeled with radioisotope dCTP (α-^{32}P) (Perkin Elmer) using Prime-It II Random Primer Labeling Kit (Agilent). After UV cross-linking, hybridization to the probes was performed using PerfectHyb Plus hybridization buffer (Sigma). The sequences of the snoRNAs are listed in table 3.5.

Chloroplast RNA-seq and AtRAP-IP RNA-seq

For chloroplast RNA-seq, 4-week-old Col-0 wild-type (WT) and *atrap-1* mutant plants were applied to chloroplast extraction and subjected to RNA extraction by TRIzol reagent

(Invitrogen). The chloroplast RNA was then treated with Ribo-Zero rRNA Removal Kit of Plant (Illumina) and subjected to RNA-seq following "NEBNext Ultra Directional RNA Library Prep Kit for Illumina" (NEB).

For AtRAP-IP RNA-seq, 4-week-old AtRAP-GFP transgenic plants under native promoter in *atrap-1* mutant background were applied to RIP. RNAs associated with AtRAP were extracted by TRIzol reagent (Invitrogen) and subjected to RNA-seq following "Illumina TruSeq RNA Sample Prep kit" (Illumina).

Accession numbers

The RNA-seq datasets were deposited to NCBI. The accession numbers are PRJNA394900, PRJNA394903, and PRJNA394905 (not released).

Nuclease treatment assay

The nuclease treatment assay was performed as previously described (Wang et al., 2010). 1 U, 10 U, and 100 U Micrococcal nuclease (MNase) was applied in the nuclease treatment assay. The intact isolated chloroplasts were pelleted and re-suspended in MNase buffer (0.6 M sorbitol, 20 mM $MgCl_2$, 5 mM $CaCl_2$, 20 mM Tris (pH 8.0) for 30 min at 27°C. The nuclease treatment was stopped by supplementing with 20 mM EGTA, and the treated chloroplasts were separated in a Percoll gradient using centrifugation. The pure chloroplasts were collected and RNA was extracted.

Primer extension assay

WT and *atrap-1* RNA was extracted using RNeasy Mini Kit (Qiagen). The RNA samples were treated with DNase I (Roche) and concentrated using RNAclean XP (Beckman Coulter). Primer extension was performed on the RNA using Primer Extension System (Promega). The target primers were end-labeled using radioisotope ATP (γ-^{32}P) (Perkin Elmer) following the manufacturer's protocol. The target primer sequences are listed in table 3.5.

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Figures and Tables

Figure 3.1 AtRAP is a chloroplast-localized protein.
(a) Phenotypes of transgenic plants compared with Col-0 WT and *atrap-1* mutants. From left to right: Col-0 WT, *atrap-1*, AtRAP-GFP, AtRAP-ΔSP-GFP, AtRAP-ΔRAP-GFP, and AtRAP-ΔOPR-GFP. The transgenic plants express native AtRAP promoter driven vectors in the *atrap-1* mutant background. (b) Western blot of AtRAP-GFP, *atrap-1*, AtRAP-ΔSP-GFP, and AtRAP-ΔRAP-GFP from both total protein and chloroplast protein. The tubulin control shows that the nuclear/cytoplasmic contamination of chloroplast protein is largely reduced. (c) AtRAP is localized in the chloroplast, and the signaling peptide (SP) is required for the subcellular localization. Confocal images show GFP fluorescence in mesophyll cells of transgenic plants expressing AtRAP-GFP, AtRAP-ΔSP-GFP, and AtRAP-ΔRAP-GFP. Chloroplasts emit red fluorescence. Bar = 25 μm.

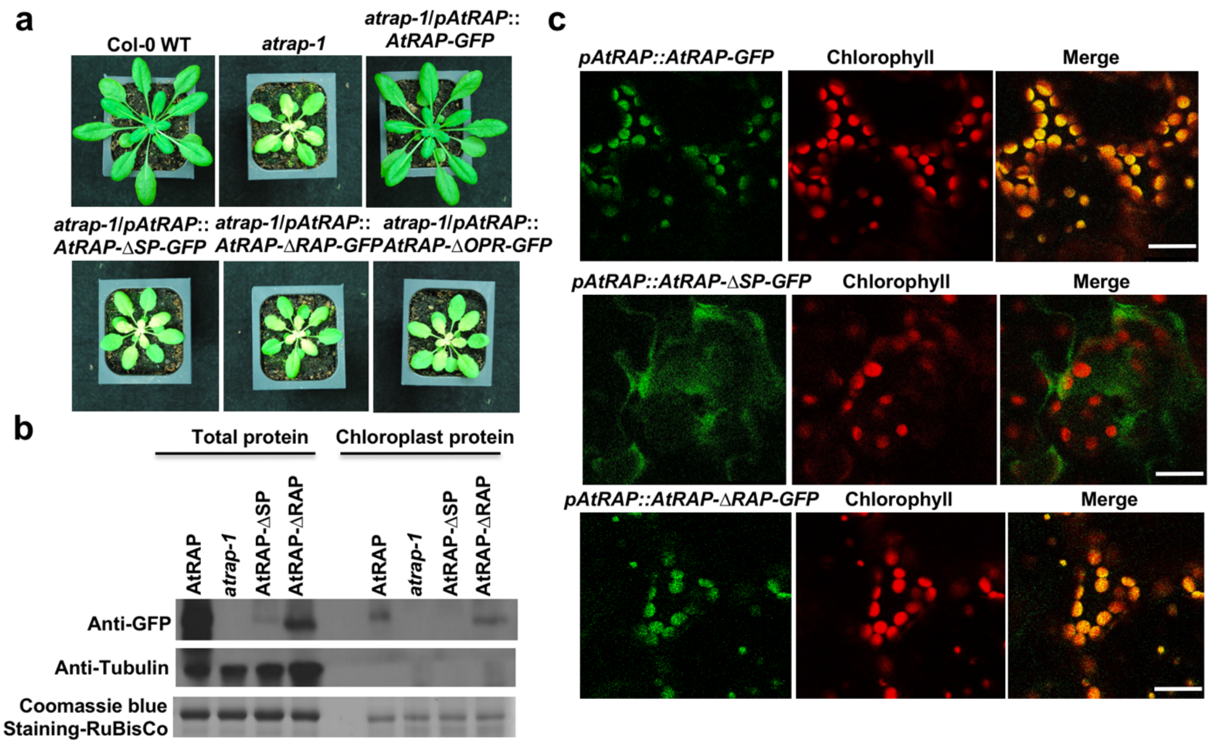
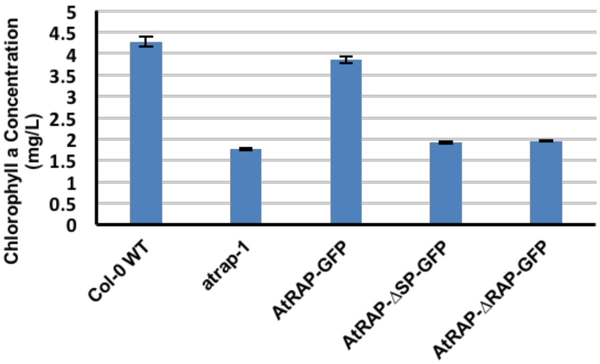

a
Col-0 WT
atrap-1
atrap-1/pAtRAP::AtRAP-GFP
atrap-1/pAtRAP::AtRAP-ΔSP-GFP
atrap-1/pAtRAP::AtRAP-ΔRAP-GFP
atrap-1/pAtRAP::AtRAP-ΔOPR-GFP
b
Total protein
Chloroplast protein
AtRAP
atrap-1
AtRAP-ΔSP
AtRAP-ΔRAP
AtRAP
atrap-1
AtRAP-ΔSP
AtRAP-ΔRAP
Anti-GFP
Anti-Tubulin
Coomassie blue Staining-RuBisCo
c
pAtRAP::AtRAP-GFP
Chlorophyll
Merge
pAtRAP::AtRAP-ΔSP-GFP
Chlorophyll
Merge
pAtRAP::AtRAP-ΔRAP-GFP
Chlorophyll
Merge

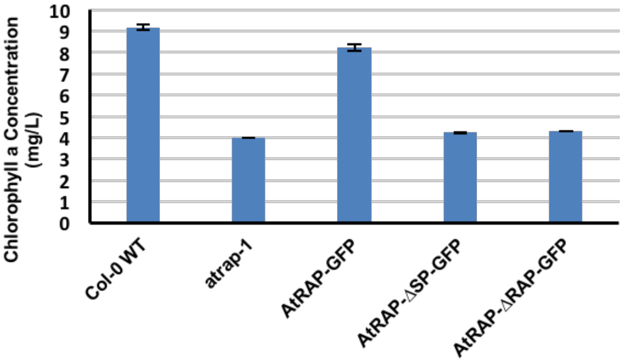
Figure 3.2. The chlorophyll parameters of the AtRAP-GFP, AtRAP-ΔSP-GFP and AtRAP-ΔRAP-GFP transgenic plants.

The chlorophyll parameters of chlorophyll a concentration (a), chlorophyll b concentration (b) and chlorophyll a+b content (c) were measured. There are obvious defects of chlorophyll in the *atrap-1* mutant plants. The AtRAP-ΔSP-GFP and AtRAP-ΔRAP-GFP transgenic plants show similar chlorophyll defects as the *atrap-1* mutant plants.

a


Chlorophyll a Concentration (mg/L)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Col-0 WT
atrap-1
AtRAP-GFP
AtRAP-∆SP-GFP
AtRAP-∆RAP-GFP

b


Chlorophyll a Concentration (mg/L)
10
9
8
7
6
5
4
3
2
1
0
Col-0 WT
atrap-1
AtRAP-GFP
AtRAP-∆SP-GFP
AtRAP-∆RAP-GFP

c

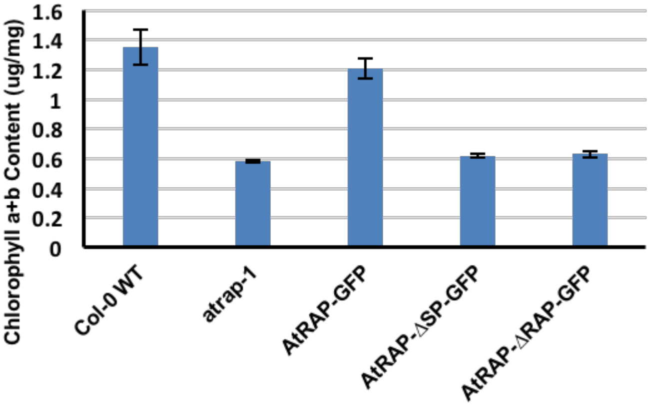
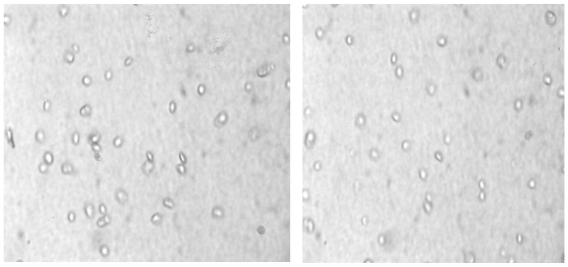

Chlorophyll a+b Content (ug/mg)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Col-0 WT
atrap-1
AtRAP-GFP
AtRAP-∆SP-GFP
AtRAP-∆RAP-GFP

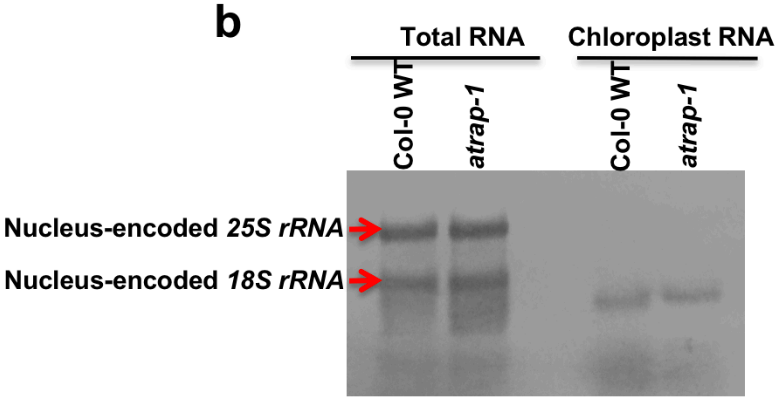
Figure 3.3. The determination of chloroplast quality.

(a) Microscope determination of chloroplast morphology after chloroplast isolation. Most chloroplasts are still intact. (b) Methylene blue staining of the total RNA and chloroplast RNA blot. The nucleus-encoded *25S rRNA* and *18S rRNA* is not present in chloroplast RNA while it is in the total RNA. (c) Northern blot analysis of the total RNA and chloroplast RNA blot. The nucleus-encoded *18S rRNA* was not detected in chloroplast RNA while it is in the total RNA.

a



b


Total RNA
Chloroplast RNA
Col-0 WT
atrap-1
Col-0 WT
atrap-1
Nucleus-encoded 25S rRNA
Nucleus-encoded 18S rRNA

c

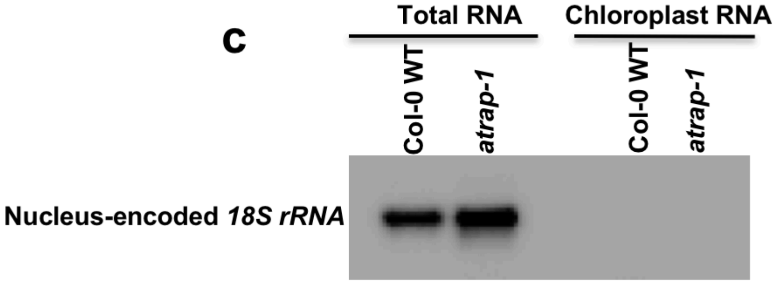

Total RNA
Chloroplast RNA
Col-0 WT
atrap-1
Col-0 WT
atrap-1
Nucleus-encoded 18S rRNA

Figure 3.4 Nucleus-encoded snoRNAs are imported into chloroplast.
(a) AtRAP is an RNA binding protein, and the RAP domain is indispensable for its binding activity. RNA molecules bound to AtRAP or AtRAP-ΔRAP from AtRAP-GFP or AtRAP-ΔRAP-GFP transgenic plants, respectively, were immunoprecipitated by GFP antibody, then subjected to radio-isotope labeling and electrophoresis. Polynucleotides bound to AtRAP showed by autoradiography signal. RNase treatment proved that polynucleotides which bound to AtRAP were RNA instead of DNA. (b, c, d) Northern blot analysis of nucleus-encoded snoRNAs on ultra-pure chloroplast RNA. Total and chloroplast RNA was extracted from Col-WT, *atrap-1*, AtRAP-ΔSP-GFP transgenic plants and AtRAP-ΔRAP-GFP transgenic plants, respectively. At least 60g plant material was applied to isolate chloroplast for each sample and only very inner part of intact chloroplast layer was taken for RNA extraction. The samples are loaded in 1.5% formaldehyde agarose gel. *18S RNA* which was detected in total RNA proved plants starting materials. The probes were SnoR Z37 (b), SnoR 77y (c) and SnoR 68 (d). Methylene blue staining and *18S rRNA* probed-blot were controls to prove the chloroplast RNA quality. The nucleus-encoded *25S rRNA* and *18S rRNA* were eliminated in methylene blue staining. The nucleus-encoded *18S rRNA* was not detected in chloroplast RNA. The snoRNA northern blot analysis, methylene blue staining and *18S rRNA* northern blot analysis are all from the same blot for each sample.

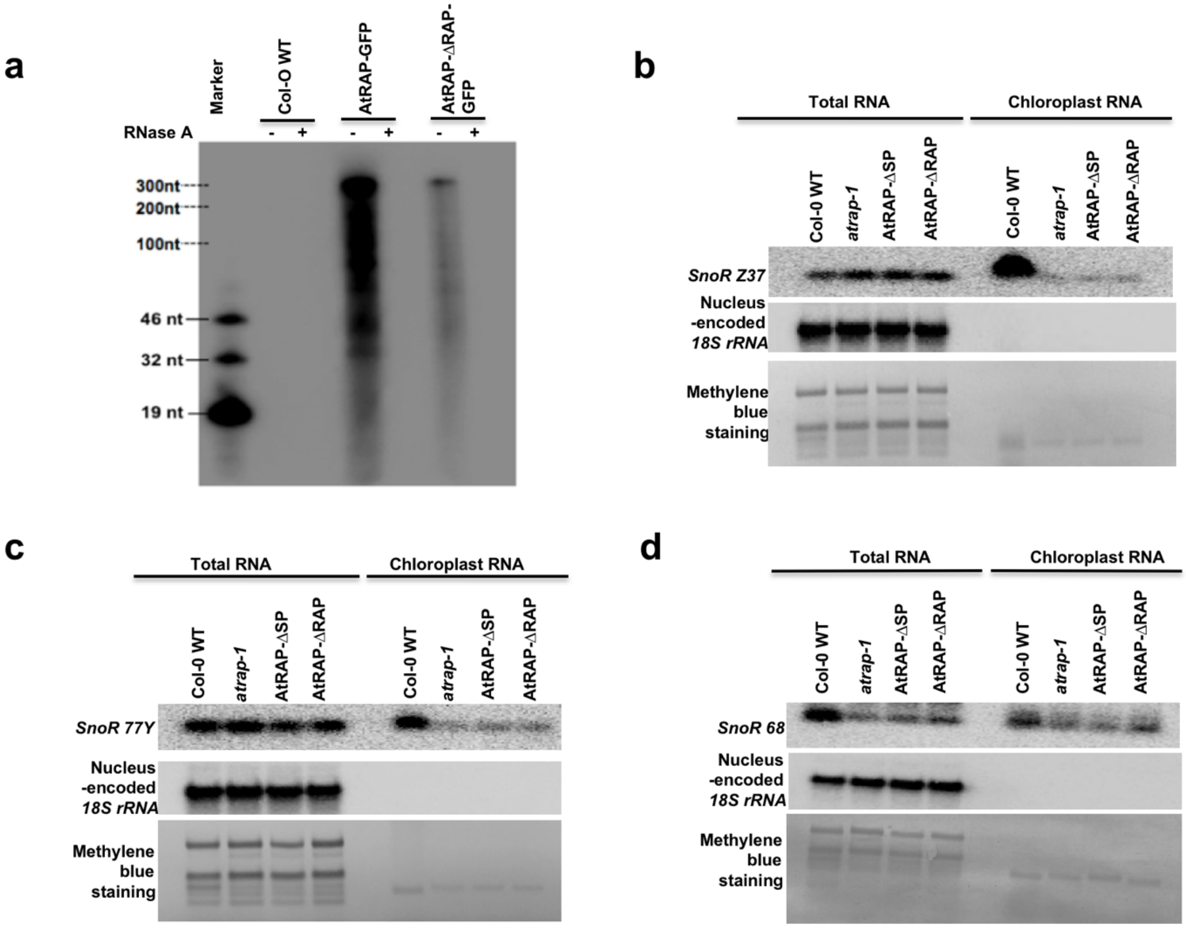

a
Marker
Col-O WT
AtRAP-GFP
AtRAP-ΔRAP-GFP
RNase A
- + - + - +
300nt
200nt
100nt
46 nt
32 nt
19 nt
b
Total RNA
Chloroplast RNA
Col-0 WT
atrap-1
AtRAP-ΔSP
AtRAP-ΔRAP
Col-0 WT
atrap-1
AtRAP-ΔSP
AtRAP-ΔRAP
SnoR Z37
Nucleus -encoded 18S rRNA
Methylene blue staining
c
Total RNA
Chloroplast RNA
Col-0 WT
atrap-1
AtRAP-ΔSP
AtRAP-ΔRAP
Col-0 WT
atrap-1
AtRAP-ΔSP
AtRAP-ΔRAP
SnoR 77Y
Nucleus -encoded 18S rRNA
Methylene blue staining
d
Total RNA
Chloroplast RNA
Col-0 WT
atrap-1
AtRAP-ΔSP
AtRAP-ΔRAP
Col-0 WT
atrap-1
AtRAP-ΔSP
AtRAP-ΔRAP
SnoR 68
Nucleus -encoded 18S rRNA
Methylene blue staining

Figure 3.5 Nucleus-encoded snoRNAs are inside of chloroplast.
Northern blot analysis of nucleus-encoded snoRNAs from extracted chloroplasts treated with Micrococcal nuclease (MNase) to remove any exterior nuclear and cytoplasmic RNAs. Chloroplast RNAs were extracted from Col-0 WT. Chloroplasts were isolated from at least 100 g of plant material was applied to isolate chloroplast, and only the most inner part of the intact chloroplast layer was extracted. The intact chloroplasts were treated with MNase, and then MNase was inactivated by EGTA. The samples were loaded on a 1.5% formaldehyde agarose gel. The blot was probed for SnoR Z37 (a), SnoR 77Y (b) and SnoR 68 (c). Methylene blue staining indicates the chloroplast RNA quality and quantity. Nucleus-encoded *25S rRNA* and *18S rRNA* are not present in the chloroplast RNA as seen in the methylene blue stained blot. The snoRNA northern blot analysis and methylene blue staining are from the same blot for each sample.

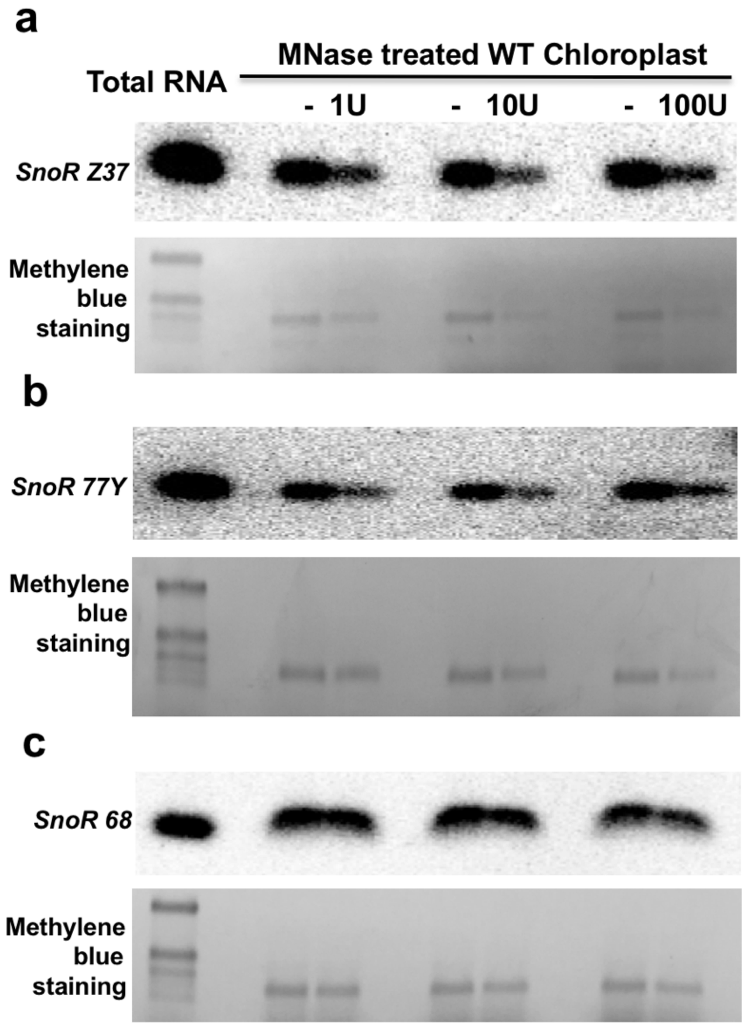

a
Total RNA
MNase treated WT Chloroplast
- 1U
- 10U
- 100U
SnoR Z37
Methylene blue staining
b
SnoR 77Y
Methylene blue staining
c
SnoR 68
Methylene blue staining

Figure 3.6 The chloroplast-localized snoRNAs regulate methylation of chloroplast-encoded RNA.
(a) Primer extension was used to detect methylation sites in predicted chloroplast-encoded RNA targets. The reverse primer was designed 30/100 bp from the predicted methylation site and subjected to ^{32}P end-labeling. During reverse transcription, in low dNTP concentration, transcription will terminate, resulting in a smaller-sized band after electrophoresis and autoradiography. Reverse transcription of SnoR Z37-targeted *23S rRNA* (b), SnoR 77Y-targeted *PETA* (c) and SnoR 68-targeted *PSBD* (d) in low dNTP concentration (0.004 mM) resulted in a shorter product, as revealed by the smaller-sized 30-nt/100-nt bands (red arrowheads). The methylation was abolished/reduced at the target site in *atrap-1*.

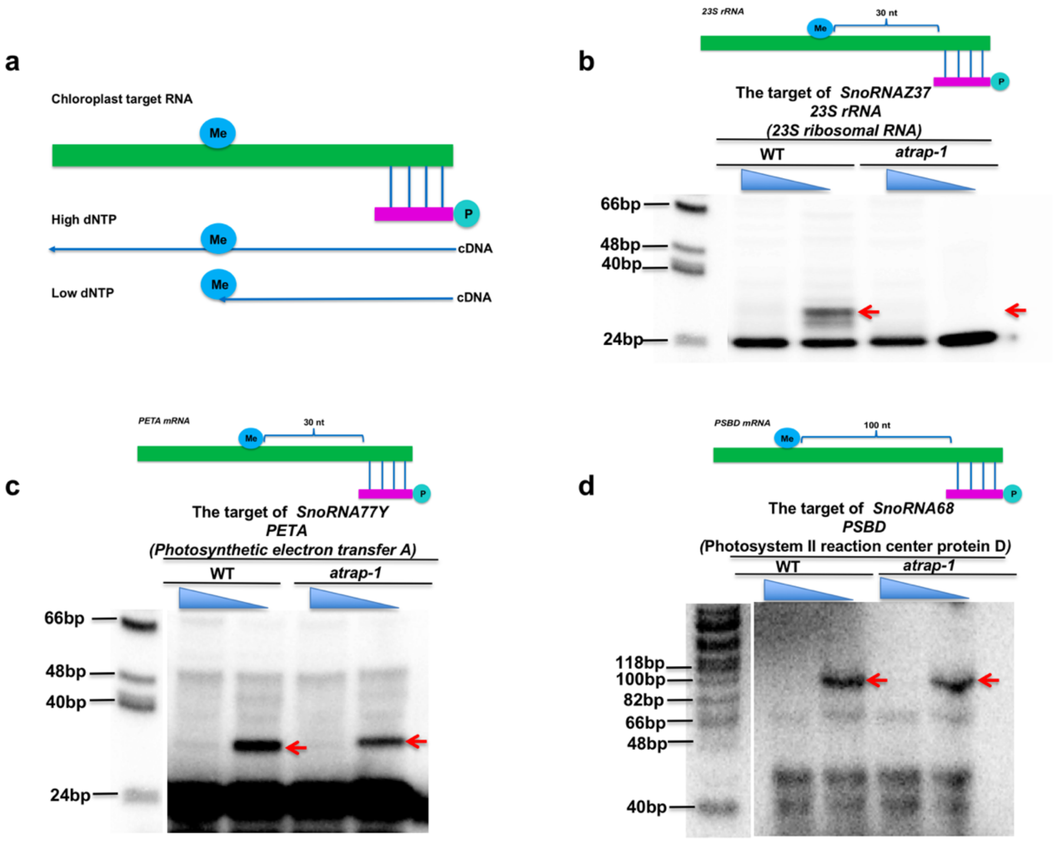

a
Chloroplast target RNA
Me
P
High dNTP
Me
cDNA
Low dNTP
Me
cDNA
b
23S rRNA
Me
30 nt
P
The target of SnoRNAZ37
23S rRNA
(23S ribosomal RNA)
WT
atrap-1
66bp
48bp
40bp
24bp
c
PETA mRNA
Me
30 nt
P
The target of SnoRNA77Y
PETA
(Photosynthetic electron transfer A)
WT
atrap-1
66bp
48bp
40bp
24bp
d
PSBD mRNA
Me
100 nt
P
The target of SnoRNA68
PSBD
(Photosystem II reaction center protein D)
WT
atrap-1
118bp
100bp
82bp
66bp
48bp
40bp

Table 3.1 Many nucleus-encoded RNAs are bound to AtRAP. 21 nucleus-encoded RNAs generated from TOP50 of AtRAP-IP RNA-seq were listed and ranked. 20 are annotated as snoRNAs. Normalized reads were given in FPKM (Fragments Per Kilobase of transcript per Million mapped reads).

AtRAP-IP			
Ranking	Normalized read counts (FPKM)	Gene	Annotation
1	39316978	ATCG00960	Chloroplast 4.5S ribosomal RNA
2	11837929	ATCG00950	Chloroplast 23S ribosomal RNA
3	8465874	ATCG00970	Chloroplast 5S ribosomal RNA
5	5337269	ATCG00920	Chloroplast 16S ribosomal RNA
7	939702	ATCG00020	Chlorophyll binding proten D1 (psbA)
8	522223	AT2G46192	Non-coding RNA-SnoR 68
9	507579	AT2G01021	Encodes Unknown protein
10	456076	ATCG00490	Encodes Large subunit of RUBISCO (RbcL)
11	135685	ATCG00280	Encodes CP43, a subunit of PSII reaction center (PSBC)
12	129496	AT2G35750	Encodes Unknown protein-SnoR Z37
14	91412	AT1G31860	Encodes A histidine biosynthesis bifunctional proteinprotein-SnoR 79
19	66593	AT1G27400	Encodes Ribosomal protein L22p/L17e family protein-SnoR 74
21	63420	AT1G72645	Encodes Unknown protein—SnoR 84
23	59291	AT5G57870	Encodes MIF4G domain-containing protein-SnoR103
25	49564	AT5G41010	Encodes DNA directed RNA polymerase-SnoR U19
26	49017	AT5G08180	Encodes Ribosomal protein L7Ae/L30e/S12e family protein-SnoR 100
27	39853	AT5G10572	Small nucleolar RNA 77
28	37454	AT2G47170	Encodeds Ras-related small GTP-binding family protein-SnoR 73
29	36397	AT3G02832	Non-coding RNA-SnoR 78
31	28887	AT3G47342	Small nucleolar RNA 19
34	28271	AT1G04480	Encodes Ribosomal protein L14p/L23e family protein-SnoR 155

39	18952	AT1G29465	Encodes Unknown protein-SnoR 5a
40	17925	AT1G26230	Encodes TCP-1/cpn60 chaperonin family protein-SnoR 95
41	17644	AT4G17390	Encodes Ribosomal protein L23/L15e family protein-SnoR 151
43	16264	AT2G33430	Encodes Differentiation and greening-like 1-SnoR 90
44	16227	AT1G67430	Encodes Ribosomal protein L23/L15e family protein-SnoR 74
47	14535	AT1G29418	Encodes Unknown protein-SnoR 5
48	13770	AT5G15022	Non-coding RNA-SnoR 13

Table 3.2 Nucleus-encoded RNAs which are bound to AtRAP are present in chloroplast fraction. 23 snoRNAs which are generated from TOP150 of chloroplast WT RNA-seq were listed and ranked. Normalized reads were given in FPKM (Fragments Per Kilobase of transcript per Million mapped reads). The corresponding ranking and normalized read counts in chloroplast *atrap* RNA-seq and AtRAP-IP RNA-seq are listed as well.

Chloroplast TOP150 SnoRNA Comparison							
Gene	Generated snoRNAs	Chloroplast WT		Chloroplast *atrap*		AtRAP-IP	
	Annotation	Ranking	Normalized read counts (FPKM)	Ranking	Normalized read counts (FPKM)	Ranking	Normalized read counts (FPKM)
AT2G05765	SnoR 77Y	1	65639.5	N/A	0	28	7560
AT4G39366	SnoR U14d-2	2	16688.7	5	932.4	50	2576
AT2G35750	SnoR Z37	3	14602.4	2	2721.3	2	129496
AT4G39364	SnoRNA	4	13520.6	7	721.1	N/A	N/A
AT3G01313	SnoR 58Y-1	5	9782.1	N/A	0	27	8125
AT4G39363	SnoR 14-3	6	9648.2	8	399.6	41	3359
AT3G24615	SnoRNA	7	7704.4	4	968.9	N/A	N/A
AT3G47348	SnoR 38	8	6707.1	3	2164.3	49	2604
AT3G21805	SnoR 37	9	5481.6	N/A	0	33	5512
AT2G20721	SnoRNA	10	2740.8	N/A	0	64	1283
AT3G27865	SnoR 1	11	2426	6	815.5	63	1289
AT4G39361	SnoR 14	12	1955.7	11	177.6	65	1279
AT1G12013	SnoR 111	13	1880.4	9	397	21	12084
AT5G54075	SnoRNA U3D	14	1762	10	179.1	N/A	N/A
AT1G29071	SnoR 105	15	1213.7	N/A	0	N/A	N/A
AT5G41471	SnoR 108	16	1212.2	N/A	0	N/A	N/A

AT5G53902	SnoR U3B	17	694.2	13	63.8	N/A	N/A
AT5G51174	SnoR 30	18	538.5	N/A	0	N/A	5773
AT1G72645	SnoR 84	19	373.2	N/A	47.6	5	63420
AT1G68945	SnoR 102	20	367.7	N/A	46	68	1105
AT1G26233	SnoR 95	21	324.9	12	92.2	N/A	N/A
AT2G46192	SnoR 68	22	318.3	14	57.2	1	522223
AT5G10572	SnoR 77	23	208.4	N/A	0	9	39853

Table 3.3 The target prediction of chloroplast RNA-seq-generated snoRNAs are listed. The target prediction of 23 SnoRNAs which are generated from TOP150 of chloroplast WT RNA-seq was listed, annotated and scored. The snoRNA targets were separately by "in chloroplast" and "in nucleus." The chloroplast photosystem II related targets were in bold and green color. The target prediction software was "SnoScan", and the cutoff was "12" (Lowe and Eddy, 1999).

Gene	Generated snoRNAs annotation	SnoRNA targets prediction in chloroplast		
		Target	Target annotation	Score
AT2G 05765	SnoR 77Y-1	ATCG00540	PETA-photosynthetic electron transfer A	15.7
		ATCG01280/ ATCG00860	YCF2.2-Chloroplast Ycf2	15.2
AT4G 39366	SnoR U14d	ATCG01180/ ATCG00950	rRNA23S-Chloroplast-encoded 23S ribosomal RNA	13.6
AT2G 35750	SnoR Z37	ATCG01180/ ATCG00950	rRNA23S-Chloroplast-encoded 23S ribosomal RNA	16.3
		ATCG00680	PSBB-PHOTOSYSTEM II REACTION CENTER PROTEIN B	15.6
AT3G 01313	SnoR 58Y-1	ATCG01310/ ATCG00830	RPL2.2-ribosomal protein L2	12.3
AT3G 24615	SnoRNA 32-1	ATCG00720	PETB-photosynthetic electron transfer B	13.3
AT3G 47348	SnoR 38	ATCG00900	RPS7-Ribosomal protein S7p/S5e family protein	14.5
		ATCG01310/ ATCG00830	RPL2.2-ribosomal protein L2	13.2
AT3G	SnoR 37	ATCG01000/	YCF1.1-Ycf1 protein	14.8

21805		ATCG01130		
		ATCG00490	RBCL-ribulose-bisphosphate carboxylases	13.1
AT2G 20721	SnoR U34c	ATCG00280	PSBC-photosystem II reaction center protein C	15.1
		ATCG01310/ ATCG00830	RPL2.2-ribosomal protein L2	13.1
AT3G 27865	SnoR 1	ATCG01300/ ATCG00840	RPL23.2 | ribosomal protein L23	18.6
		ATCG00960/ ATCG01170	rRNA4.5S-Chloroplast-encoded 4.5S ribosomal RNA	16
AT1G 29071	SnoR 105	ATCG00650	RPS18-ribosomal protein S18	17.5
		ATCG01250/ ATCG00890	NDHB.2-NADH-Ubiquinone/plastoquinone (complex I) protein	13.9
AT5G 51174	SnoR 30	ATCG01180/ ATCG00950	rRNA23S-Chloroplast-encoded 23S ribosomal RNA	13.9
		ATCG01310/ ATCG00830	RPL2.2-ribosomal protein L2	12.5
AT1G 68945	SnoR 102	ATCG00270	PSBD-photosystem II reaction center protein D	17.2

		ATCG01280/ATCG00860	YCF2.2-Chloroplast Ycf2	14.7
AT2G46192	SnoR 68	ATCG00270	PSBD-Photosystem II reaction center protein D)	13.6
AT4G39364	SnoR U14c	No predicted target (Cutoff score: 12)		
AT4G39363	SnoR U14b	No predicted target (Cutoff score: 12)		
AT4G39361	SnoR U14a	No predicted target (Cutoff score: 12)		
AT1G12013	SnoR 111	No predicted target (Cutoff score: 12)		
AT5G54075	SnoR U3D	No predicted target (Cutoff score: 12)		
AT5G41471	SnoR 108	No predicted target (Cutoff score: 12)		
AT5G53902	SnoR U3B	No predicted target (Cutoff score: 12)		
AT1G72645	SnoR 84	No predicted target (Cutoff score: 12)		
AT1G	SnoR 95	No predicted target (Cutoff score: 12)		

26233		
AT5G10572	SnoR 77	No predicted target (Cutoff score: 12)

Table 3.4 The target sequence prediction of chloroplast-localized snoRNAs in chloroplast are listed. SnoRZ37, SnoR 77Y, and SnoR68 which are three experimental confirmed nucleus-encoded and chloroplast-localized snoRNAs were applied to target sequence prediction (Lowe and Eddy, 1999). The target sequences were listed, and the modification sites were in bold and red color.

SnoRNA	SnoRNA generated from Gene	Chloroplast target	Target score	Target modification site
SnoR Z37 (C/D)	AT2G35750 (unknown protein)	ATCG00950 (Chloroplast encoded 23S rRNA)	16.3	GUCCC**G**AGGGACGG
SnoR 77Y (C/D)	AT2G05765 (SnoR77Y)	ATCG00540 (PETA: Photosynthetic electron transfer A)	15.7	AACGG**U**ACUUCC
SnoR 68 (C/D)	AT2G46192 (non-coding RNA)	ATCG00270 (PSBD: Photosystem II reaction center protein D)	13.6	CGA**U**CUGUUCAAUUGCGA

Table 3.5 The oligos and primers which were used in this study are listed.

Primer	Sequence
AtRAP Native Promoter F	CACCAGGAAGAGAAACGTCGGAGAGAAAC
AtRAP Native Promoter R	GCCGGAATGGAACTACACACTCCATcttcttctatctgagaattttaatg
AtRAP Native Promoter R'	CCGAGTTACCAATCACAGACGCCATcttcttctatctgagaattttaatg
AtRAP CDS F	cattaaaattctcagatagaagaagATGGAGTGTGTAGTTCCATTCCGGC
AtRAP CDS R	TATGCAGCCGGTGAGAATCTCCCTC
AtRAP-ΔSP CDS F	cattaaaattctcagatagaagaagATGGCGTCTGTGATTGGTAACTCGG
AtRAP-ΔSP CDS R	TATGCAGCCGGTGAGAATCTCCCTC
AtRAP-ΔRAP CDS F	cattaaaattctcagatagaagaagATGGAGTGTGTAGTTCCATTCCGGC

AtRAP-ΔRAP CDS R	TATGCAGCCGGTGAGAATTTTCTTCTCGACCAGA
AtRAP-ΔOPR CDS F1	cattaaaattctcagatagaagaagATGGAGTGTGTAGTTCCATTCCGGC
AtRAP-ΔOPR CDS R1	AGGCAAATGACCACAATAGTTGAGCCAATTCACCTCCAATTTTGGATAAT
AtRAP-ΔOPR CDS F2	ATTATCCAAAATTGGAGGTGAATTGGCTCAACTATTGTGGTCATTTGCCT
AtRAP-ΔOPR CDS R2	TATGCAGCCGGTGAGAATCTCCCTC
SnoRZ37 Full length	TAGCCAATGAGGACATCAGATTATAAAAGAACACCATCTTTCGGGACTGATTAACATTGATGATACTTCTGCAATTCTGAGGCTA
SnoR68 Full length	GTTAATATCGGAAATAAACAGATCGTGGGACAAATCTCGTCTCGCGAGTTGATGTCCGATAATATTATGGTTCGTATTCGCTGAG
SnoR77Y	ACCGATGATGATTATTGCTAAACTATGGAATTACCGTCTGAG

Full length	TTTATTCTTTGACGAGAAAAACGCTGGCTTACTGAGG
SnoRZ37 F	TAGCCAATGAGGACATCAGATTATA
SnoRZ37 R	TAGCCTCAGAATTGCAGAAGTATCA
SnoR68 F	GTTAATATCGGAAATAAACAGATCG
SnoR68 R	CTCAGCGAATACGAACCATAATATT
SnoR77Y F	ACCGATGATGATTATTGCTAAAC
SnoR77Y R	CCTCAGTAAGCCAGCGTTTTTCT
23S Primer extenison R	ATTTTCTCTACCCCTTCTTACCCTG
PSBD Primer extenison R	ATCGAAATATAGCCGCTACACCAAA
PETA Primer extenison R	TTGTTTTAGTTGCATATCATAAGGA

Primer	Purpose
AtRAP Native Promoter F	Amplify AtRAP Native Promoter (with CACC for pENTR cloning)
AtRAP Native Promoter R	Amplify AtRAP Native Promoter (with overlapping PCR primer to fuse AtRAP-CDS/AtRAP-ΔRAP/AtRAP-ΔOPR CDS)
AtRAP Native Promoter R'	Amplify AtRAP Native Promoter (with overlapping PCR primer to fuse AtRAP-ΔSP CDS)
AtRAP CDS F	Amplify AtRAP CDS (with overlapping PCR primer to fuse Native Promoter; same with AtRAP-ΔRAP CDS F and AtRAP-ΔOPR CDS F1)
AtRAP CDS R	Amplify AtRAP CDS (without stop codon for GFP tag translation; same with AtRAP-ΔSP CDS R and AtRAP-ΔOPR CDS R2)
AtRAP-ΔSP CDS F	Amplify AtRAP-ΔSP CDS (with ATG for starting translation; with overlapping PCR primer to fuse Native Promoter)
AtRAP-ΔSP CDS R	Amplify AtRAP-ΔSP CDS (without stop codon for GFP tag translation)
AtRAP-	Amplify AtRAP-ΔRAP CDS (with overlapping PCR primer to fuse

ΔRAP CDS F	Native Promoter)
AtRAP-ΔRAP CDS R	Amplify AtRAP-ΔRAP CDS (without stop codon for GFP tag translation)
AtRAP-ΔOPR CDS F1	Amplify AtRAP-ΔOPR CDS first part (with overlapping PCR primer to fuse Native Promoter)
AtRAP-ΔOPR CDS R1	Amplify AtRAP-ΔOPR CDS first part (with overlapping PCR primer to fuse AtRAP-ΔOPR first part and second part together)
AtRAP-ΔOPR CDS F2	Amplify AtRAP-ΔOPR CDS second part and (with overlapping PCR primer to fuse AtRAP-ΔOPR first part and second part together)
AtRAP-ΔOPR CDS R2	Amplify AtRAP-ΔOPR CDS second part (without stop codon for GFP tag translation)
SnoRZ37 Full length	SnoRNA sequence
SnoR68 Full length	SnoRNA sequence

SnoR77Y Full length	SnoRNA sequence
SnoRZ37 F	Amplify SnoRZ37 Full length for random labeling
SnoRZ37 R	Amplify SnoRZ37 Full length for random labeling
SnoR68 F	Amplify SnoR68 Full length for random labeling
SnoR68 R	Amplify SnoR68Full length for random labeling
SnoR77Y F	Amplify SnoR77Y Full length for random labeling
SnoR77Y R	Amplify SnoR77Y Full length for random labeling
23S Primer extension R	Primer extension
PSBD Primer extension R	Primer extension
PETA Primer extension R	Primer extension

GENERAL CONCLUSIONS and PERSPECTIVES

We studied the roles of the AtRAP protein from two aspects: in plant immunity and in RNA transport into the chloroplast. More importantly, we made the novel discovery that nucleus-encoded RNAs are located inside chloroplasts and investigated their the function and translocation. Chloroplasts are semi-autonomous organelles that produce sugars and oxygen, which are essential for all life on Earth. Understanding anterograde trafficking and the function of nuclear RNAs inside organelles will advance our knowledge of the communication and regulation between the nucleus and semi-autonomous organelles. Furthermore, a number of neuromuscular and metabolic diseases and accelerated aging problems are due to mutations in and malfunctioning of the human mitochondria. Understanding anterograde RNA trafficking will ultimately aid in development of effective therapeutic strategies for these diseases. There are several approaches which we can take for future study:

Determination of whether snoRNA anterograde trafficking is protein-dependent

In the null *atrap-1* chloroplasts, there is still a small amount of snoRNAs (Chapter 2), suggesting that there must be other pathways or components involved in snoRNA transport. SnoRNAs are likely to be dependent on other proteins for translocation, but it is also possible that some of them can move without assistance from protein facilitators. To address this question, we will take advantage of mutants defective in the Toc-Tic translocon pathway that have blocked anterograde trafficking of proteins localized in the chloroplast (Jarvis, 2008). On the chloroplast outer membrane surface, the protein precursors are recognized by the GTPase Toc159 (Ma et al., 1996; Perry and Keegstra, 1994). Tic20 and Tic21 are hydrophobic proteins essential for the function of inner chloroplast membrane channels (Chen et al., 2002; Kouranov et al., 1998; Teng et al., 2006). The AtToc1-9 mutant *ppi2* (from Felix Kessler's lab) and Tic20 Tic21 double mutant *tic20 cia5* (from Hsou-min Li's lab) display an albino phenotype, indicating that the import of most of the proteins is blocked. We can extract the chloroplasts from these mutants and examine the snoRNAs by Northern blot analysis. If no snoRNA signal is detected, this indicates that snoRNA import is protein-dependent and proteins other than AtRAP could also facilitate snoRNA import. If there is still detectable snoRNAs present in the mutant chloroplasts, we can examine a non-canonical protein import pathway.

Very few chloroplast-localized proteins, such as Tic32, chloroplast envelope quinone-oxidoreductase homolog (ceQORH), and NADPH-dependent protochlorophyllide oxidoreductase isoform A (prPORA), do not need Toc-Tic translocon for their import into chloroplasts (Aronsson et al., 2003; Miras et al., 2007; Nada and Soll, 2004; Philippar et al., 2007; Pollmann et al., 2007). prPORA can be imported by the only known Toc-Tic independent translocon-outer membrane protein 16 (OEP16) (Aronsson et al., 2003; Philippar et al., 2007; Pollmann et al., 2007). In Arabidopsis, *OEP16.1* is the major isoform of OEP16, and its T-DNA mutant (SALK_024018 obtained from Arabidopsis Biological Resource Center, ABRC) is normal in continuous white light but has a rapid onset bleached phenotype resulting in death in continuous darkness. We can examine the snoRNAs in this mutant to determine if protein-independent RNA trafficking exists. If necessary, an *AtToc159* and *OEP16.1* double mutant will be generated, although it would be difficult to select and maintain the double mutants due to the strong pleiotropic phenotype.

Visualization of snoRNAs and other RNAs imported into chloroplasts

Visualization of snoRNAs *in vivo* would be the best method to demonstrate snoRNA chloroplast localization and nucleus-chloroplast partitioning. It would also help us determine what mechanisms of trafficking are being used to import the snoRNAs into the chloroplasts. Fluorescein-labeled synthetic RNAs combined with a bombardment delivery method were successfully used for visualizing the movement of small RNAs within plants (Dunoyer et al., 2010). Our lab has successfully used fluorescein-labeled RNAs to study fungal RNA uptake during infection. Therefore, we can adapt this approach to visualize and study the localization and import of snoRNAs.

Briefly, we can use Fluorescein RNA labeling Mix (Roche) and T7 polymerase to generate fluorescently labeled snoRNAs. The labeled snoRNAs can be delivered into plant cells by pneumatic particle gun bombardment (which is available in the Plant Transformation Facility on our campus). The labeled RNAs will be visualized by confocal microscopy 20–40 hours after bombardment. The localization of the RNAs and nucleus-chloroplast partitioning will be examined and measured in Col-0 WT, *atrap-1*, *ppi2, tic20cia5,* and *oep16.1* mutants. This experiment will greatly help us understand snoRNA anterograde trafficking.

Validation of new nucleus-encoded RNAs localized in chloroplasts

In order to gain more insight into the role of nucleus-encoded RNAs, we can focus on protein-coding mRNAs or other classes of non-coding RNAs for functional analysis. We can first perform Northern blot analysis on these newly identified chloroplast-localized nuclear RNAs. Then, we can identify knockout mutants from T-DNA insertion populations, or generate mutants using CRISPR/CAS or artificial miRNA strategies. The function of these genes inside the chloroplasts will be assessed using these mutants. The photosynthetic efficiency, stress response, translation efficiency, etc., will be measured accordingly.

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