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Jacob Kim April 13, 2021

Deciphering the role of ACTIN-RELATED PROTEIN8 in *Arabidopsis thaliana* using CRISPR-Cas9 mutagenesis and coimmunoprecipitation (Co-IP)

by

Jacob Kim

Roger Deal
Adviser

Biology

Roger Deal

Adviser

Arri Eisen

Committee Member

Levi Morran

Committee Member

Frank McDonald

Committee Member

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Jacob Kim

Roger Deal

Adviser

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Abstract

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The actin protein family consists of cytoskeletal actin as well as actin related proteins (ARPs). The role of cytoskeletal ARP is well known, but the role of nuclear ARPs is not as clear. Nuclear ARPs are known to be related to nuclear actin in that they play a role in transcriptional regulation and are often found as components of chromatin remodeling complexes. *Arabidopsis thaliana* encodes six nuclear ARP proteins, one of which is ARP8. ARP8 is a plant-specific nuclear ARP, and it is unique in being localized exclusively in the nucleolus. It also contain an F-box domain, which is involved in protein degradation via the recruitment of ubiquitin ligase, and is not found in any other ARPs. Despite its unique characteristics, the role of *Arabidopsis* ARP8 is unclear. Here, we generated *Arabidopsis* ARP8 homozygous mutants using CRISPR/Cas9 to introduce a premature stop codon and examine whether the loss of ARP8 protein affects plant development. Yeast-two hybrid screening had previously identified ASK1 and RPL14p proteins as potentially interacting with ARP8. ASK1 protein is a component of ubiquitin ligase and RPL14p protein is a ribosomal protein, so determining ARP8's interaction with these proteins would help us better understand its potential function. We therefore tested for direct protein-protein interaction between ARP8 and the proteins ASK1 and RPL14p by performing in vitro coimmunoprecipitation (Co-IP) to get a better understanding of the molecular role of ARP8. We found that phenotypes of ARP8 homozygous mutant plants are variable, with some appearing highly similar to wild-type and others showing reduced size and potentially late flowering. The ARP8 transcript level has not been affected in the mutant plants, but the plants likely lack the ARP8 protein due to the premature stop codon. Moreover, the co-IP suggests that ARP8's interaction with ASK1 is weak, but the results are inconclusive. Future studies including determination of ARP8 protein levels in the mutant plants and *in vivo* protein interaction of ARP8 with ASK1 and RPL14p would further assist in characterizing ARP8's potential role in *Arabidopsis thaliana.*

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Table of Contents

Introduction

Actin is part of the actin protein family which consists of cytoskeletal actin as well as actin related proteins (ARPs). Cytoskeletal actin is essential for maintaining cellular shape as it provides structural support. While the role of cytoskeletal/cytoplasmic actin is well understood, the role of nuclear actin has not been well characterized. Emerging evidence suggests that nuclear actin has many functions, including transcription, mRNA processing, and chromatin remodeling (Chen and Shen 2007). Specifically, β-actin, a nuclear actin, is a component of an RNA polymerase complex (Chen and Shen 2007). How nuclear actin contributes to chromatin remodeling complexes is unknown, but it is thought that their functions are closely associated with that of nuclear ARPs. ARPs are proteins that are related to actin and they are named from 1-10 based on their degree sequence similarity with actin. ARP1 is the most similar and ARP10 is the least similar to actin (Poch and Winsor 1997). ARPs are conserved across all eukaryotic organisms due to their vital functions in the cell. For instance at least eight of the ten ARP subfamilies are conserved from human to yeast (Chen and Shen 2007). In these organisms, ARP1-3 and ARP10 encode cytoplasmic ARPs that interact with the actin filaments in the cytoskeleton. ARP4-9 are localized in the nucleus, and are thus called nuclear ARPs, and are known to have roles in chromatin remodeling. For example, it is known that yeast ARP4 is a component of INO80, a chromatin remodeling complex, and it binds directly to histones and thus modifies chromatin structure (Chen and Shen 2007).

The model plant *Arabidopsis thaliana* encodes nine ARP proteins, six of which are nuclear ARPs. Most *Arabidopsis* ARPs have yeast orthologs that share high amino acid sequence identity. The functions of these ARPs are predicted to be conserved with those in yeast. However, there are also plant-specific ARPs such as ARP7 and ARP8 which do not have

yeast or animal orthologs and appear to be plant-specific. Specifically, plant ARP7 has 39% and ARP8 has only 29% amino acid sequence identity to yeast actin (McKinney et al. 2002). Moreover, the presence of larger regions of poor conservation make plant ARP8 the most highly divergent ARP sequence in *Arabidopsis,* which is why it is known as a plant-specific orphan ARP (McKinney et al. 2002).

In addition to the low amino acid sequence identity to other ARPs, there are other features that make plant ARP8 an interesting gene to study. ARP8 protein has a unique subcellular distribution and is exclusively localized to the nucleolar compartment (Kandasamy et al. 2008) whereas other nuclear ARPs cannot be found in the nucleolus but are rather found only in the nucleoplasm. Additionally, the ARP8 protein contains a distinct F-box domain, (Kandasamy et al. 2008) which typically provides substrate specificity for ubiquitin ligases that target proteins for proteolytic degradation. The F-box domain is unique since it is not found in any other ARPs. The primary function of the nucleolus is in generating and processing ribosomal RNA (rRNA) for ribosome production, so the location of ARP8 leads to the hypothesis that its role might be related to regulating ribosome production at the level of rRNA transcription. The presence of an F-box domain also suggests that ARP8 might perhaps have a role in ribosomal protein quality control via protein degradation. However, nothing is known about the specific role that ARP8 plays in the plant, but such unique features lead us to think that it might have an important role within the organism.

The main goal of this paper is to try and decipher the possible role ARP8 might have on the plant. One approach in an attempt to understand ARP8's role in the plant is to see what effect ARP8 null mutation would have in *Arabidopsis thaliana*. Therefore, we generated CRISPR/Cas9 mutations in ARP8 in order to generate a null mutation and observe its effect on the plant. Previous attempts to reduce ARP8 transcript levels via RNAi and T-DNA insertion

have been unsuccessful (data not shown). To date, no reports of ARP8 mutants have been published. Here, we took advantage of the fairly recent CRISPR/Cas9 technology to generate multiple frameshift mutants of ARP8 in an attempt to successfully reduce its expression level *in vivo*. We then observed the phenotype of the mutant plants to see if there are any detectable developmental abnormalities relative to wild-type plants. If ARP8 is essential, then homozygous mutations would result in death of the plant during embryogenesis, leaving us with no viable homozygous plants. If not essential, phenotypes of homozygous mutants would not result in death but might show some developmental defects. Genotyping of the CRISPR/cas9 mutant ARP8 led us to discovering a heterozygous mutant plant in the first generation. Planting that particular line and after genotyping the plants, one plant in the third generation was revealed to have a homozygous mutation containing a 1bp addition and a 1bp deletion in two of the target regions in the ARP8 gene. This introduced a frameshift mutation in the gene, leading to a generation of a premature stop codon. However, the transcript level of endogenous ARP8 of homozygous mutant plants did not change compared to wild-type plants, which was an unexpected result. Despite the high levels of transcript levels, it shouldn't encode a proper ARP8 protein, so the plants are likely null mutant plants. Moreover, the phenotypes of the mutant plants show high variability throughout various developmental stages. The growth of some mutants are slower than wild-types while other mutants show no significant difference.

Another approach we took to understand ARP8's role in the plant is testing which proteins ARP8 interacts with. Yeast two-hybrid assays performed previously in our lab (data not shown) suggested that ARP8 interacts with various proteins including Arabidopsis SKP1 homologue (ASK1), Ribosomal protein L 14p (RPL14p), and ARP8 itself (data not shown). SKP1 is a component of the SCF family of E3 ubiquitin ligases that associates with an F-box

protein which provides specificity for the substrate protein to be ubiquitinated. RPL14p is a ribosomal protein for the large ribosomal subunit. To see whether these protein interactions are direct, we performed in vitro co-IP between ARP8 or ASK1/RPL14p. The proteins were produced by inserting the coding sequences derived from wildtype *Arabidopsis* cDNA into a vector plasmid and performing in vitro transcription and translation. Since functions of the ASK1 and RPL14p proteins are well known, interaction tests would give more information on the possible roles of ARP8 in the plant. Co-IP results indicate that ARP8 might possibly interact with ASK1, but its interaction is weak. The results of RPL14p interaction are inconclusive. Therefore, the interaction of ARP8 with both proteins remains unclear and requires further testing.

Result

Generating homozygous ARP8 mutant Arabidopsis thaliana plant using CRISPR/Cas9 mutagenesis

The *Arabidopsis* ARP8 gene is ~3.4kb in length and contains 12 exons. I designed three guide RNAs (gRNAs), which is a ~20 nucleotide sequence that is complementary to the region of interest, targeting the first exon for the cas9 protein to cut (Figure 1A). We hope that by targeting the first exon, we will produce a frameshift mutation (+/-1bp) that will throw off the coding sequence of the whole rest of the protein, making it nonfunctional. Conversely, if you put the mutations in later exons, the n-terminal section of the protein might remain fully or partially functional. We used three different cut sites in order to increase the likelihood of generating a mutation. Following DNA digestion from the CRISPR/Cas9 complex, the non-homologous end-joining DNA repair system should repair the cut and introduce either an extra base pair(s), delete a base pair(s), or repair faithfully (Figure 1C). With a single cut site, although indel mutations are often generated, introduction of multiple cut sites would further

increase the probability of generating frameshift mutations or even larger deletions.

One type of mutation that could occur following DNA digestion is large deletions in between two cut sites. I have designed two vector plasmids, each of which have CRISPR/Cas9 proteins that cut two out of the three cut sites. In other words, one vector plasmid is designed to cut at target cut site 1+2, and the other at cut site 1+3. A large deletion would occur if two of the cut sites are cut and the entire section in between the two cut sites is not retained by the repair system (Figure 1B). I designed primers that flank the cut sites in the first exon and performed a PCR reaction to analyze DNAs that were extracted from several ARP8 mutant candidate plants. Gel electrophoresis results of the PCR products tells us the size of the mutant candidates' ARP8 DNA, indicating whether or not a deletion occurred. PCR products from all of the 12 candidates were ~370bp, suggesting that big deletions have not occurred (Figure 2). With a large deletion, the mutant ARP8 will be 50~200bp shorter than WT and the difference in band position is detectable through agarose gel electrophoresis. However, all mutant candidate PCR products were similar in size to WT, indicating that no large deletions had occurred.

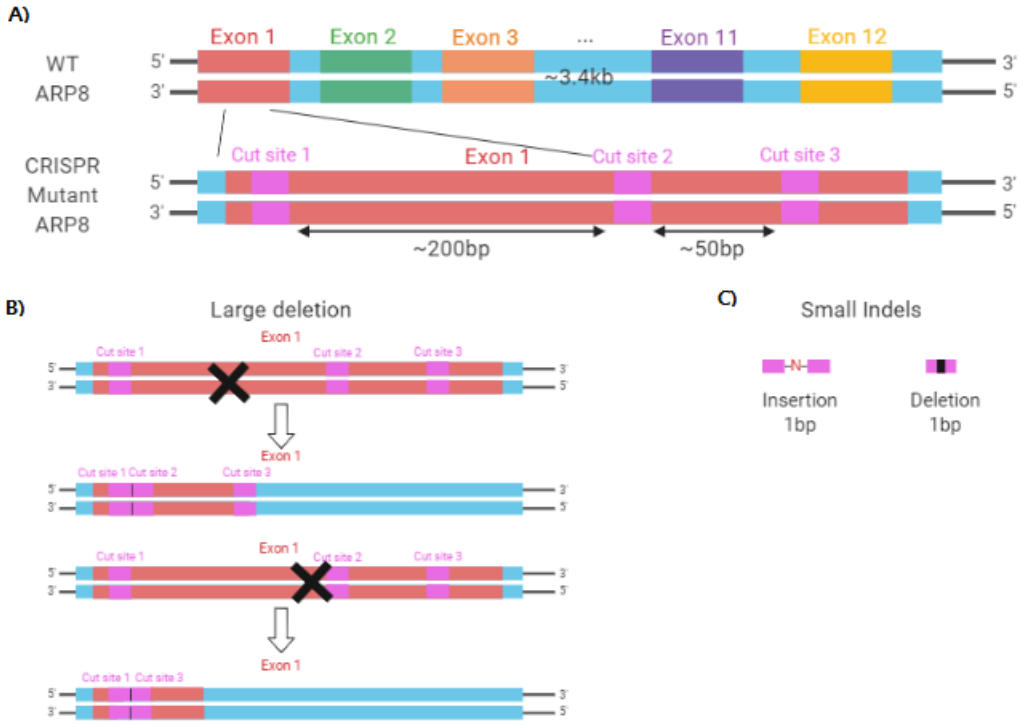

Figure 1: General schematic of the experimental concept of generating an ARP8 mutation in *Arabidopsis*. A) Representation of the *Arabidopsis* ARP8 gene and the location of the three cut sites we designed in the first exon. B) Schematic showing how a large deletion, in which the whole portion in between the two cut sites that produced by CRISPR/Cas 9 cuts is lost, would result in a significant loss of the gene C) Small indels (single nucleotide insertions/deletions) produced by CRISPR/Cas9 cuts would not have a significant effect on gene size.

Figure 2: Photo of gel electrophoresis comparing the length of ARP8 genes between ARP8 mutants and wildtype *Arabidopsis* (control). The bands in the gel electrophoresis are in the same location for both WT and ARP8 mutants, suggesting that mutations were not large deletions, but rather small indels. The length of the bands shown is ~370bp.

The plants were then genotyped by amplifying the ARP8 region via PCR and sequencing them. After genotyping all of the plants in the first generation, most plants did not have any mutations. However, we were able to detect one plant, which had the 1+3 cut site vector, that contained a heterozygous mutation in both desired cut sites. We then collected the seeds from the plant, and grew the next generation plants. In the third generation, one plant was revealed to contain a homozygous mutation in both target sites. More specifically, it had a

single base pair insertion in the first target site and a single base pair deletion in the third site. Analyzing the sequence data for this mutant allele indicates that introduction of the mutations should introduce a premature stop codon in the middle of the first exon, after the first cut site. This suggests that a null mutation has been successfully generated. As mentioned earlier, a premature stop codon would disrupt normal expression of the ARP8 protein, and therefore if ARP8 is vital for plant development, phenotypic observations should indicate a significant difference from wildtype *Arabidopsis*. The seeds from the particular homozygous mutant plant were collected and planted for the fourth generation plants. All mutant plants germinated successfully, and were confirmed to be homozygous for the mutation, strongly suggesting that the gene is not essential.

Phenotypes of homozygous ARP8 mutant plants vary compared to wild-type plants

The phenotypes of the homozygous ARP8 mutant plants were then compared with wild-type plants. All seeds were planted at the same time, stratified and grown under identical conditions. As mentioned above, all plants have successfully generated, but variation in phenotypes were observable. Out of the many mutant plants, some mutants such as #3 - 5 in Figure 3 had smaller sized leaves and their growth was relatively slower compared to wildtype plants before the flowering stage (Figure 3). However, growth speeds varied among the plants because #3 was smaller than #2 in early developmental stages (Figure 3A), but in later developmental stages, the growth of #3 had progressed further than #2 (Figure 3C). Moreover, there were also many mutants - such as mutant #1 - that did not show pronounced phenotypic differences compared to the wild-type plants including the size, color and shape of the leaves as well as growth speed.

Figure 3 Phenotype comparison between ARP8 mutant and wild-type *Arabidopsis thaliana* plant at different growth stages. Phenotypes of the ARP8 homozygous mutant plants (1~5) were compared to wild-type plants (WT). A) is the picture of the plants 30 days after planting the seeds, before they entered the flowering stage. B) is taken 3 days after A). C) is 2 weeks after B), after WT plants have entered the flowering stage. Sizes of the plants that are placed side by side are scaled relative to each other. All plants were planted at the same time.

mRNA levels in homozygous ARP8 mutant plants do not differ from wild-type plants

We then examined the level of ARP8 mRNA transcripts in the mutant plants from Figure 3 to see if the phenotypic variations that are observed could be explained by the ARP8 expression level in these plants. We extracted RNA from the plant leaf tissue, and using reverse transcriptase, converted the RNA into cDNA. Then, we performed PCR with APR8-specific primers to amplify the ARP8 region encompassing the CRISPR-targeted sites and ran a gel electrophoresis. Surprisingly, the transcript levels of ARP8 between wildtype and mutant *Arabidopsis* plants did not differ significantly as the intensity of the bands were nearly identical (Figure 4). Our expectation was that mutant plants would have significantly decreased level of ARP8 mRNA levels because of the nonsense-mediated mRNA decay pathway, which is a surveillance pathway for eukaryotes to degrade mRNAs that contain premature stop codons. However, that doesn't seem to be the case here. This suggests that the molecular effect of the null mutation of ARP8 is not visible in the level of mRNA, and might only be visible in the level of proteins.

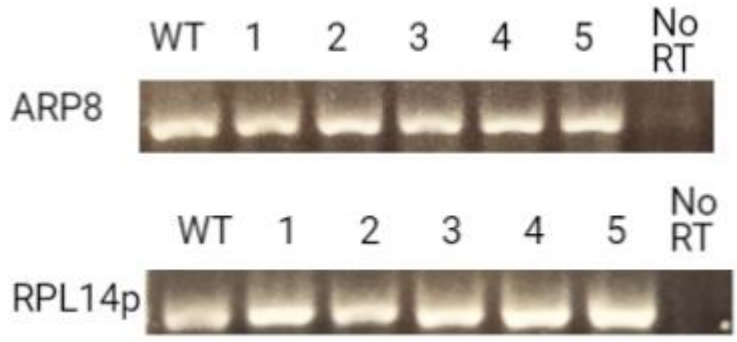

Figure 4 Gel electrophoresis of RT-PCR comparing ARP8 transcript levels between wildtype and mutant plants RT-PCR results show the ARP8 mRNA levels between wildtype (WT) and ARP8 mutant plants (1~5). Intensity of the bands of the mutant plants are the same as the WT (upper gel), indicating that the *in vivo* transcription level of ARP8 do not differ. Absence of bands in the sample that did not contain reverse transcriptase (No RT) shows that no DNA contamination occurred and confirms that the bands shown are amplified from the cDNAs that were generated. Amplification of RPL14p using RPL14p-specific primers (bottom gel) was used as an internal control. Presence of bands means that RNA was successfully extracted from the plant.

Results of in vitro protein-protein interaction testing between ARP8 and ASK1 or RPL14p are inconclusive

Nuclear ARPs in yeast and humans are mostly found as part of chromatin remodeling complexes. Plant-specific nuclear ARPs such as ARP7 are also known to participate as a component of chromatin remodeling complexes (Kandasamy et al. 2005). Therefore, it is possible that ARP8 is also part of a protein complex, or it interacts with other proteins when carrying out its role in the plant. Previous yeast two-hybrid assays from our lab (data not shown) suggested that ARP8 interacts with ASK1 and RPL14p. Therefore, in vitro co-IP was performed to verify the interaction between the ARP8 protein and ASK1, as well as the RPL14p protein.

First, the ARP8, ASK1 and RPL14p coding sequences were amplified from the wildtype *Arabidopsis* cDNA stock by designing primers which targeted each coding regions. The primers also encoded epitope tags in the N-terminus so that the expressed proteins can be detected by antibodies in the western blot after co-IP. ARP8 contained the FLAG epitope tag (DYKDDDDK) and ASK1 and RPL14p contained the Myc epitope tag (EQKLISEEDL). The epitope tag-containing coding sequences were then cloned into the multiple cloning site (MCS) of the in vitro expression vector plasmid pT7CFE-CHis. After confirming that the genes were successfully cloned by sequencing the plasmids, in vitro transcription and translation was performed for protein production.

After in vitro translation, the co-IP assay was performed. The proteins were mixed together (ARP8 + ASK1 and ARP8 + RPL14p) to allow for interaction to occur, and pulled down using anti-FLAG magnetic beads. The use of anti-FLAG M2 magnetic beads for pulldown allows only the ARP8 protein to be extracted from solution. Therefore, only a strong enough intermolecular interaction with ARP8 allows the myc tag-containing ASK1 or RPL14p

proteins to be extracted also. Following the extraction via anti-FLAG magnetic beads, the proteins were eluted with an excess of 3X FLAG peptide, and a western blot was performed on an SDS-PAGE gel to examine the eluted products. The blot was probed using anti-FLAG and anti-Myc antibodies, so that a band in the region that was probed with anti-Myc would be visible only if a strong interaction allowed the ASK1 and RPL14p proteins to be extracted together. We also included controls to help us interpret the results. To ensure the presence of the proteins, mixtures of ARP8/ASK1 or ARP8/RPL14p protein was loaded to the western blot directly without going through co-IP, acting as a positive control. Moreover, to ensure that the myc-tag proteins doesn't get pulled down by the anti-FLAG M2 beads, we let just the myc-tagged protein ASK1 and RPL14p go through the co-IP which acted as a negative control.

The result of the western blot show protein bands in the ARP8/ASK1 interaction well – in the region that was probed with anti-myc antibody – whose size is analogous to the ASK1 (Figure 5A). This suggests that ASK1 possibly interacts with ARP8. However, when looking at the interaction results with RPL14p (Figure 5A), the presence of a band in the negative control and in the ARP8/RPL14p interaction well – in each case the band size was different from RPL14p and the same as ASK1 – indicating that something else is going on. A possible hypothesis is that the two bands were from the light chain and heavy chains from the antibodies of the anti-FLAG M2 magnetic beads, whose sizes are 25kDa and 50kDa, respectively. This would also cast doubt on the bands of the ARP8/ASK1 interaction well since size of ASK1 and ARP8 proteins are similar to the light and heavy chain of the M2 antibody, respectively, and the bands might be the antibody bands and not ASK1.

To verify the hypothesis, I repeated the co-IP assay but included two modifications. One modification was to use a new loading dye which lacked 2-mercaptoethanol, a reducing agent which is a component of the protein loading dye that is used during western blot. Lack

of reducing agent would not reduce the disulfide bonds connecting the antibody heavy and light chains and therefore prevent them from dissociating. Therefore, if the bands were indeed the antibodies, using the new loading dye would change the current band locations to move upwards due to its heavier molecular weight. This would then reveal any bands that were shadowed by the antibody bands. Results show that the previous bands in the ~50kDa is removed and a new band in the ~200kDa region, suggesting that the bands were indeed the antibody bands (Figure 5B). Moreover, the ARP8/ASK1 interaction did not show any bands, meaning that ASK1 did not get pulled down together with ARP8. ASK1 proteins were detected in the supernatant of the wash after ARP8 proteins were captured with the beads (Figure 5B). The presence of the ASK1 protein band in the wash implies that ASK1 either did not bind to ARP8 or was washed away after being pulled down, and therefore its intermolecular interaction with ARP8 is weak or non-existent. Another possibility is that the interaction between ARP8 and ASK1 does occur, as indicated by the yeast-two hybrid results, but their interaction is not direct and might involve intermediate proteins.

Another modification was to repeat the co-IP assay, but use only the secondary antibody to probe during western blot. All primary antibodies and the anti-flag antibody on the M2 magnetic beads are monoclonal mouse antibody. Therefore, the secondary antibody used to probe the western blot would also be able to detect antibodies that dissociated from the magnetic beads. Therefore, by excluding the use of the primary antibody, I would be able to eliminate the possibility of the secondary antibody detecting the epitope-tagged proteins ASK1 and RPL14p. Thus, if protein bands still appear after performing the western blot, it indicates that the bands are for sure the heavy and light chain antibody bands. This is exactly what the results show us (Figure 5C). Since the band sizes of the heavy and light chains correspond to the band locations that were observed in the first western blot (Figure 5A), we could confirm

that the bands were indeed the antibody bands.

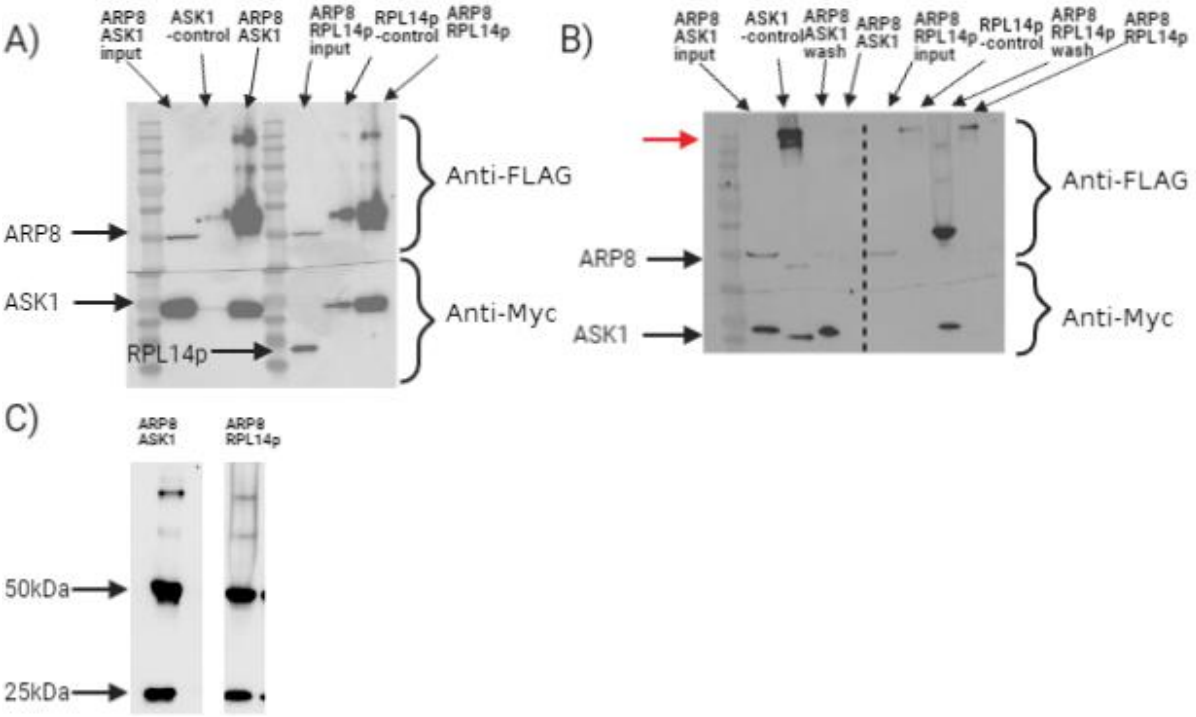

Figure 5. Western blot of Coimmunoprecipitation between ARP8 and ASK1/RPL14p A) Interaction test between ARP8 and ASK1 or RPL14p. Input controls contain mixtures of ARP8/ASK1 or ARP/RPL14p proteins without going through the co-IP assay, acting as a positive control. Negative controls contain only myc-tag proteins going through co-IP assay. ARP8/ASK1 and ARP8/RPL14p is the experimental well where the proteins were allowed to interact and had gone through co-IP. Arrows on the side of the gels indicate the location of the proteins from the positive control, and brackets represent the type of antibody used to probe the blot. **B)** Western blot without the use of 2-mercaptoethanol. Labeling of wells are the same as explained in A). Addition of two extra wells are the supernatant of the wash after ARP8/ASK1 or ARP8/RPL14p proteins were pulled down with beads. Red arrow indicates the location of the non-reduced antibody bands. **C)** Western blot results of probing only with the secondary antibody. Band locations that are identical to bands in A) indicate that bands shown were indeed the anti-FLAG antibody bands from the magnetic beads.

Discussion

To characterize the role of a certain gene in an organism, a common approach that is taken first is to reduce its expression levels to see what effect it has on the organism. In the past, efforts in reducing the expression levels of ARP8 in *Arabidopsis thaliana* had been made with RNA interference as well as using transgenic plants carrying T-DNA insertions in the gene. However, such approaches have been unsuccessful at reducing the ARP8 transcript and protein, for unknown reasons. Therefore, we are using another approach by generating null mutations in the ARP8 gene via the CRISPR/Cas9 system. We hope that utilization of the fairly modern CRISPR/Cas9 technology would lead us to successfully decrease its expression level in the organism.

While designing the vectors that have the CRISPR/Cas9 cut sites, I had designed three vectors that each cut a pair of the cut site- cut site 1+2, 1+3 and 2+3. The vector which had the cut site 2+3 didn't grow, with reasons unknown, only allowing me to use the other two vectors. Moreover, after transforming the plants with the vector plasmids, sequencing results indicate that the vast majority of plants did not contain any mutations. However, we were ultimately able to detect a plant that was transformed with the 1+3 target site vector that contained a homozygous mutation in both the first and third target regions. According to the sequence data, a premature stop codon was generated in between the two target sites, which indicates that, in theory, a null mutation occurred.

Modifying levels of ARP7, another plant-specific nuclear ARP, is known to cause various effects to the plant. Reducing the expression levels of ARP7 via RNA interference or T-DNA insertions produced plants that show developmental defects (Kandasamy et al. 2005). Moreover, homozygous mutations of ARP7 is embryonically lethal, meaning the plant does not survive embryogenesis (Kandasamy et al. 2005). Therefore, since ARP8 is also a plant-specific

nuclear ARP and considered an orphan ARP in *Arabidopsis*, it is possible that similar phenotypic effects in homozygous ARP8 mutant organisms might be observable. However, the homozygous mutant plants have successfully germinated, indicating that the gene is not essential for embryonic development. When comparing the phenotypes between mutant plants and wildtype plants throughout various developmental stages, some mutant plants showed slower growth which was seen by their having smaller leaf sizes and entering the flowering stage later than the wild-type (Figure 3). There were also many mutants which did not show significant phenotypic differences compared to wild-type plants throughout growth. Although we were able to observe some mutants which had some noticeable differences in their growth compared to wild-type plants, no consistent growth abnormalities were observable among the mutant plants. We hoped that such phenotypical variation that was observed might be explained by ARP8 expression levels in the mutant plants. However, the level of ARP8 mRNA expression was found to be identical between wild-type and mutant *Arabidopsis,* leading us to confusion, since we had expected the nonsense-mediated mRNA decay pathway to have degraded ARP8 mRNAs in the mutant plants. Since the level of ARP8 mRNA are the same, the next step would be to identify the level of ARP8 proteins found in the mutant plants. The premature stop codon should, in theory, disrupt translation of the ARP8 proteins, and therefore the mutant plants' level of ARP8 proteins should differ significantly compared to wild-type plants. Therefore, future experiments could use anti-ARP8 antibodies to detect ARP8 proteins in the plant tissue and its quantity analyzed via western blot. If all homozygous mutant plants lack functional ARP8 proteins, a possible explanation of the variable phenotypes that was observed is that stronger phenotypical expression might be only observed in the mutants under stress conditions. Since the plants were grown under ideal conditions for this experiment, stressful environmental conditions such as hotter/colder temperature and drought could lead to homozygous ARP8

mutant plants developing a more distinct phenotype, different from the inconsistent phenotypes that were observed here.

The presence of an F-box domain and exclusive localization in the nucleolus is what makes ARP8 unique. ASK1 and RPL14p are related to the F-box motif and the nucleolus, respectively, and correspond to the two characteristics of ARP8. Therefore, understanding the interaction of ARP8 with these proteins would aid in explaining its potential function in the plant. However, the result of the co-IP is inconclusive about the interaction between ARP8 protein with ASK1 and RPL14p proteins. At first, it seemed like a strong interaction was occurring between ARP8 and ASK1 (Figure 5A), but repeating the western blot by including modifications of the protein loading dye to exclude the reducing agent (Figure 5B) and probing with only the secondary antibody (Figure 5C) confirmed that the initial bands were the M2 beads' anti-FLAG antibody. Although no strong, direct interaction occurred between ARP8 and ASK1 or RPL14p, this does not mean there aren't any interaction involved at all. Being able to detect the ASK1 protein in the wash liquid after the protein pulldown might indicate that only weak intermolecular interactions are involved between the proteins. It can also be that ARP8's interaction between the proteins are not through a direct interaction, but rather involves intermediate proteins to carry out functions. Moreover, since the protein interaction test for this experiment was performed *in vitro,* the interaction results might be different when observed *in vivo.* The various cofactors and intermediate proteins present in the plant that ARP8 might be associated with could lead to stronger interaction between the proteins *in vivo.*

The results up to date provide an inconclusive result regarding the potential role of ARP8 in *Arabidopsis thaliana*. However, we believe that we have laid a significant foundation with regard to the various approaches that were utilized in an attempt to understand the possible function of ARP8 in *Arabidopsis thaliana*. Like many nuclear ARPs, which are found as part

of chromatin complexes, ARP8 could also be part of a protein complex. Due to its distinct localization in the nucleolus, it is logical to think that ARP8 might have roles related to rRNA transcriptional control. Furthermore, the presence of an F-box motif suggests that the regulation might involve protein degradation. Future experiments that might involve modifications of the experiments used above or other novel techniques that leads to conclusive results would aid in characterizing the role of ARP8. We believe that while the data that we currently have might be inconclusive on its own, it could be interpreted together with data obtained from future experiments and serve as important foundational information. Such findings would be significant since nothing is known about ARP8 in the plant so far, and revealing the role of ARP8 has the potential to unravel previously unknown information about plant development that could be crucial for the field of biology.

Methods

Golden Gate Cloning for CRISPR/Cas9 mutation

For the Golden Gate cloning, we used the protocol described in "*A CRISPR/Cas9 toolkit for multiplex genome editing in plants*" (Xing et al. 2014). Since we wanted to have two cuts in each ARP8 gene to generate mutants (1+2, 1+3), we needed two gRNAs for each vector plasmid. Therefore, we followed the protocol of designing two gRNAs described in the paper. To assemble two gRNAs, before the Golden Gate reaction, the two target sites that we selected were incorporated into both PCR forward and reverse primers. Plasmid vector pCBC-DT1T2 (one of the two binary vectors used) was used to amplify the PCR fragment. The PCR product was purified and inserted into the other binary vector plasmid. Restriction-ligation reaction using BsaI and T4 DNA Ligase was set up to insert the PCR product. Then, the Golden Gate reaction was taken place, which was incubating the reaction in a thermocycler for 5 hours at 37°C, 5min at 50°C and 10 min at 80°C. The reaction mixture was transformed into *E.coli* competent cells, and positive clones were selected on kanamycin LB agar plates.

Primer design of gRNAs

gRNAs were designed by following the methods used by "*A CRISPR/Cas9 toolkit for multiplex genome editing in plants*" (Xing et al. 2014). Primer design was specified in "Sequence of DT1T2-PCR with Targets 1 and 2 for dicots", which was found in Additional file 3, Methods S2, pg. 7.

DT1-BsF: ATATATGGTCTCGATTGGAAAGTATGGGGATCGGTGGTT

DT1-F0: TGGAAAGTATGGGGATCGGTGGTTTTAGAGCTAGAAATAGC

DT2-R0: AACTGGGACTCCATTTTCTTCGCAATCTCTTAGTCGACTCTAC

DT2-BsR: ATTATTGGTCTCGAAACTGGGACTCCATTTTCTTCGCAA

Underlined region represents the sequence of the target sites.

Transforming the vector plasmid to *Arabidopsis*

The clones that were selected from the Golden Gate reaction was then transformed into

Arabidopsis (just before they were flowering) using the floral dip method.

DNA extraction from *Arabidopsis* plant

DNA was extracted from the ARP8 mutant seedlings using DNA extraction protocol which uses Edwards buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). 1~2 leaves were selected per seedling, and frozen using liquid nitrogen. The leaves were then lysed using a pestle and Edwards buffer was added to the lysed leaves. The mixture was centrifuged, and the supernatant was moved to a new tube. Equal volumes of phenol was added to the supernatant, mixed vigorously and centrifuged. A clear layer at the top formed, and the clear layer was carefully pipetted into a new tube. Equal volume of isopropanol was added, incubated for 5 min and centrifuged. The supernatant was then discarded and we were left with a DNA pellet on the bottom of the tube. 70% ethanol was added, incubated for 1~2 min, centrifuged, and the ethanol was removed for washing purposes. Then, the pellet was air dried until completely dry and resuspended in distilled water.

Verification of possible candidates of ARP8 mutants

After DNA extraction, we ran a PCR reaction of the DNA using primers which flank the ARP8 gene. The PCR products were then run through gel electrophoresis to confirm that the gene has been properly amplified. Since the difference in length of the ARP8 gene between the wildtype and the mutants were not detectable through gel electrophoresis, we used Tape Station. D1000 Tape Station was used to detect the base pair length of the ARP8 gene, and once we saw a difference in the base pair numbers in the ARP8 gene between wildtype and mutants, we gel purified the PCR fragments and sequenced them.

Protein production by cloning Wildtype *Arabidopsis* cDNA into PT7CFE1-CHis vector plasmid

We designed primers to amplify ARP8, ASK1, and RPL14p regions from *Arabidopsis* cDNA stock via PCR. The PCR products were then inserted into PT7CFE1-CHis vector plasmid using restriction enzyme BamHI & Sac I (Sequential digestion with two incompatible enzymes) and T4 DNA ligase. The plasmids were then transformed into E. Coli by culturing in LB media containing Ampicillin. The plasmids were then extracted via Qiagen Miniprep, and In vitro translation was performed by following the 1-Step Human Coupled IVT Kit from Thermo Fisher Scientific.

ARP8 Primers

Forward: ATGATCCTGAAGAAAGTATGGGGAT

Reverse: CCACATGAGTCTTGACTTGCG

ASK1 PCR Primers

Forward: ATGTCTGCGAAGAAGATTGTGT

Reverse: TTCAAAAGCCCATTGGTTCTCT

RPL14p Primers

Forward: ATGTCGAAGCGAGGGCGTGGA

Reverse: GACAATGGCATTGGCAGCACT

Coimmunoprecipitation Protocol for protein-protein interaction

5µL of FLAG tagged ARP8 was mixed with 5µL of Myc-tagged proteins (ASK1 or RPL14p) from the IVT reaction. The protein samples (except for input) were then diluted to 100µL of 1X PBS. Then, 10µL equivalent of compact beads (ANTI-FLAG M2 Magnetic Beads from Sigma-Aldrich) were added to the protein mixture. The beads were washed by resuspending in PBS buffer and capturing them on a magnet before adding it to the mixture. Beads were allowed to incubate in the mixture for 1 hour at 4℃ on rotator. Then, the beads were captured and washed with 1X PBS by resuspending it and capturing it on a magnet. The wash was repeated

a total of 3 times, and the supernatant of the first wash was kept for western blot analysis. After the wash, the captured beads were eluted with 10μL of 3X FLAG peptide (500ng/μL) for the protein product.

RT-PCR for identifying transcript levels of ARP8

RNA was extracted from mutant and wild-type *Arabidopsis thaliana* leaf tissue using Qiagen RNeasy Plant mini kit. Size of leaves were all <100mg. The extracted RNA was then treated with DNase using Ambion Turbo DNase. The reaction volume that was used for DNase treatment was 10μL, and 4μL of the extracted RNA was used. cDNA of the DNase treated mixture was prepared using Superscript III reverse transcriptase. PCR reaction was performed using the cDNAs that were prepared as the template and ARP8 primers. NEBNext High-Fidelity 2X PCR Master Mix was used for the PCR reaction. We followed the protocols that were provided in the kits.

Kits used:

- Qiagen RNeasy Plant Mini Kit (cat # 74903)
- Ambion Turbo DNA-free kit (cat # AM1907)
- Invitrogen Superscript III first strand synthesis kit (cat # 18080-051)
- NEBNext High-Fidelity 2X PCR Master Mix (cat # M0541)

ARP8 Primer

Forward: CGAGAAAAAACAAAAATCCGCAAACTCGAC

Reverse: CCGGAGAGGATAACCAGAACGCAAA

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