UNIVERSITY OF CALIFORNIA SAN DIEGO

Utilizing Genetic Diversity of Artificially Induced Micro RNAs and Naturally Diverse Ecotypes to Investigate the Transcription Factors Involved in Abscisic Acid Response in *Arabidopsis Thaliana*.

A Thesis submitted in partial satisfaction of the requirements
Master of Science

in

Biology

by

Everett Weinstein

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The Thesis of Everett Weinstein is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

To my family and friends, to those who have stopped at nothing to go above and beyond in their support of me, I thank you from the bottom of my heart. I will always consider myself extremely lucky to have people in my life that stand by my side no matter the situation. To my parents who pushed me from childhood until now, who motivated me and propped me up when I needed it, who gave me the scaffolding I needed to build from, thank you. To my brother who gave me advice and was always positive and welcoming, and was always someone I could turn to, thank you. To my entire family who reach out with their love and support, even when I myself am not great at reaching out to them, thank you. To my friends who gave me a near infinite number of hilarious stories and who I created thrilling adventures with, I thank you. It has been an absolute pleasure to work in the Schroeder Lab, and a privilege to be surrounded by fellow scientists who possess such immense brilliance and humor and dedication. To Dr. Schroeder thank you for allowing me the wonderful opportunity to work in your lab, it has been a remarkable two years that I will always cherish. And to Dr. Charles Seller, my direct mentor, thank you for your guidance and all that you have taught me, I could not have made this thesis half of what it was without your help over the past couple of years. To everyone I know, thank you for being a part of my life, it has been a genuine pleasure so far and I look forward to everything to come with excited anticipation. Enjoy the read and wishing you all well!!!

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LIST OF ABBREVIATIONS

ABA	Abscisic Acid.
AIF 1	ATBS1 interacting factor 1, Basic helix-loop-helix protein 150, bHLH transcription factor 150.
AIF 4	ATBS1 interacting factor 4.
amiRNA	artificial micro-RNA.
ATBS1	Activation-Tagged Bri1-Suppressor1. Also known as PRE3, bHLH135, & TMO7.
BIK-1	*Arabidopsis thaliana* native to Bikfaiya, Lebanon.
bHLH	Basic helix-loop-helix transcription factor.
CIRY-13	*Arabidopsis thaliana* native to Ciry-le-Noble, France.
Col-0	*Arabidopsis thaliana* native to Columbia, Missouri, USA.
HsMyo	Col-0 lineage of *Arabidopsis thaliana* with an amiRNA targeting the *Homo sapiens* myosin gene.
MS	Murashige and Skoog media.
UBQ10pr::mCherry-AIF1	*Arabidopsis thaliana* with transgene that overexpresses AIF1 using the Ubiquitin 10 promoter to produce the fusion protein mCherry-AIF1.

ACKNOWLEDGEMENTS

Figures were generated using BioRender.com and Microsoft Excel. Measurements of primary root growth were conducted using Fiji software (Schindelin et al., 2012). This thesis contains unpublished material coauthored with Dr. Seller, Charles. Pogliano, Sofia. and Sama, Andrea. The thesis author was the primary author of this thesis.

ABSTRACT OF THE THESIS

Utilizing Genetic Diversity of Artificially Induced Micro RNAs and Naturally Diverse Ecotypes to Investigate the Transcription Factors Involved in Abscisic Acid Response in *Arabidopsis Thaliana*

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Abscisic Acid (ABA) is a phytohormone that is crucial for regulating the stress response pathway in plants. Accumulation of ABA can be triggered by many types of environmental stress, and buildup of ABA can cause changes in plant germination, the elongation and prioritization of primary and lateral root growth, and more. Though we know ABA is an extremely important hormone for plant stress response, we know little about the transcription

factors involved in the ABA regulatory pathway. In this thesis we utilize two different sources of genetic diversity in *Arabidopsis thaliana* to investigate the transcription factors behind the ABA stress response pathway. The first source of genetic diversity comes from artificial micro RNAs (amiRNAs). By screening through a library of seeds containing amiRNAs that target transcription factors, we identified two transcription factors, AIF1 and AIF4, that we believe are involved in the ABA stress response pathway. The second source of genetic diversity comes from naturally occurring ecotypes of *Arabidopsis thaliana* found in various regions across the globe. By screening through seeds collected from different countries around the world, we found two lineages of *Arabidopsis thalian*a, one from Bikfaiya, Lebanon, and another from Ciry-le-Noble, France, that demonstrate ABA hypersensitivity compared to Col-0, the current standard for *Arabidopsis thaliana* research native to Columbia, Missouri, USA. By continuing to analyze these ecotypes of *Arabidopsis thaliana*, we may be able to discern what naturally occurring genetic variations granted the increased ABA sensitivity we observed during our experimentation.

INTRODUCTION

There are many different environmental stresses that can impact a plant's development, and many of these stresses share the commonality of triggering the abscisic acid (ABA) pathway. ABA is a phytohormone found in plants that accumulates in tissues to trigger developmental and physiological changes that promote survivability in the face of abiotic stresses. ABA is responsible for the regulation of thousands of genes in plants and can modify the behavior of everything from stomatal apertures, to seed germination, to primary and lateral root growth (Finkelstein R., 2013). While we know a great deal about what the outcomes of ABA synthesis and ABA concentration increases are, we know little about the transcription factors involved in the ABA response pathway. To identify more of the transcription factors responsible for the ABA response, we sought to identify genes that impact ABA-mediated germination delay and root growth inhibition.

Previous studies have shown that exogenous ABA can delay seed germination in *Arabidopsis thaliana*, as well as impeding existing root elongation by arresting growth. Germination is the process during which a plant breaks free from its seed coating and the embryonic root begins to grow. Germination is delayed or outright inhibited in the presence of exogenous ABA based on concentration (Garciarrubio et al., 1997). Both cell proliferation and elongation are essential aspects of root growth, and auxin is a critical phytohormone involved in those processes. Auxin, another plant hormone, forms a gradient heavily concentrated within the stem cell niche that determines the fate of apical meristem cells, which guides root development and is associated with cell elongation in shoots (Sun et al., 2018). ABA is able to arrest root

growth in part by controlling auxin since auxin signaling is involved in the shaping of both the primary root and stimulates the initiation and growth of lateral roots, meaning that multiple phenotypic outcomes are possible when investigating ABA's interactions with auxin-mediated root development (Du & Scheres., 2018). Small concentrations of ABA are found to bolster the growth rate of *Arabidopsis*, and even increase the initiation of lateral root growth (Harris J. M. 2015). This is because at low concentrations of ABA accumulation, ABA seems to increase auxin transport and signaling in the apex of the root which bolsters root growth (Xu et al., 2013). At higher concentrations of ABA accumulation, auxin levels are reduced in roots, in part due to ABA's ability to destroy mRNA transcripts for auxin receptors, which leads to the arrest of root growth (Harris, M. 2015). Auxin plays a major role in the pathways responsible for the increased root growth at low ABA concentrations, as well as the inhibitory effects on root growth at higher concentrations of ABA (Li et al., 2017).

While exogenous ABA at low concentrations can encourage growth or disrupt only lateral root initiation, the general trend in *Arabidopsis thaliana* is that higher ABA concentrations inhibit germination, as well as primary and lateral root growth (Li et al., 2017). Exposure to higher ABA concentrations creates a phenotype which is more drastic and therefore easier to score for analysis than lower ABA concentrations. Because of this, we instead opted to use primary root growth as our reference for ABA susceptibility, and used 0.25μM ABA for germination assays only while increasing ABA concentrations to up to 20μM for root growth assays.

To learn more about the transcription factors that facilitate ABA response, two genetic approaches were pursued to identify novel genes required for ABA responses in the model plant

Arabidopsis thaliana. The first approach involves the use of artificial microRNAs (amiRNAs) targeting potentially redundant genes (Hauser et al., 2013). Micro RNAs are, on average, around 21 nucleotides long and are extremely useful for down-regulation or post-transcriptional supression of targeted gene products (Elbashir et al., 2001). Plants have their own endogenous miRNAs that are used for facilitating regulation and development (Napoli & Jorgensen.,1990; Reinhart et al., 2002), and as we have learned more about miRNA function we have learned how miRNAs can be used to silence specific genes (Chuang & Meyerowitz., 2000). Artificial miRNAs can be made that target mRNA transcripts and either degrade the associated mRNA, or stop the mRNA from being translated by the ribosome (Agrawal et al., 2013). Because the amiRNAs act as post-transcriptional regulators they result in a knockdown of the gene responsible for producing the mRNA. This knockdown which reduces mRNA levels makes miRNA-based approaches valuable for their capacity to study genes that are lethal if completely turned off like they would be in a gene knockout. Additionally, because miRNAs can target closely homologous sequences, miRNAs can target similar regions of different genes that may have overlapping functions allowing them to silence multiple genes at once (Bezanilla et al., 2005). Plants have larger gene families than known genome sequences of other organisms. As a result, highly similar sequences within gene families are more common in plants when compared to animals, so using miRNAs to circumvent the obstacle of redundant gene function provides a powerful approach for forward genetic screening (Hauser et al., 2013 Plant Cell; Hauser, Ceciliato et al., 2019).

The laboratory has computationally derived over 2 million amiRNA sequences that are predicted to target diverse combinations of homologous subsets of genes (Hauser et al., 2013).

Furthermore, sub-libraries from 22,000 amiRNAs were synthesized (Hauser et al., 2013) and T2 generation seeds were generated for over 14,000 genes for forward genetic screening (Hauser, Ceciliato et al., 2019 J; Y. Takahashi et al. 2020).The amiRNA library consists of many different sub-libraries, and the sub-library we used for our experiment was the amiRNA transcription factor library, which also includes DNA and RNA binding proteins, that targets 8,611 genes across 2964 unique amiRNAs (Hauser et al., 2013). To account for the presence of an amiRNA in our controls, our first amiRNA experiment control featured HsMyo, a Col-0 line of *Arabidopsis thaliana* containing an amiRNA targeting the human myosin gene. Because the miRNA library contains many seedlings that may be heterozygotes or may have failed to take up and express the miRNA construct, it is important to select for the control and experimental seeds that actually express an amiRNA. To test for miRNA presence, a basta resistance selection can be used because the amiRNA seeds from the transcription factor library and the HsMyo control amiRNAs are resistant to phosphinothricin (Hauser et al., 2013). For later experiments when a permanent AIF1 mutant was created, Col-0 was used as a control instead of HsMyo as there was no longer a need for an amiRNA in our control.

The second approach will feature naturally occurring lineages of globally diverse *Arabidopsis thaliana* ecotypes. Natural populations (ecotypes) of *Arabidopsis thaliana* can be found across the world and, due to unique environmental conditions, possess a new source of genetic variation that may affect ABA responses (Weigel D., 2012). Here we show two of these ecotypes that are native to different environments and investigate their ABA responses compared to Col-0 as a standard. The objective of the two lines of experimentation will then be to determine the transcription factors and other critical rate-limiting mechanisms involved in ABA-

mediated root growth inhibition. Understanding plant stress response pathways starting from a genetic level can grant us additional insight into how flora develop in adverse environmental conditions.

RESULTS

We started by screening the massive library of previously generated seeds containing artificial miRNAs targeting possibly redundant genes, specifically predicted transcription factors along with other DNA and RNA binding proteins (Hauser et al., 2013). From this library, we conducted an initial screen where we germinated over 1,400 seeds from this transcription factor library onto 0.5MS agar plates infused with 0.25μM ABA. We chose our initial concentration of 0.25μM ABA to delay germination because at this concentration lateral root growth was strongly inhibited showing almost no budding of new lateral roots (De Smet et al.,2003), and at the same time the concentration of ABA was not so highly concentrated that we observed excessive failure to germinate. While we had originally planned to score lateral root growth as our phenotypic indicator of resistance or susceptibility to ABA, we quickly found that impacts to lateral root growth were more subtle compared to differences in germination delay and the growth of the primary root which we decided to use when scoring for ABA sensitivity (Figure 1A). While we would later use control plates that were supplemented with EtOH to match the volume of ABA added to the experimental replicates, the initial screen of the amiRNA library only looked at ABA infused 0.5MS plates partially due to the volume of seeds being screened, and also because there was no way to confirm that the seeds we put in the control were the same as the ones in our experimental ABA replicate until we established and propagated lines. Seedlings that demonstrated a differential sensitivity to ABA when compared to the other seedlings in the transcription factor library were identified as possible candidate mutants. What constitutes differential sensitivity was measured qualitatively by measuring the primary root growth 2 weeks after the seeds were sown onto ABA plates and comparing the growth of the amiRNA seedlings to our control for either strong resistance or sensitivity to ABA (Figure 1A).

After the initial screen, possible candidates were allowed to grow to maturity and propagated. Seeds were collected from the possible candidates and rescreened, performing the same assay by comparing the primary root growth of the amiRNA seedlings against wild-type Col-0 when grown on 0.5MS agar plates infused with 0.25μM ABA (Figure 2A). This time only a handful of the possible candidates advanced to the next phase of the experiment by demonstrating a repeat phenotype of differential ABA expression.

Out of this pool of candidates, we found one amiRNA seed lineage that appeared to display consistent sensitivity to ABA (Figure 2A), and that the corresponding gene targets of this amiRNA were AIF1, and AIF4. These two targets, ATBS1 interacting factor 1 and ATBS1 interacting factor 4 respectively, are both basic helix-loop-helix transcription factors. The amiRNA targeting AIF1 and AIF4 showed heightened sensitivity to ABA by way of delayed germination and reduced cotyledon emergence on ABA infused media (Figure 2 B, C, & D). From there we set out to attain AIF1 and AIF4 mutants that had permanent alteration to the genes. AIF4 mutants were attained as an existing tDNA line allowed for purchase of the AIF4 mutant genotype, however the AIF4 tDNA was in the promoter sequence for the AIF4 gene, and the insertion into the promoter alone was not sufficient to cause any abnormal ABA response when compared to a wild type control. No tDNA lines existed for AIF1, so we performed an agrobacterium mediated transformation using CRISPR Cas9 to target the AIF1 and AIF4 sites in the hopes of making separate single mutants for each transcription factor, as well as a double mutant with a knockout of both transcription factors. Two gRNA cassettes, one designed to target AIF1 and another to target AIF4, were transformed into a pHEE401E mCherry Vector (Wang et al., 2015) containing CRISPR Cas9 (Figure 3A). This vector contained a hygromycin

resistance gene which was used for an initial screen to test for seeds that successfully integrated the plasmid vector containing the gRNA cassette. The seeds were then subjected to agrobacterium mediated transformation by floral dipping. The transformed seeds were sown onto hygromycin plates to see which seeds grew unimpeded by the antibiotic, and therefore expressed the vector. Seedlings that demonstrated growth on hygromycin were taken, potted to allow for propagation, and screened for successful Cas9 edits. During their growth, DNA was extracted from the plants and tested for mutations in the AIF1 gene. This was accomplished by taking leaf cuttings from the plants, extracting DNA, amplifying with PCR, and then using a restriction enzyme on an agarose gel to see if AIF1 was mutated (Figure 3C). We used the restriction enzyme HpyCH4III (Figure 3B) to easily screen through the plants that had been subjected to the agrobacterium mediated transformation and find the ones that had received the AIF1 edit.

The HpyCH4III restriction enzyme (Figure 3B) shows if there was a successful edit on a gel because the site where the restriction enzyme cuts is 3bp upstream of the PAM, which is also where Cas9 is supposed to cut. Our final PCR product should total around 350bp without the HpyCH4III restriction enzyme cutting. When we run a PCR, plants where the plasmid was integrated but Cas9 failed to cut will have the restriction enzyme work properly, and cleave the DNA in half, resulting in two overlapping bands at roughly 175bp each, or one at 175 and one at 350 if we have a heterozygote (Figure 3C). If the integrated plasmid successfully cuts the AIF1 gene and there is a mutation, then the restriction enzyme will not recognize the cutting site and will fail to cut, so the PCR product should show up on the gel as a band of 350bp, indicating a successful Cas9 mutation of AIF1. After using the HpyCH4III restriction enzyme screening, we isolated candidate seedlings that were heterozygous for our AIF1 mutation due to having two

distinct bands on our agarose gel, one at 175bp and the other at 350bp (Figure 3C). From there, heterozygotes for the AIF1 mutant were self-fertilized to attain a homozygous AIF1 mutant.

The mutation in the AIF1 gene as a result of our Cas9 edits was the insertion of a thymine (Figure 3D), causing a frameshift mutation that resulted in a few early stop codons being created in the gene (Figure 3E). What is important about the location of the early stop codons are that they appear just before a basic helix-loop-helix (bHLH) binding domain (Figure 3E). AIF1 is a basic helix-loop-helix transcription factor, meaning it relies on its bHLH domain to successfully bind to DNA (Jones S., 2004). Because of the Cas9 edits to the AIF1 gene, a number of early stop codons were introduced to the gene before the bHLH sequence, truncating the final protein before the bHLH motif could be translated. This early termination deforms the resulting AIF1 protein and renders it non-functional.

We wanted to determine if the ABA hypersensitivity we saw in the AIF1/4 amiRNA line was attributable to the amiRNA targeting both genes or, alternatively, if only one of the two transcription factors needed to be eliminated to confer the ABA hypersensitivity. To that ends, the homozygous AIF1 mutant was subjected to both germination assays (Figure 1A) and root growth transfer assays (Figure 1B) to assess its susceptibility to ABA (Figure 4). When the AIF1 mutant seeds underwent a germination assay, we found that while the AIF1 mutant didn't have drastically reduced cotyledon emergence when compared to Col-0 (Figure 4A), there was a noticeable delay in germination, enough that the final growth of the primary root was noticeably shorter in the AIF1 mutants when compared to the control (Figure 4B, 4C, & 4D). Additionally, when the AIF1 mutants underwent a root growth transfer assay, they also showed ABA hypersensitivity in the form of reduced primary root elongation after being transferred to media plates containing 15μM ABA (Figures 5A, 5B, & 5C). Due to the AIF1 mutants displaying

hypersensitivity to ABA in both germination and during root elongation, it appears that AIF1 may contribute at least in part to the extreme sensitivity we saw in the AIF1/4 double mutant.

Since a knockout of AIF1 appears to confer ABA hypersensitivity, we were interested to see if AIF1 overexpression could confer ABA resistance. To that ends we created a UBQ10pr:mCherry-AIF1 line designed to use the UBQ10 promoter to overexpress the mCherry-AIF1 fusion protein. Although we were able to generate a line homozygous for this transgene and use a confocal microscope to confirm AIF1's localization as a nuclear transcription factor (Figure 6A), the expression levels of our transgene were not sufficient for further testing.

Due to the success of the germination assay in helping us narrow down the amiRNA library, we chose to use the same assay to screen through a selection of accessions of *Arabidopsis thaliana* from across the world. Though most of the ecotypes we looked through did not appear to be statistically distinct from our Col-0 standard when exposed to ABA, two of the natural accessions stood out as promising due to their heightened ABA sensitivity. The first of these accessions is Bik-1, an ecotype of *Arabidopsis thaliana* native to Bikfaiya, Lebanon. When exposed to ABA during germination, Bik-1 seedlings demonstrated significantly reduced cotyledon emergence (Figure 7A, 7C, & 7D), as well as drastically reduced primary root growth from the seeds that did manage to germinate (Figure 7B, 7C & 7D). Due to the significant hypersensitivity of Bik-1 to ABA during germination, we conducted a root growth transfer assay with Bik-1 seedlings using no ABA, 5μM ABA, and 20 μM ABA (Figure 8). We saw that Bik-1 was slightly shorter, but still an acceptable match for Col-0 in our control plates without any ABA (Figure 8A, & 8D). However, when the Bik-1 seedlings were transferred to 0.5MS media plates containing 5μM ABA, we immediately saw a decrease in primary root elongation greater than the reduction seen in Col-0 (Figure 8B, & 8D). The pattern of ABA hypersensitivity

continued in the 0.5MS media plates infused with 20μM ABA, with Bik-1 showing a further reduction in primary root elongation with a lower standard deviation compared to Col-0 (Figures 8C, & 8D.

The other ecotype of *Arabidopsis thaliana* we observed to have noticeable ABA sensitivity during our screen of accessions was CIRY-13, a lineage of *Arabidopsis thaliana* native to Ciry-le-Noble, France. When the germination assay was performed comparing CIRY-13 seeds with Col-0, we observed significant levels of ABA hypersensitivity from the CIRY-13 accession (Figure 9). On our control plates, CIRY-13 had slightly better cotyledon emergence than Col-0 (Figure 9A, & 9B), however when germinated on 0.25μM ABA, the percentage of cotyledon emergence recorded in CIRY-13 dropped well below that of Col-0 (Figure 9A, & 9C) indicating that CIRY-13 possesses hypersensitivity to ABA when undergoing germination. Of the seeds that did germinate, CIRY-13 had significantly reduced primary root growth on ABA when compared to Col-0 (Figure 9A, & 9C). Taken together, these observations in both Bik-1 and CIRY-13 imply an underlying genetic distinction in these accessions of *Arabidopsis thaliana* that render these lines as ABA hypersensitive when compared to Col-0.

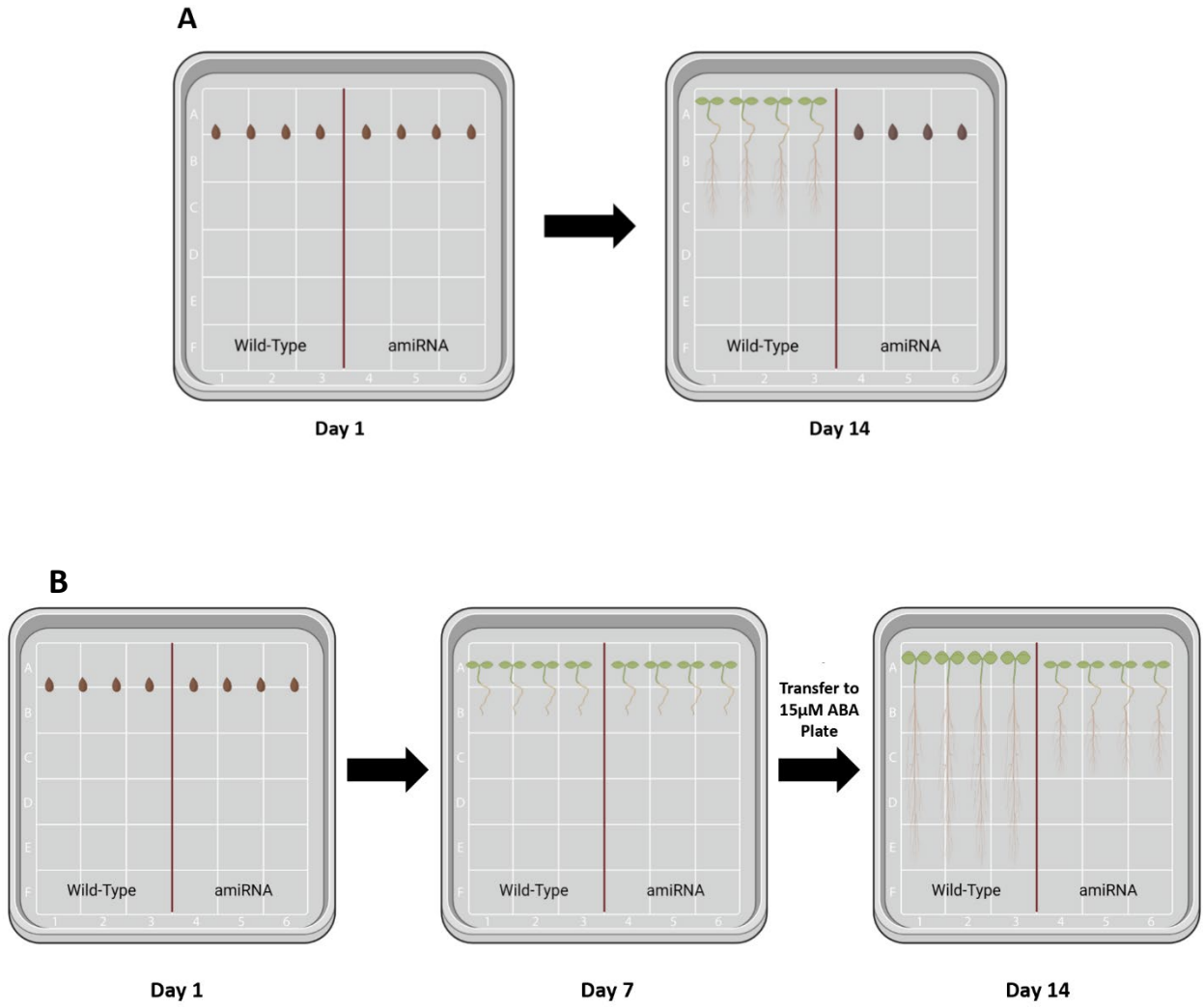

Figure 1: Protocols used to assess ABA sensitivity. (A) Protocol for germination assay. In this protocol seeds are sown directly onto 0.5MS media infused with ABA. For this assay, cotyledon emergence is used to score the percentage of successful germination, while primary root length is measured after 14 days for seeds that manage to germinate on the ABA plates. (B) Protocol for root growth transfer assay. In this protocol seeds are sown onto 0.5MS plates and allowed to grow for 7 days before being transferred to a new 0.5MS plate containing ABA to see how root elongation is impacted by ABA once seeds have already germinated and begun to grow. Seedlings are allowed to grow for 10 days before the length of the primary roots is recorded.

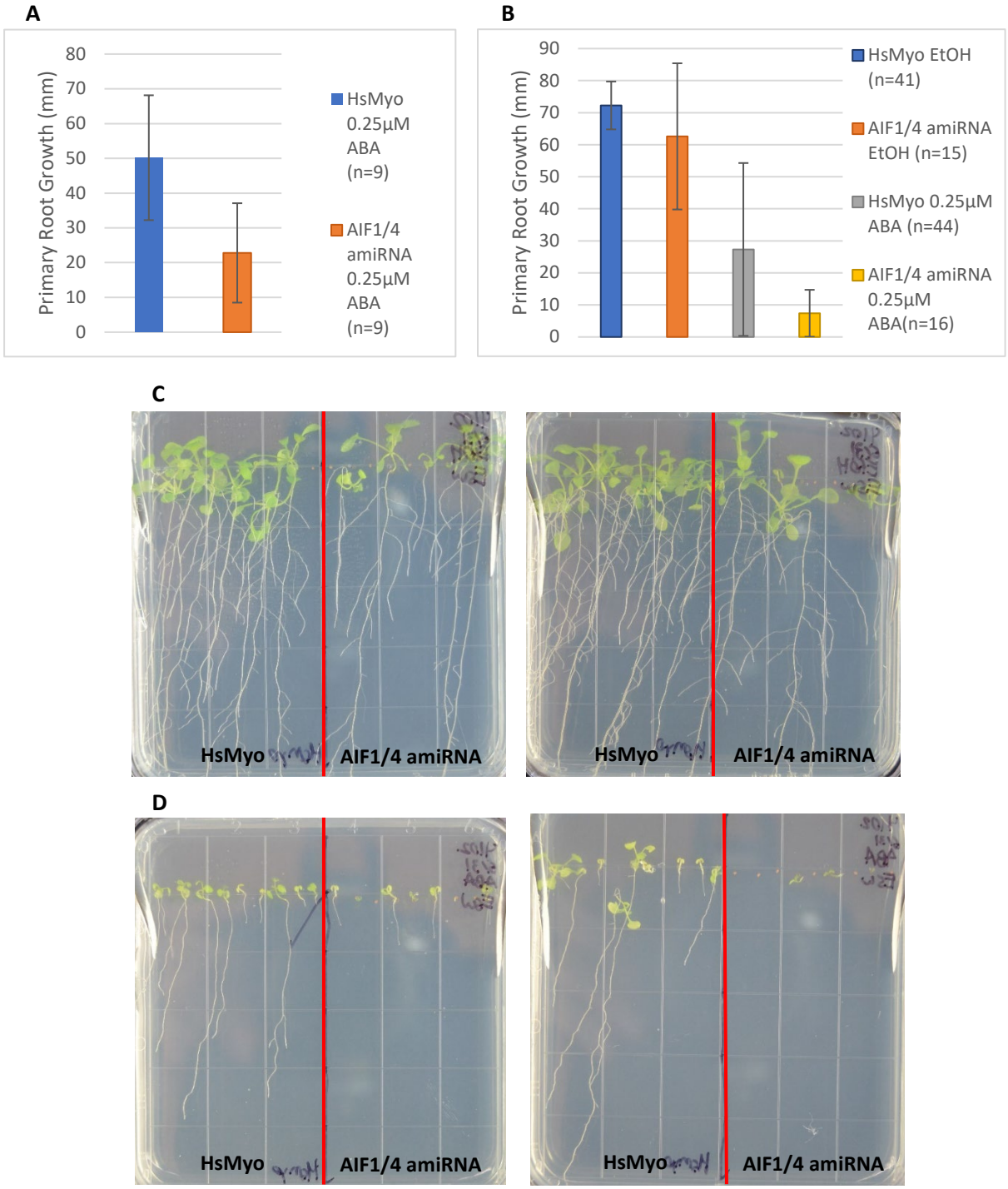

Figure 2: AIF1/4 amiRNA seeds show heightened sensitivity to ABA during germination. (A) AIF1/4 amiRNA T2 Generation Primary Root Growth After 14 Days Germinating on 0.25µM ABA. (B) AIF1/4 amiRNA T3 Primary Root Growth After 14 Days Germinating on 0.25µM ABA. (C) Images of AIF1/4 amiRNA T3 generation seeds on EtOH plates 14 days after sowing. (D) Images of AIF1/4 amiRNA T3 generation seeds on 0.25µM ABA plates 14 days after sowing.

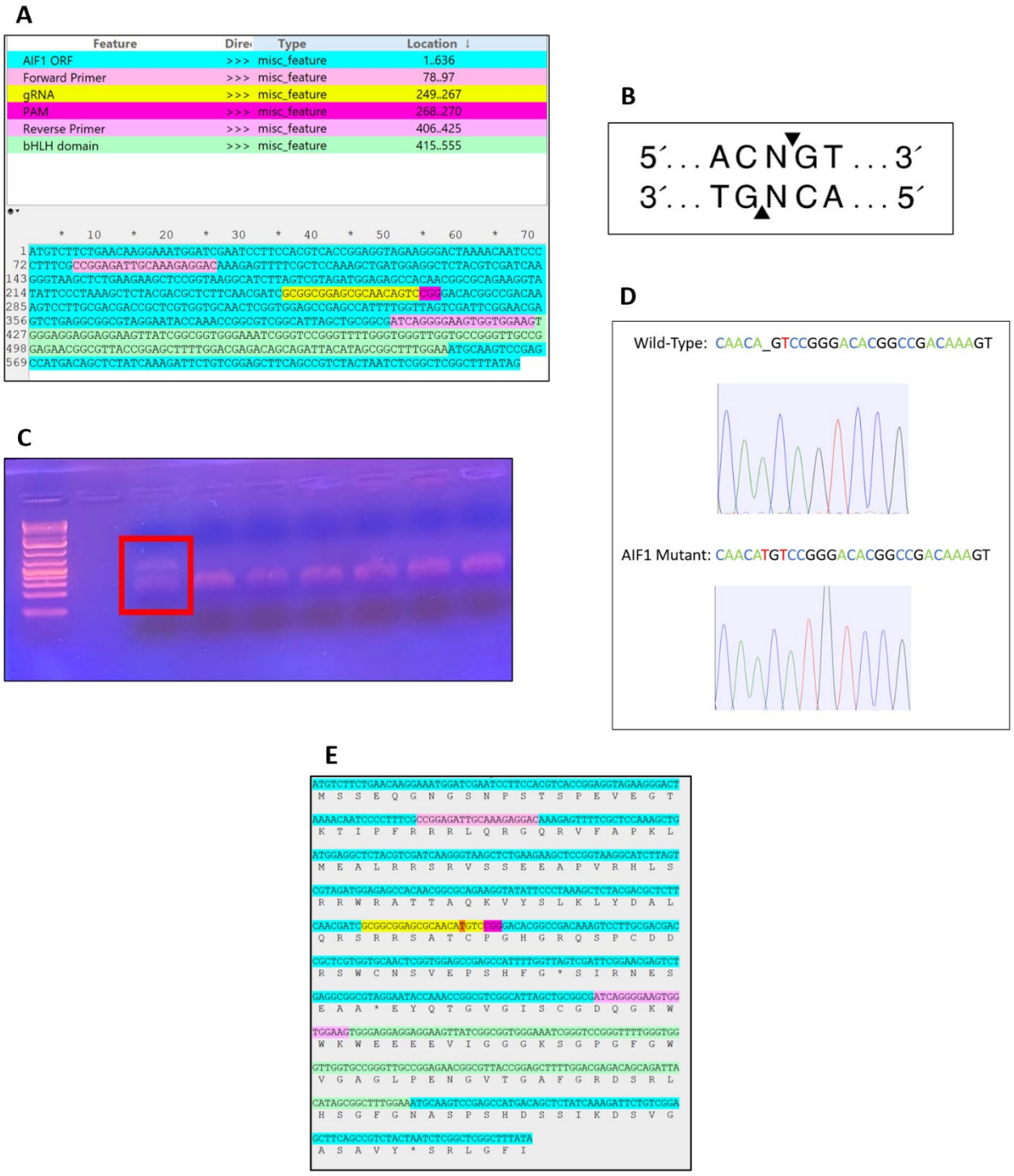

Figure 3: Creation of an AIF1 mutant using CRISPR Cas9. (A) Open reading frame of AIF1 gene (light blue), including primers (light pink), guide RNA (yellow), location of the PAM site (dark pink), and the basic helix-loop-helix binding domain (light green). (B) Cut sequence for the HpyCH4III restriction enzyme. (C) DNA of heterozygous AIF1 mutant on a gel, showing up as a double band with one band at ~350bp and one band at ~175bp. (D) Chromatogram of AIF1 homozygous mutant. (E) Amino acid sequence of AIF1 mutant, with thymine insertion (orange).

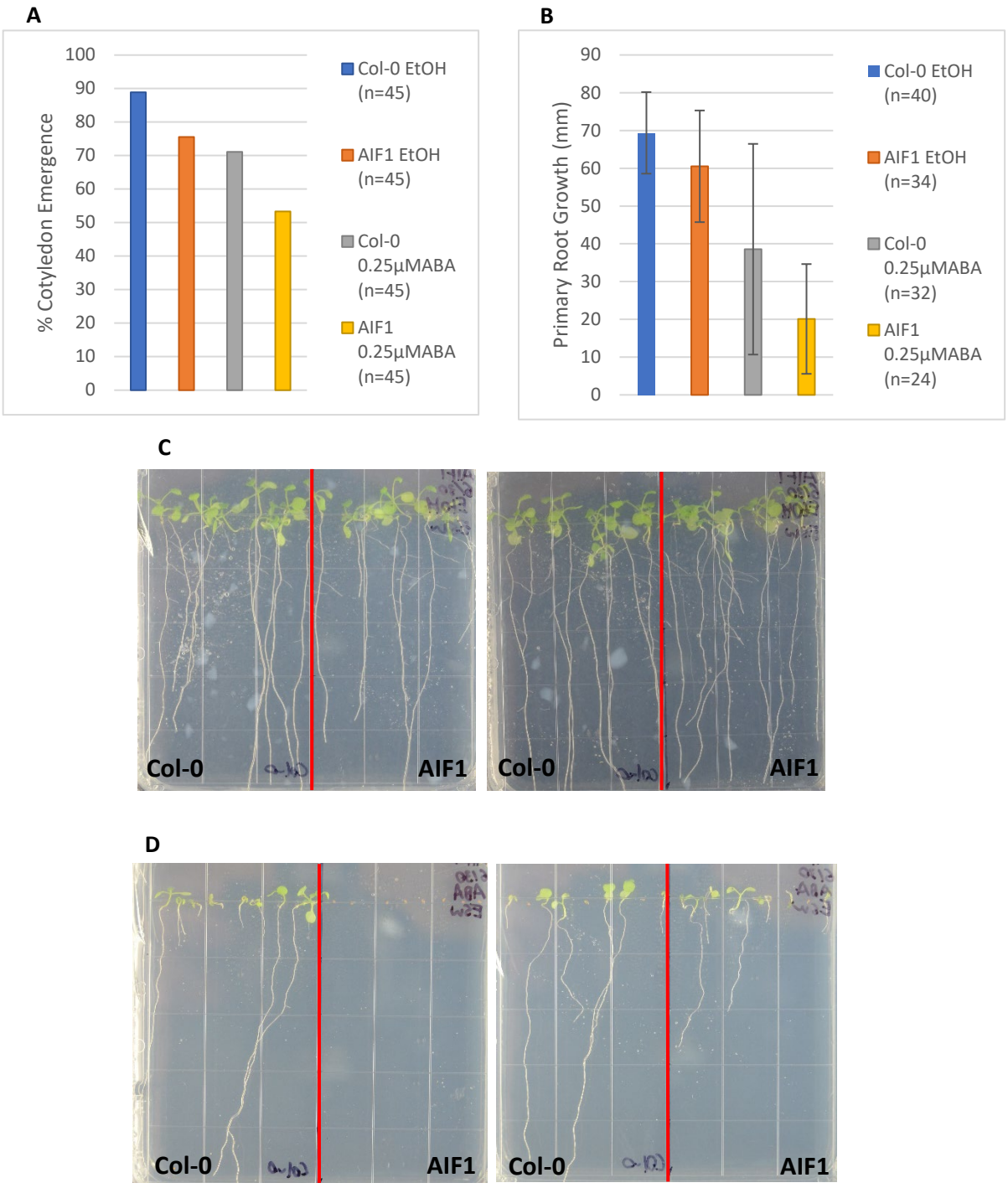

Figure 4: AIF1 mutant shows heightened sensitivity to ABA during germination. (A) Germination percentage of AIF1 mutant on 0.25µM ABA. (B) Primary root growth of AIF1 mutant after 14 days germinating on 0.25µM ABA. (C) Images of AIF1 mutant 14 days after germinating on 0.5MS plates infused with EtOH. (D) Images of AIF1 mutant 14 days after germinating on 0.5MS plates infused with 0.25µM ABA.

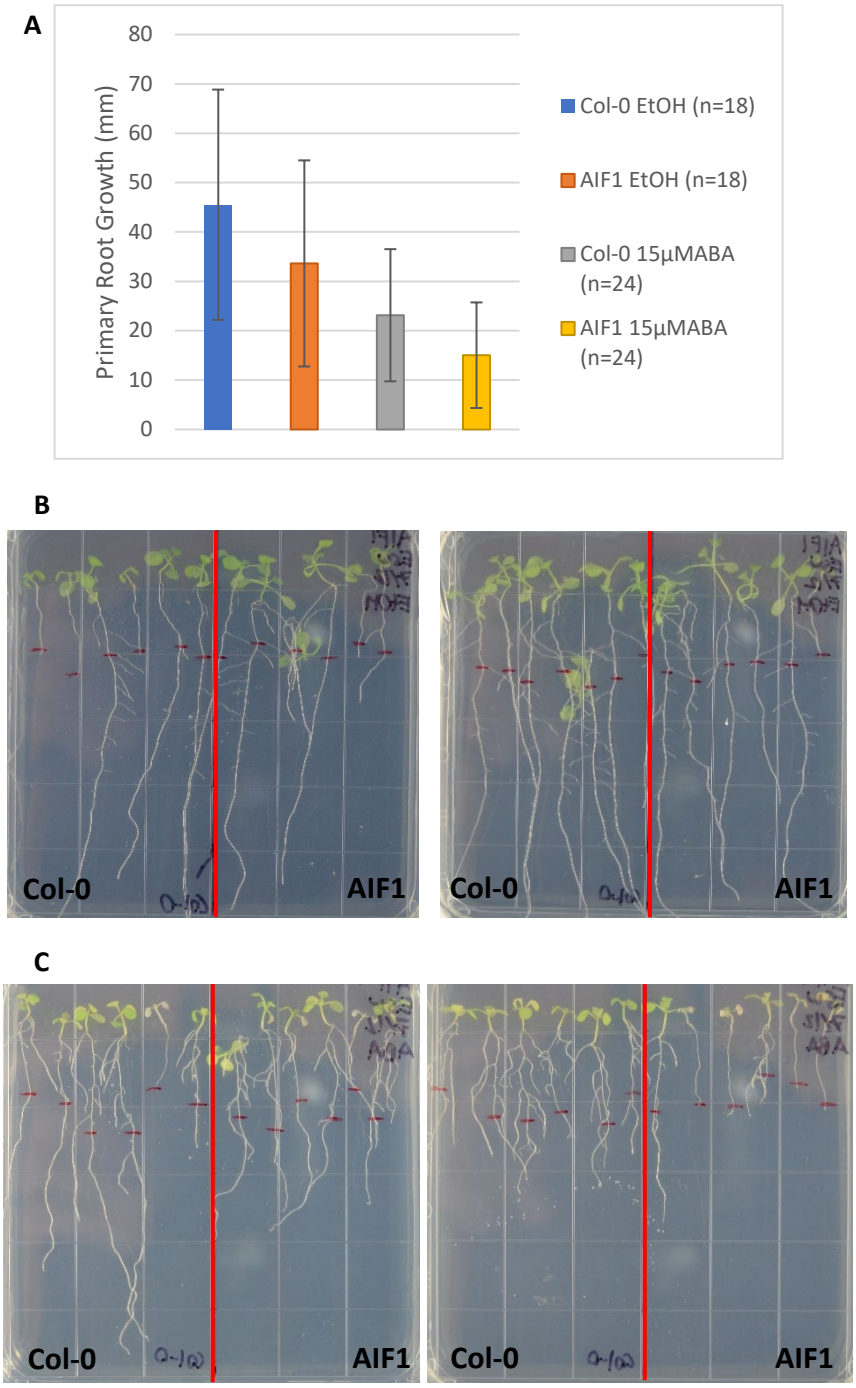

Figure 5: AIF1 mutant shows heightened sensitivity to ABA during root elongation. (A) AIF1 mutant primary root growth 10 days after transfer to ABA plates. (B) Images of AIF1 mutant alongside Col-0 grown on 0.5MS plates infused with EtOH (red lines indicate length of the root when first transferred to the new plate). (C) Images of AIF1 mutant alongside Col-0 grown on 0.5MS plates infused with 15µM ABA (red lines indicate length of the root when first transferred to the new plate).

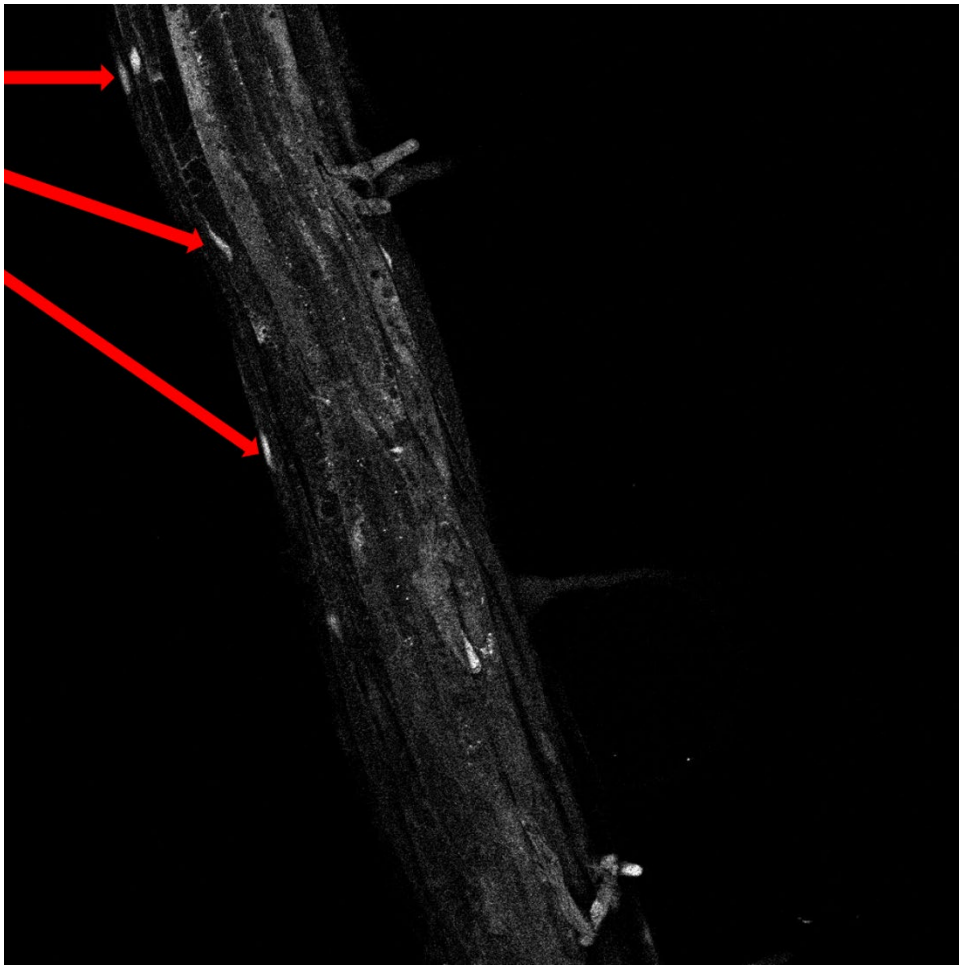

Figure 6: Analysis of a UBQ10pr::mCherry-AIF1 mutant. (A) mCherry-AIF1 fusion protein (red arrows) localized in nucleus of root.

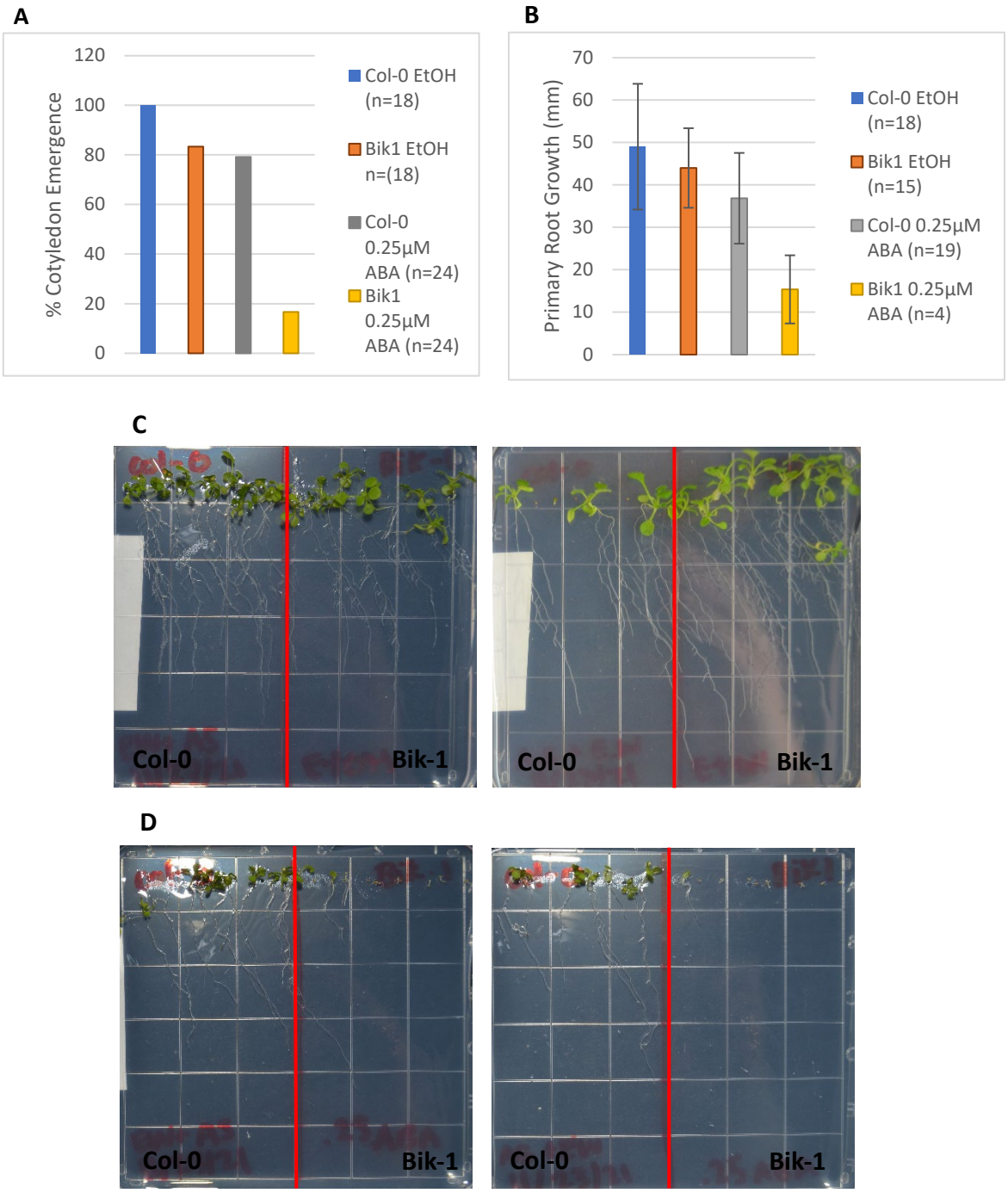

Figure 7: Bik-1 shows heightened sensitivity to ABA during germination. (A) Bik-1 germination percentage measured by cotyledon emergence after 14 days growing on 0.25µM ABA. (B) Bik-1 primary root growth 14 days after transfer to 0.25µM ABA plates. (C) Images of Bik-1 seeds on EtOH control plates 14 days after sowing. (D) Images of Bik-1 seeds on 0.25µM ABA plates 14 days after sowing.

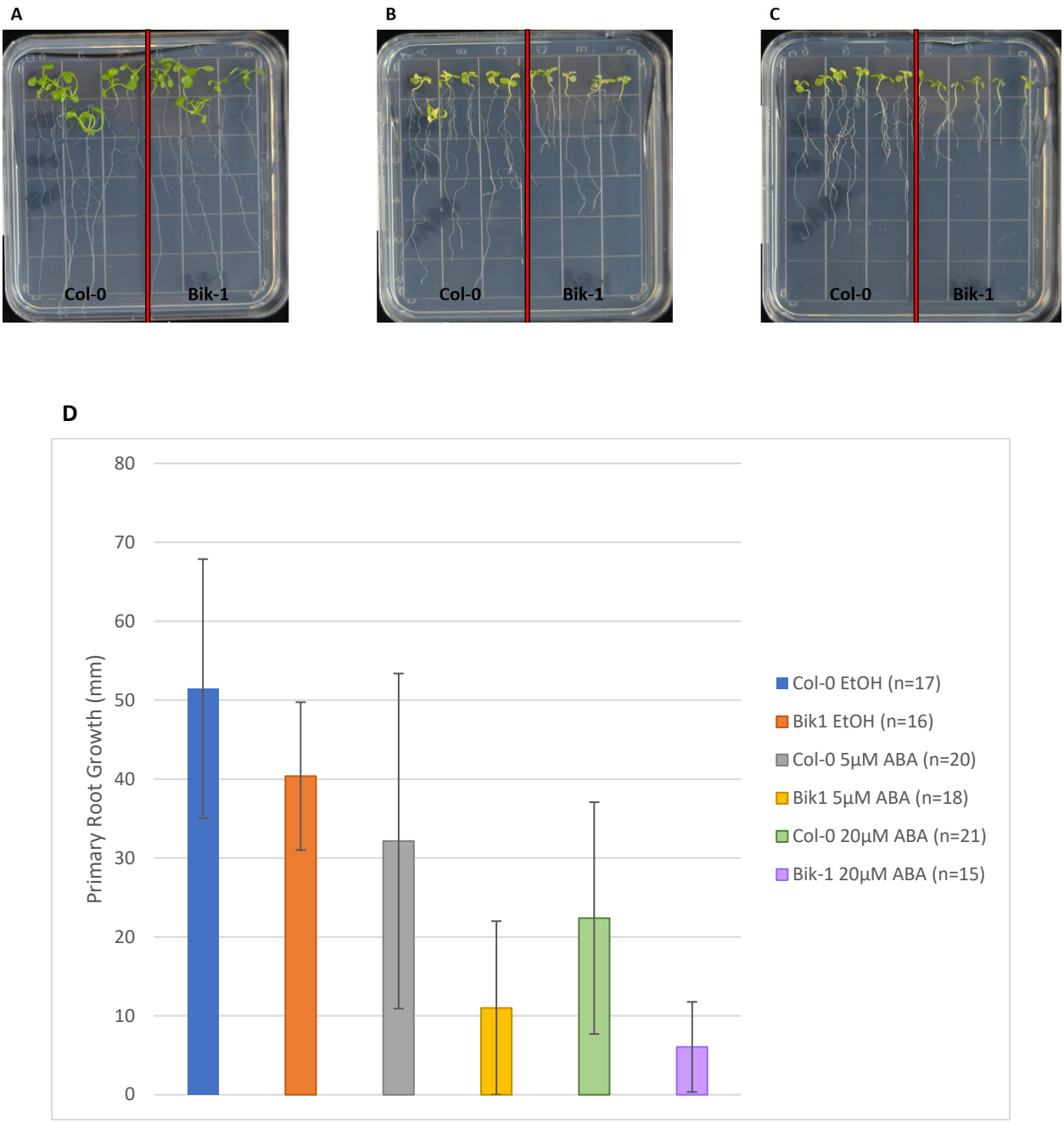

Figure 8: Bik-1 shows heightened sensitivity to ABA during primary root elongation. (A) Images of Bik-1 seeds 10 days after transfer to control plates containing EtOH. (B) Images of Bik-1 seeds 10 days after transfer to plates containing 5µM ABA. (C) Bik-1 seeds 10 days after transfer to plates containing 20µM ABA. (D) Primary root growth of Bik-1 seeds 10 days after transfer to plate containing EtOH, 5µM ABA, and 20µM ABA.

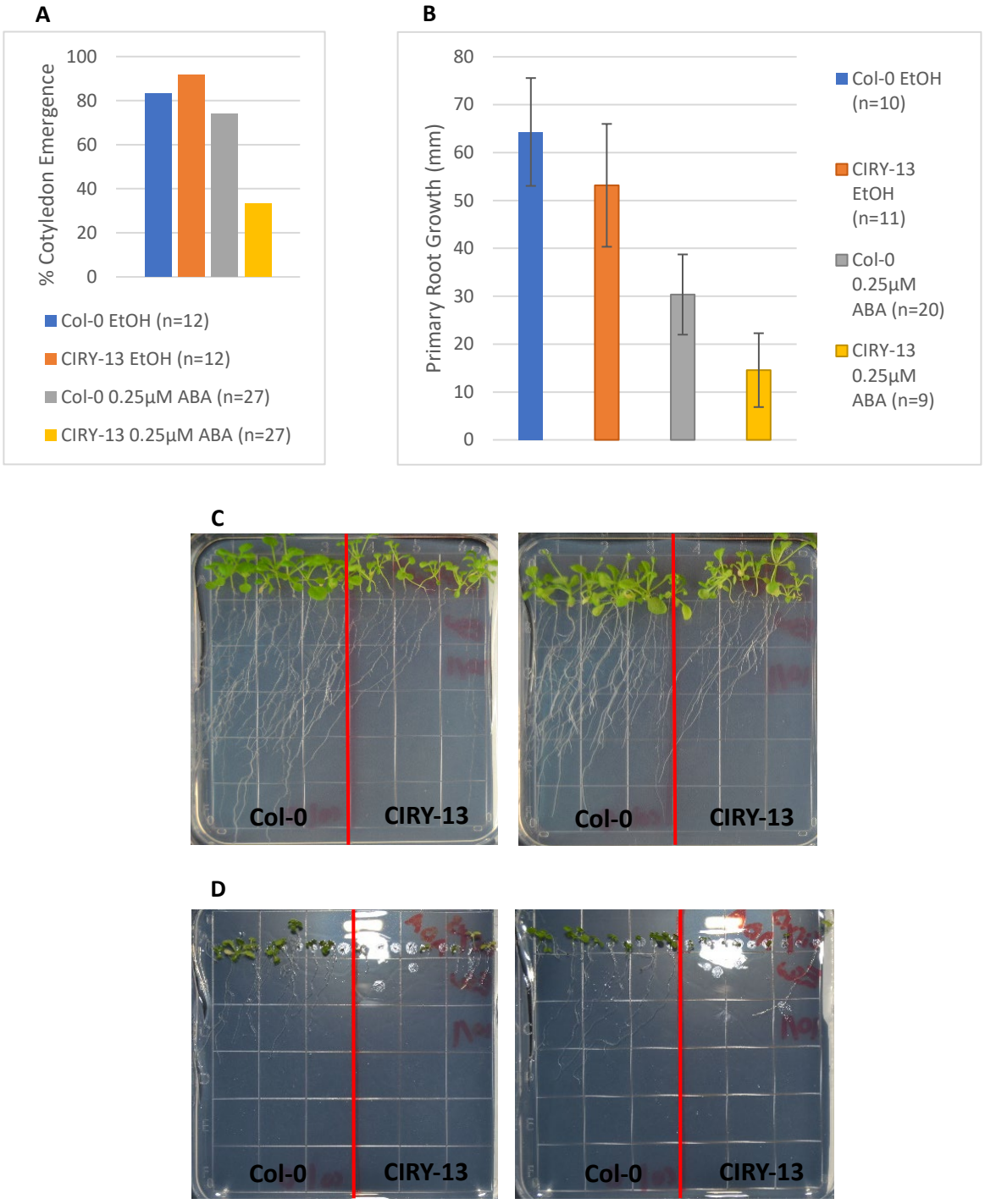

Figure 9: CIRY-13 shows heightened sensitivity to ABA during germination. (A) Germination % of CIRY-13 on 0.25µM ABA. (B) CIRY-13 Primary Root Growth After 14 Days Germinated on 0.25µM ABA. (C) Images of CIRY-13 14 days after germinating on 0.5MS plates infused with EtOH. (D) Images of CIRY-13 14 days after germinating on 0.5MS plates infused with ABA.

DISCUSSION

Summary

We aimed to learn more about the transcription factors involved in the ABA stress response pathway by using naturally occurring genetically diverse ecotypes of *Arabidopsis thaliana*, while also using artificial miRNAs to screen for new genes and homologous gene combinations involved in the ABA response pathway. We collectively isolated the natural ecotype lines CIRY-13 and Bik-1 as demonstrating increased sensitivity to ABA, as well as using amiRNAs to find that AIF1 and AIF4 are transcription factors also implicated in ABA sensitivity.

AIF and amiRNA Experiments

Using the artificial miRNA approach, we isolated candidate mutants that cause ABA hypersensitivity in the form of delayed germination and inhibited primary root elongation. We found that targeting AIF1/4 with an amiRNA was able to cause increased ABA sensitivity by disrupting germination. Additionally, when testing a single AIF1 mutant, there seemed to be a notable ABA sensitivity increase in terms of reduced cotyledon emergence and delayed germination when compared to our control, though the same could not be said for only disrupting the promoter of AIF4. During our testing, we noticed that during both germination assays and root growth assays that a single outlier replicate was usually responsible for larger standard deviations in the data we collected. We observed that in most instances when collecting 4-5 replicates for our germination experiments, we often found a single outlier replicate that would appear to possess a phenotype somewhere in between our 0.5MS media plates infused with EtOH and our 0.5MS media plates infused with ABA. This observation resulted in some

replicates generating results as if they had received a lower concentration of ABA despite mixing the ABA infused media thoroughly before pouring our 0.5MS plates. This is why in our photos of the AIF1 knockout line, we see that the AIF1 mutant seeds are more sensitive to ABA during primary root elongation on average when compared to Col-0 (Figures 5B & 5C), but the graph showing primary root growth has a large standard deviation since some plates appeared to have higher ABA concentrations than others (Figure 5 A), though AIF1 was always more sensitive than its control counterpart. Similarly, sometimes control plates would have inhibited germination or root elongation when all other control plates grew as expected. It is possible that these anomalous plates received a slightly different angle of light or disproportionate humidity causing them to have unequal conditions that affected the outcome of experiments.

The original AIF1/4 amiRNA line was difficult to screen as each successive generation had less potent expression of the amiRNA silencing. The phenomenon that the RNAi suppression grows weaker over successive generations is an occurrence that has been observed in *Caenorhabditis elegans* for some time but was recently documented in *Arabidopsis thaliana* as well (Hu et al., 2020). It becomes difficult to screen our artificial miRNA seeds for repeat phenotypes when each subsequent generation has different levels of amiRNA silencing, trending towards the loss of RNAi silencing, resulting in an unstable lineage with a notable deviation in phenotypes. Therefore, future research will be needed to determine how extensive the reduction in amiRNA silencing becomes after each generation since transgene expression may be increasingly silenced after every propagation to a new generation of seeds. Additionally, future experiments need to be conducted after retransformation with the original amiRNA line to start.

While we did see that our AIF1 mutant line demonstrated sensitivity to ABA, it was not as strong as the sensitivity we perceived originally with the AIF1/4 amiRNA. One source of variability in our data comes from the enduring presence of Cas9 and the guide RNAs in the AIF1 mutants. The persisting Cas9 means that it may continue to cut at the AIF4 sites due to the present gRNA. This means that while all the seeds have been verified through sequencing to be AIF1 mutants, some of the seeds in the AIF1 mutant line may be AIF4 heterozygotes, some may have a wild type AIF4 gene, and others still could potentially be AIF4 homozygous mutants in addition to being an AIF1 mutant. Because the CRISPR Cas9 remains active, there is even the potential for off target cutting. This could potentially cause us to see different phenotypes between individual seeds, generate outliers, and cause a shift in the perceived efficacy of a single AIF1 mutation.

As expected, we observed that ABA delays germination and inhibit root growth which agrees with prior studies (Finkelstein R., 2013; Garciarrubio et al., 1997). Additionally, when investigating a UBQ10pr::mCherry-AIF1 line, we saw the mCherry-AIF1 fusion protein localized in the nucleus of root tissue, which corroborates existing research that AIF1 is a transcription factor within the nucleus (Wang et al., 2009). However, our findings on AIF1's impact on plant growth is in opposition to existing research.

Our control replicates sown onto EtOH infused 0.5MS media plates show that the knockdown and knockout of AIF1 resulted in slightly shorter root length in our control conditions than average, with a much greater reduction in root length when exposed to ABA. This disagrees with previous studies that AIF1-4 were negative regulators of *Arabidopsis* cell

elongation, though those studies only look at leaf tissue and not root length (Ikeda et al., 2013, Wang et al.,2009). Prior experimentation has reported that an AIF1 knockdown was able to partially rescue a Br1 dwarf mutant, and that AIF1 overexpression in wild-type *Arabidopsis thaliana* was able to create a dwarf phenotype (Wang et al., 2009), however our research has generated results in opposition to that of older studies. AIF1 was formerly shown to be a negative regulator of brassinosteroid which has a positive impact on cell elongation (Wang et al.,2009) meaning AIF1 would have a negative impact on cell elongation that corroborates another paper implicating AIFs in negatively regulating cell elongation in *Arabidopsis thaliana* (Ikeda et al., 2013). Our studies have shown that AIF1 knockouts result in slightly shorter root length than wild-type Col-0 for our control replicates. In supplemental figures provided in "Regulation of *Arabidopsis* Brassinosteroid Signaling by Atypical Basic Helix-Loop-Helix Proteins" (Wang et al.,2009) a photo was included demonstrating that the use of RNAi to silence AIF1 in wild type *Arabidopsis thaliana* resulted in no perceivable phenotypic change, while our current experiments that have a full AIF1 knockout show a decrease in root growth.

Though we previously mentioned the variability brought about by the enduring Cas9 in our AIF1 mutants, and the variability from the gradual transgenerational silencing of the amiRNA in our AIF1/4 amiRNA screen, there are two other sources for variance in our data. One source of variation comes from the natural discrepancy in how seeds germinate, even when comparing seeds of the same lineage grown in the same conditions. Even in our wild-type controls, there is variation in how seeds germinate and grow, so those variations only grow as we include more variables in the form of external ABA, and gene knockouts (Abley et al., 2021).

Additionally, based on our observations and the data collected it also appears that the root transfer assay may cause unquantified stress to plants during the transition between plates. This stress is likely caused by a mix between the mechanical transfer of the seedlings and the sudden drop in humidity as they are transferred between controlled environments, but another combination of factors may be at play as well. Even when seedlings are taken from a normal 0.5 MS plate and placed onto our control EtOH 0.5 MS plate, there is a significantly higher standard deviation in the root transfer assay's control when compared to the germination assay's control. Similarly, if we look at the Bik-1 vs Col-0 experiments, and the AIF1 vs Col-0 experiments, the control data for different seed lines in the EtOH plates is closer for the germination assays when compared to the root transfer assays which have a larger standard deviation on average. The stress of transferring the seedlings between plates for both control and experimental replicates is a source of variability that should be taken into account when interpreting the data collected.

Natural Accessions Experiments

Both CIRY-13 and Bik-1 are ecotypes of *Arabidopsis thaliana* that demonstrated heightened ABA sensitivity when compared to Col-0. Both Bik-1 and CIRY-13 showed lower frequencies of cotyledon emergence and reduced primary root growth when germinated directly on 0.25μM ABA compared to our control. Additionally, when grown on normal 0.5 MS media plates and then transferred to plates infused with ABA after 7 days, we found that Bik-1 showed inhibited root elongation when compared to the Col-0 control ecotype. These results are subject to some of the considerations mentioned above in the section on the AIF and amiRNA experiments. This includes the discrepancy of 0.5MS plates sometimes receiving slightly more or less ABA than intended, the natural disparity in the rate at which seeds of the same lineage and

age germinate, as well as the limitations for the experimental procedure of the root transfer assay causing unquantified stress that results in the control and experimental replicates growing worse after the transfer.

Conclusions and Future Directions.

During our experiments we found that silencing AIF1 and AIF4 using an artificial miRNA resulted in an increased sensitivity to ABA. Furthermore, knocking out AIF1 on its own conveyed a partially enhanced ABA sensitivity when compared to that of the double AIF1/4 knockdown, though it would be beneficial to generate more replicates to verify existing data. Several future experiments can be designed from the data generated in this thesis, delving deeper into both the AIF transcription factors, as well as the natural ecotypes of *Arabidopsis thaliana*. Regarding the screen of the transcription factor portion of the amiRNA library, further research will be needed to uncover the relationship between the AIFs and ABA responses. We need to test an AIF4 single mutant, as well as an AIF1/4 double mutant. This will illuminate whether only one of the two AIFs mentioned in this thesis was responsible for the amiRNA-silenced phenotype we observed, or if both AIF1 and AIF4 need to be knocked out to achieve the ABA sensitivity phenotype. Any further testing of the AIF1/4 amiRNA should be done after retransformation with the original amiRNA as the silencing phenotype appears to grow weaker with each subsequent generation. Finally, though the amiRNA identified in this thesis achieved a phenotype by only targeting AIF1 and AIF4, in the future it would be worth investigating AIF2 and AIF3 to also illuminate interesting relationships between the other ATBS1 Interacting Factors and the ABA response pathway.

For the natural variation approach to genetic diversity, a root transfer experiment with CIRY-13 should be conducted to see if the ABA sensitivity phenotype is exclusive to germination, or if it also applies to root growth like what we observed with Bik-1. An experiment should be developed and pursued to compare the transcriptome of CIRY-13, Bik-1, and Col-0 to gain further insights into how the ABA hypersensitivity may be mediated. To continue the natural variation approach to uncovering more about how ABA sensitivity differs in naturally occurring ecotypes, the next steps will be to create hybrids of our current lineages of interest. This will be accomplished by crossing Bik-1 with Col-0, as well as CIRY-13 with Col-0. The progeny of the original cross will be self-pollinated to create recombinant inbred lines to reduce heterozygosity, and then tested for an ABA sensitivity phenotype like the one we witnessed in the original Bik-1 and CIRY-13 lines respectively. If the Bik-1/Col-0 hybrid and CIRY-13/Col-0 hybrid lines still demonstrate sensitivity to ABA, then the genes conferring that sensitivity still exist within the hybrid. The hybrids will then be crossed with Col-0 again to attain a new hybrid with a genome more closely resembling Col-0. That new hybrid will be self-fertilized to reduce heterozygosity further and to attain another hybrid, now possessing a greater portion of Col-0 genome, but still having some elements of the CIRY-13 or Bik-1 genomes once again. The resulting hybrids will again be tested for ABA sensitivity and, if ABA sensitivity is still present, then we can assume the genes conferring sensitivity in these hybrid plants come from their original Bik-1 or CIRY-13 lines. This process can be repeated until the genome of the hybrid sufficiently resembles Col-0 enough that we could compare the genomes of the hybrids directly to the genome of Col-0. This would generate a list of genes seen in the hybrid that stand out as being irregular to the typical Col-0 genome and could be responsible for the ABA sensitivity attributable to the original Bik-1 or CIRY-13 lineages. From there the genes can be tested

individually to see if they are responsible for conferring the ABA sensitivity observed in these naturally diverse ecotypes of *Arabidopsis thaliana*.

In the present thesis, genetic screens were conducted to identify new mechanisms that cause ABA hypersensitivity in seeds and seedling roots. An amiRNA screen, that uses combinational supression to co-silence homologous transcription factors, led to the identification of transcription factors that have not been previously implicated in ABA signaling. These two transcription factors, AIF1 and AIF4, may play a crucial role in the ABA-mediated delay of seed germination and root elongation. Furthermore, a screen of *Arabidopsis* accessions identified two ecotypes that showed an increase in ABA sensitivity. These two accessions, Bik-1 and CIRY-13, were observed to be more sensitive to ABA during both germination and root elongation when compared to Col-0. Further research will be needed to dissect the mechanisms and relevance of the isolated mutants, ecotypes, and genes.

MATERIALS AND METHODS

Seed Acquisition

The artificial microRNA (amiRNA) library of seeds is all Col-0 in background and are derived from "A genomic-scale artificial microRNA library as a tool to investigate the functionally redundant gene space in *Arabidopsis*" (Hauser et al., 2013), and the library was received from Dr. Charles Seller (UCSD). The creation of the plasmid vector and cas9 construct for transforming the *Arabidopsis thaliana* seeds to mutate AIF1 and AIF4 was created by Dr. Charles Seller, and the process of agrobacterium mediated transformation was also carried out by Dr. Charles Seller. The generation of AIF1 homozygotes by self-fertilizing heterozygous plants was carried out by Dr. Charles Seller. The creation of the UBQ10pr::mCherry-AIF1 line was created by Dr. Charles Seller. The different natural variant lines of *Arabidopsis thaliana* including Bik-1, CIRY-13, and Col-0 were obtained from the Arabidopsis Biological Resource Center (ABRC) and the Salk Institute from Dr. Wolfgang Busch's laboratory.

Seed Sterilization

10mL of sterilization solution is made by combining 5mL of bleach, 500µL of 10% tween-20, and 4.5mL of water. Seeds are added to 1.5mL Eppendorf tubes, and 1mL of sterilization solution is added to each tube. Tubes are vortexed briefly and then placed on a rotator for 15 minutes. Next, under a sterile hood, the sterilization solution is removed from the seeds by pipetting. The seeds are washed with 1mL of autoclaved water, pipetting up and down to thoroughly rinse the seeds and remove the sterilization solution before removing the water

wash. Repeat the water wash step 4 times, leaving the 4th wash to leave the seeds suspended in water. The seeds are then placed in the refrigerator in a dark container for 3 days to stratify.

Making 0.5MS Media

To make 500mL of 0.5MS media, 1.1g of MS Salts, 0.25g MES, 2.5g sucrose, and 5g of phytoagar are mixed with water to just under 500mL. KOH is added as needed to bring pH to 5.7, and then any remaining water is added to bring final volume to 500mL. When making 0.5MS media for the root growth or seed germination portions of an assay sucrose is excluded due to its counteracting effect on ABA (Finkelstein and Lynch, 2000).

Seed Germination Assay

Working under a sterile hood, 0.5MS media lacking sucrose is split equally into two containers. To one container, ABA will be added to achieve a final concentration of 0.25µM, to the other container EtOH will be added to match the volume of ABA added to the first container. 30mL of 0.5MS media is poured into petri dishes and allowed to cool and solidify for 40 minutes. Next, sterilized seeds have their water wash removed from them, and 1mL of 0.1% agarose is used to resuspend the seeds so they stay separated for ease of disruption on the agar plates. Using a 1,000µL pipette, 360µL of the seed suspension is extracted into a 1mL pipette tip. The tip, still containing the suspension, is removed from the pipette. Next, by making contact between the 0.5MS plate and the narrow opening of the pipette tip, the seed suspension will flow and gradually deposit seeds onto the plate. Seeds are deposited in a single line, and agar plates are divided in half with one side dedicated to the control seeds and the other dedicated to the

experimental seeds. After all seeds are sown onto the plate, the lid is replaced, and the perimeter of the plates are sealed by double wrapping with micropore tape. Once all plates are sealed, they are transferred to a room with a 16-hour light/8-hour dark cycle for 14 days at 22°C. After 14 days measurements are taken of seedling growth.

Root Growth Transfer Assay

Working under a sterile hood, 30mL of 0.5MS media is poured into petri dishes and allowed to cool and solidify for 40 minutes. Next, sterilized seeds have their water wash removed from them, and 1mL of 0.1% agarose is used to resuspend the seeds so they stay separated for ease of disruption on the agar plates. Using a 1,000µL pipette, 360µL of the seed suspension is extracted into a 1mL pipette tip. The tip, still containing the suspension, is removed from the pipette. Next, by making contact between the 0.5MS plate and the narrow opening of the pipette tip, the seed suspension will flow and gradually deposit seeds onto the plate. Seeds are deposited in a single line, and agar plates are divided in half with one side dedicated to the control seeds and the other dedicated to the experimental seeds. After all seeds are sown onto the plate, the lid is replaced, and the perimeter of the plates are sealed by double wrapping with micropore tape. Once all plates are sealed, they are transferred to a room with a 16-hour light/8-hour dark cycle for 7 days. After seeds have been allowed to grow for a week they are returned to the sterile hood to be transferred to ABA plates. Working under a sterile hood, 0.5MS media lacking sucrose is split equally into two containers. To one container, ABA will be added to achieve a final concentration of 15µM, to the other container EtOH will be added to match the volume of ABA added to the first container. 30mL of 0.5MS media is poured into petri dishes

and allowed to cool and solidify for 40 minutes. Using forceps, seedlings are taken from the initial 0.5MS plates and transferred to the new 0.5MS plates containing either EtOH or ABA. The petri dishes are divided in half with one side dedicated to the control seedlings and the other dedicated to the experimental seedlings. After all seedlings are transferred onto agar plates, the lid is replaced, and the perimeter of the plates are sealed by double wrapping with micropore tape. A sharpie is used to mark the current root lengths immediately after transfer. Once all plates are sealed and marked, they are transferred to a room with a 16-hour light/8-hour dark cycle for 7 days at 22°C. Last, the final length of the root is compared to the originally marked length to measure the growth of the roots after the 7 days.

Primary Root Measurement and Graph Generation

Using Fiji, a modification of ImageJ software (Schindelin et al., 2012), the lengths of primary roots were calculated by tracing the end of the root tips to the top of the hypocotyl, just below the cotyledon. Graphs were generated using Microsoft Excel, and diagrams were generated using BioRender.com.

Primers and amiRNA Loop Sequence

Table 1.1

amiRNA Genotyping Primer (Forward)	AGAGAACACGGGGGACGAG
amiRNA Genotyping Primer (Reverse)	AAACCGGCGGTAAGGATCTG
AIF1 Cut Site Primer (Forward)	CCGGAGATTGCAAAGAGGAC
AIF1 Cut Site Primer (Reverse)	ATCAGGGGAAGTGGTGGAAG

Table 1.2

amiRNA Loop Sequence for Identifying Target	CAGGTCGTGATATGATTCAATTAGCT TCCGACTCATTCATCCAAATACCGAG TCGCCAAAATTCAAACTAGACTCGTT AAATGAATGAATGATGCGGTAGACA AATTGGATCATTGATTCTCTTTGA

This thesis contains unpublished material coauthored with Dr. Seller, Charles. Pogliano, Sofia. and Sama, Andrea. The thesis author was the primary author of this thesis.

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