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UNIVERSITY OF CALIFORNIA SAN DIEGO

High-throughput Screening of a Genetic Knockdown Collection Related to Iron-Deficiency in

*Arabidopsis thaliana*

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

in

Biology

by

Kane Wu

Committee in charge:

Professor Wolfgang Busch, Chair  
Professor Martin F. Yanofsky, Co-Chair  
Professor Julian I. Schroeder

2024

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University of California San Diego

2024

## DEDICATION

I would like to dedicate this thesis to my ever-loving parents, my amazing and supportive girlfriend Clara, and to the friends and family who have fervently and continuously supported me which has kept me driven through all of my years in college and during my Master's program at UCSD.

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## LIST OF ABBREVIATIONS

$\frac{1}{2}$  MS:  $\frac{1}{2}$  Murashige and Skoog Medium

AmiRNA: Artificial microRNA

Ami-CT: AmiRNA Control line

gRNA: guide RNA

RSA: Root Structure Architecture

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## ABSTRACT OF THE THESIS

High-throughput Screening of a Genetic Knockdown Collection Related to Iron-Deficiency in

*Arabidopsis thaliana*

by

Kane Wu

Master of Science in Biology

University of California San Diego, 2024

Professor Wolfgang Busch, Chair  
Professor Martin F. Yanofsky, Co-Chair

Iron plays an essential role in the growth, development, and biological functions of all plants. Its importance extends from being involved in processes from photosynthesis and chlorophyll biosynthesis to plant growth and development. In environments where iron is

present in low abundance, dicotyledonous plants such as *Arabidopsis thaliana*, have developed efficient strategies to acquire iron from the soil to overcome this. Some of these strategies include transcriptional changes, altering root structure architecture (RSA), and iron-translocation mechanisms to respond to environmental changes. As such, strict iron homeostasis must be maintained for proper growth and development. Vast networks of genes facilitate the proper response from environmental stimuli. These network of gene families encode for proteins with similar or overlapping functions. This feature is called gene redundancy and serves many purposes; mainly to confer robustness to biological functions to ensure that a single-gene mutation would not affect essential functions of the plant. However, this acts as an obstacle when trying to conduct a forward genetic screen. In this study, a mutant collection utilizing amiRNAs to combinatorially co-silence closely homologous gene cluster members was used to circumvent this issue. AmiRNAs are single-stranded 21-mer RNAs whose sequence can target and silence multiple genes within the same family. From this collection, 12 candidate lines with observed unique primary root growth phenotypes under iron-deficient conditions, were selected and studied for further characterization. This will enable future studies to elucidate the mechanisms involved by associating the genes targeted in the candidate lines in connection to iron homeostasis.

## INTRODUCTION

### **The Role of Nutrients on Plant Root Structure Architecture**

In plants, the root is essential for nutrient uptake to enable root growth, development, and biological functions. The root is also important for adaptation in the face of environmental stresses and conditions (Giehl et al., 2014). In the paper, Giehl discusses that plant root systems display developmental plasticity in accordance to the perception and reaction to environmental information thus altering root progression and maturation. Morphological changes of the root due to nutrient bioavailability can be observed in the primary and lateral roots.

In plants, the nutrients that are uptaken affects its primary root developments by three major cellular biological processes: 1.) the rate of cell division, 2.) the rate of cell differentiation, and 3.) the elongation of cells (Giehl et al., 2014). In lateral roots, nutrients availability influences development throughout their different chronological stages, from initiation from pericycle cell to their elongation (Péret et al., 2009). There is a need to sustain progressive growth and development and so plants need to constantly monitor and react to fluctuations of soil nutrients in a spatial and temporal manner (Giehl et al., 2014). Signals that are triggered by soil nutrient detection, induces physiological and biological responses to initiate nutrient mobilization, storage, uptake, and allocation (Alvarez et al., 2012). Such nutrient signals induces changes in gene or protein expressions within root developmental program to cause specific root development based on the conditions in the soil (Giehl et al., 2014). Among those nutrients, iron is one that have been extensively studied because of its biological importance in plants.

## Iron Uptake in Plants

In plants, iron is a micronutrient that is essential for many major biological functions, such as cellular respiration, metabolism, oxygen transport, photosynthesis, and defense mechanisms within plants (Li & Lan, 2017). Although iron is relatively abundant within the earth's crust and soil, iron bioavailability to plants is exceptionally low (Morrissey & Guerinot, 2009). In nature, iron is present in two forms, the ferrous ( $\text{Fe}^{2+}$ ) and ferric ( $\text{Fe}^{3+}$ ) forms. Ferric iron ( $\text{Fe}^{3+}$ ) is the predominant form in soil, however it cannot be readily absorbed by plant roots, hence iron is not bioavailable to plants (Naranjo & Bauer, 2016). Plants utilize two main strategies to uptake iron and maintain iron homeostasis; Strategy I, for non-graminaceous plants and dicots, and Strategy II, for graminaceous plants and monocots (Celma et al., 2013). Dicotyledonous plants utilize Strategy I to uptake iron by first acidifying the plant rhizosphere which is done by the *H<sup>+</sup>-translocating P-type ATPase 2, AHA2*, to increase the availability of Fe(III)-chelate concentration (Li & Lan, 2017). Subsequently, available ferric iron, Fe(III), is reduced down to Fe(II) through the *Ferric-Chelate Reductase 2, FRO2*, by which Fe(II) is taken up into the plant by the *Iron Regulated Transporter 1, IRT1* (Murata et al., 2015; Figure 1). In Strategy II, monocotyledonous plants synthesize and release iron-chelating molecules, phytosiderophores (PS), through the *Transporter of Mugineic Acid 1, TOM1*, transporter to bind with insoluble Fe(III) in the soil and taken up by the plant as a Fe(III)-PS complex through the YS1 transporter.

## Iron Responses on Root Structure Architecture in Plants

On one end, excessive iron can generate oxidative stress in plants cells and cause iron toxicity for the plant (Wu and Ling., 2019). On the other, iron deficiency can trigger signals in plants to alter root development and induce variations in biological and morphological responses

(Giehl et al., 2014). These responses allow the plant to direct resources to ensure iron sufficiency for development and growth along with the facilitation of directional root growth towards more favorable patches of soil (Li et al., 2016). Under iron deficiency, some of the plants' responses can trigger increase of root hair formation to expand the root surface area to uptake more iron (Li et al., 2016). Additionally, iron-deficient stress also triggers the formation of ectopic root hairs at locations of the root that are normally occupied by non-hair cells (Schmidt et al., 2000). Other iron responses also include alterations of the lateral roots in the face of iron-deficiency. It is suggested that an increase of lateral root number and density is triggered under moderate Fe deficiency (Gruber et al., 2013). Studies have also shown that moderate iron limitations affects the RSA by regulating root-tip hormones and iron content, however, under severe iron deficiency, plant elongation of lateral roots are repressed (Giehl et al., 2012a; Giehl et al., 2012b; Giehl et al., 2014). Primary roots affected by iron-deficiency are one of the more commonly observed effects as a result of insufficient iron in its environment. Under moderate iron-deficiency, it was suggested that the primary root lengths are increased, however under severe deficiency of iron, the decrease and inhibition of primary root length was exhibited (Gruber et al., 2013; Satbhai et al., 2017). These morphological changes are a result of the plants' regulative response pathways to maintain iron homeostasis for iron uptake, translocation, and storage (Wu and Ling., 2019). In plants, transcription factors play essential roles towards regulating the transcriptional levels of genes under iron deficiency. As such, the master regulator *FIT* (*FER-like Iron Deficiency-Induced Transcription Factor*) gene encodes for a basis-helix-loop-helix transcription factor that plays a crucial role in regulating iron-uptake genes within the root epidermis upon iron-deficiency (Mai et al., 2015; Sivitz et al., 2012; Wild et al., 2016). As a result, this poses a challenge when trying to identify candidate genes in traditional forward genetic screenings.

## **The Issue of Redundancy**

Forward genetic screenings are powerful tools that are used to identify new genes. However, one of the issues that traditional forward genetic screens run into is genetic redundancy. Genetic redundancy describes the situation when two or more genes perform the same function and upon inactivation or mutation of one gene would then have mitigated or no effect on the biological phenotype (Nowak et al., 1997). This results from the extensive overlapping functions of closely homologous genes mediated by the large-gene families present in the plant genome (Arabidopsis Genome Initiative, 2000). In fact, a genome-wide analysis study have shown that within the *Arabidopsis* genome, an estimate of 75% of the genes are members of gene families (Xie et al., 2021).

Different methods have been developed to circumvent this issue. One such method employs artificial microRNAs (amiRNAs) that are designed to target different endogenous messenger RNAs and synthesized to combinatorially co-silence closely homologous gene clade members (Schwab et al., 2006; Hauser et al, 2013). AmiRNAs are single-stranded 21mer RNAs processed from amiRNA precursors which were designed using the endogenous miRNA backbone (Ossowski et al., 2008). A seed collection of amiRNA lines was generated with the intention to target and genetically silence regions that are conserved between gene family members (Hauser et al., 2019). In this amiRNA collection, 139 subpools of seeds consisting of 22,000 amiRNAs were synthesized in 10 sublibraries, each targeting defined functional protein classes (Hauser et al, 2013; Hauser et al, 2019).

In this thesis, a forward genetic screening was conducted using the amiRNA library collection. From this collection, 12 candidate lines presenting interesting phenotypes were selected and their putative target genes were identified. Two putative candidate lines, P11-1-1 and P74-1-1 were then chosen for having the strongest and most reproducible phenotypes of

“iron-tolerant” and “iron-sensitive” primary roots when grown under iron-deficient conditions, respectively. Under iron-deficient conditions, P11-1-1 had roots that were “iron-tolerant” (i.e., longer primary roots) compared to the amiRNA control line (ami-CT). The putative target genes were identified to be the genes *PKP1*, *PKP2*, and *PKP3*, which are proteins that are a part of the protein kinase superfamily. In contrast, P74-1-1 had roots that were “iron-sensitive” (i.e., shorter primary roots) when compared to the ami-CT. The putative target genes were identified to be the genes *APL2*, *APL3*, and *APL4*, which encodes for a large subunit of ADP-glucose pyrophosphorylase that plays a regulatory role in catalyzing the first rate-limiting step of starch biosynthesis in plants (Figueroa et al., 2022).

## RESULTS

### **Traditional Forward Genetic Screening vs. amiRNA Forward Genetic Screening**

Traditional forward genetic screens are powerful tools for identifying candidate genes. However, there are limitations caused by the overlapping functions of closely homologous “redundant” genes that are mediated by the large gene families within the plant genome (Arabidopsis Genome, 2000). As a result of this phenomenon, it is difficult to observe phenotypes from the effects of loss of function single-gene mutations (Lloyd & Meinke, 2012). To address these issues, a library collection of artificial microRNAs (amiRNAs) was developed that targets a diverse combination of genes within sub-clades of genes. This library collection facilitated forward genetic screens to identify new genes that are involved in CO<sub>2</sub> and ABA signaling from large gene families (Hauser et al., 2019). As a result, an amiRNA library collection that is specifically designed to co-silence closely homologous gene clade members would prove to be an invaluable tool for forward genetic screens. From this amiRNA library, a screening was conducted to identify putative target genes through observable phenotypes in response to iron-deficiency. Through the identification of putative target genes, further understanding between the network of genes that play essential roles within the iron-deficiency pathway can be elucidated.

### **AmiRNA Candidate Lines**

The amiRNA library collection consists of 12,000 transformed T3 lines that express amiRNAs from 10 sublibraries. From these sublibraries, a total of 139 subpools exists that target defined classes of protein families. The 139 subpools were then screened through three additional generations resulting in 48 lines that had presented unique root phenotypes observed under iron-

deficient conditions when compared to the amiRNA control (ami-CT) line, ami-HsMYO; the amiRNA sequence of ami-HsMYO targets the human *MYOSIN2* gene that has no target gene in *Arabidopsis*. From the 48 lines, 12 candidate lines consisting of 33 genes were identified that exhibited robust and reproducible root phenotypes (*Table 1.*). Two main categories of root phenotype traits were identified from the candidate lines grown on iron-deficient Gruber media (75  $\mu$ M Ferrozine). The first category includes “iron-tolerant” lines which have longer primary root lengths compared to the ami-CT. As such, these candidate lines are observed to be “tolerant” against the effects of iron deficiency in root growth assays. In contrast, the second category includes “iron-sensitive” lines that were observed to have shorter primary root lengths when compared to the ami-CT. In root growth assays, these candidate lines are “sensitive” to the effects of iron deficiency.

From this forward genetic screening, one candidate line from each of the two categories of root phenotypic traits were selected for their robustness and reproducibility. For the “iron-tolerant” category, P11-1-1 was chosen as the primary root lengths were measured to be significantly longer than the ami-CT in both iron-sufficient and iron-deficient conditions. The putative amiRNA target genes for the candidate line, P11-1-1, were identified to target three genes that encodes for proteins within the protein kinase superfamily. As the names of these genes have not been officially published, the unofficial names of the three genes, *PKP1*, *2*, and *3* were given to easily refer to the putative target genes (*Table 1.*). The *PKP1*, *2*, and *3* genes are located in tandem on chromosome 2. Meanwhile, for the “iron-insensitive” category, P74-1-1 was the chosen candidate line as the primary root lengths were similar to the ami-CT under iron-sufficient conditions, however it was significantly shorter than the ami-CT in iron-deficient conditions. The putative target genes for P74-1-1 were identified to target the genes *APL2*, *3*, and *4* which encodes for a large subunit of ADP-glucose pyrophosphorylase that plays a regulatory

role in catalyzing the first rate-limiting step of starch biosynthesis in plants (Figuerola et al., 2022). The *APL2*, *3*, and *4* genes are located separately on chromosomes 1, 4, and 2, respectively.

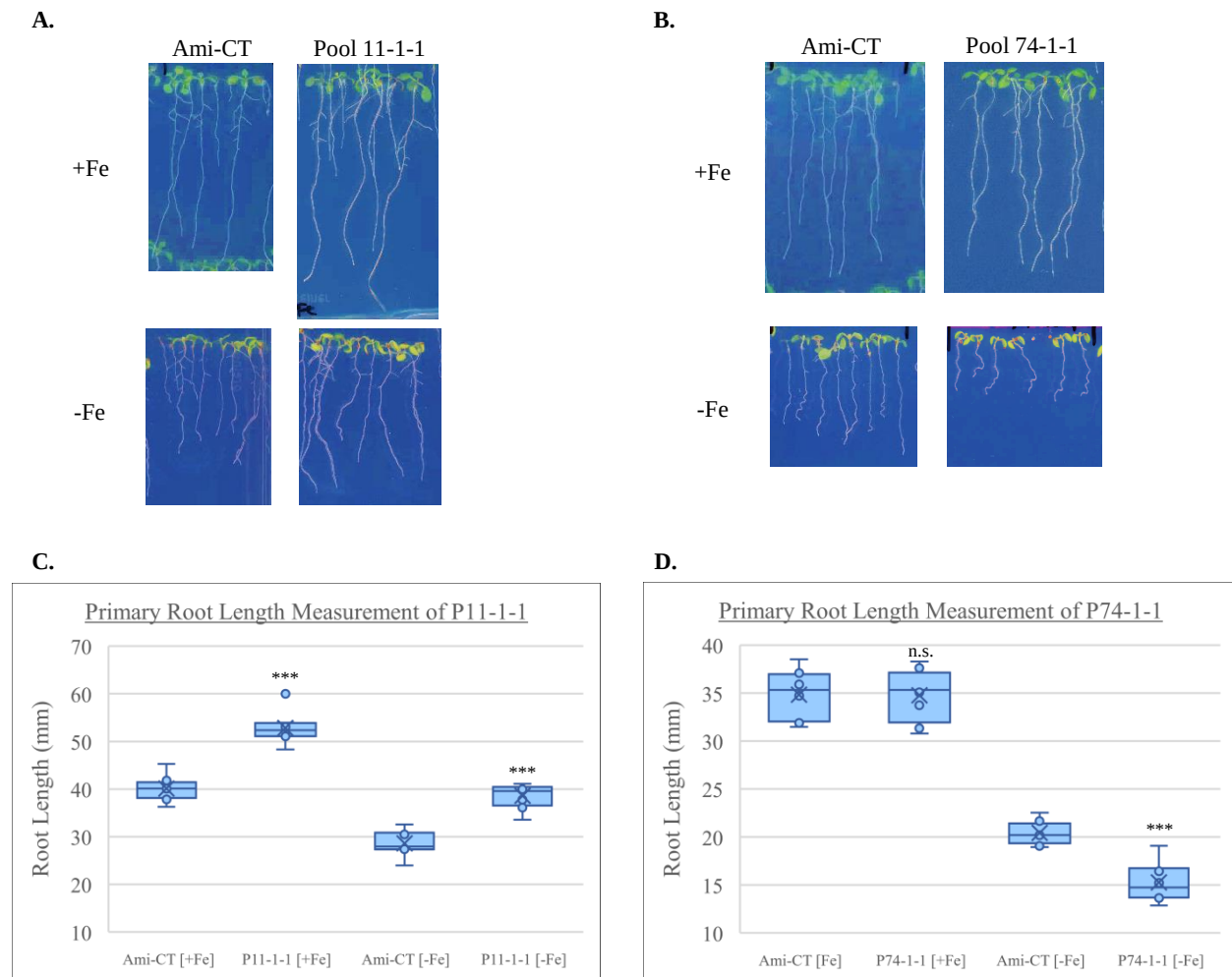


**Figure 1. Phenotype and Root Length Measurements of amiRNA Candidate Lines P11-1-1 and P74-1-1 .** Candidate line seeds were grown on either iron-sufficient (25  $\mu$ M of Na-Fe-EDTA; +Fe) or iron-deficient (-Fe) Gruber media. Seedlings were grown for 11 days. Candidate lines were chosen based on observations of primary root lengths with interesting observable traits and selected through the course of three generations. **(A.)** Phenotypic traits of candidate line P11-1-1. **(B.)** Phenotypic traits of candidate line P74-1-1. **(C.)** Primary root length measurements of P11-1-1 seedlings were manually measured using the ImageJ software. Statistical significance was determined by using a two-sample student's t-test. Data represents a boxplot of P11-1-1 seedlings (n=8). \*\*\* =  $P \leq 0.001$ . **(D.)** Primary root length measurements of P74-1-1 seedlings were measured using the ImageJ software. . Statistical significance was determined by using a two-sample student's t-test. Data represents a boxplot of P74-1-1 seedlings (n=8). n.s. = not significant, and \*\*\* =  $P \leq 0.001$ .

**Table 1. Identified Candidate Lines with Putative Target Genes.** Phenotypes of candidate lines were recorded and validated through multiple generations of screening. AmiRNA sequences were identified and the target gene IDs and target gene descriptions were obtained. **(A.)** Table of candidate lines that displayed seedling primary root length tolerance on iron-deficient media (i.e., longer roots) when compared to the ami-CT. **(B.)** Table of candidate lines that displayed seedling primary root length sensitivity on iron-deficient media (i.e., shorter roots) when compared to the ami-CT.

**A.**

| ID                           | Phenotype                                                                                                                             | AmiRNA Sequence       | Target Genes ID                             | Target Gene Description                                                                                                                                                                                                         | Validated in T3 | Validated via Retransformation          |
|------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|-----------------------|---------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|-----------------------------------------|
| <b>P11-1-1</b>               | <b>Root Length;</b><br><br>More tolerant than ami-CT on +Fe and -Fe media                                                             | TTTTTAGATCGCATTGGACAT | AT2G05060<br><br>AT2G41910<br><br>AT2G42550 | Protein kinase superfamily protein (PKP1)*<br><br>Protein kinase superfamily protein (PKP2)*<br><br>Protein kinase superfamily protein (PKP3)*<br><br>*Gene names are not official; used to easily refer to the target gene IDs | Yes             | In-Progress via CRISPR Retransformation |
| <b>P112-4-2 and P112-4-5</b> | <b>Root Length;</b><br><br>More tolerant than ami-CT on +Fe and -Fe media<br><br><b>Shoot Size;</b><br><br>Bigger shoots on -Fe media | ACCTGACATATCTTCAGCT   | AT5G05940<br><br>AT5G02010                  | ROP guanine nucleotide exchange factor 5 (ROPGEF5)<br><br>RHO guanyl-nucleotide exchange factor 7 (ROPGEF7)                                                                                                                     | Yes             | No                                      |
| <b>P132-1-7 and P132-1-9</b> | <b>Root Length;</b><br><br>More tolerant than ami-CT on +Fe and -Fe media<br><br><b>Shoot Size;</b><br><br>Bigger shoots on -Fe media | ATTGGTATCTACACACGCTA  | AT2G16650<br><br>AT5G60430                  | Proteinaceous Rnase P 2 (PRORP2)<br><br>Drug transmembrane transporters; antiporters                                                                                                                                            | Yes             | No                                      |
| <b>P133-2-3 and P133-2-6</b> | <b>Root Length;</b><br><br>Very tolerant than ami-CT on +Fe and -Fe media                                                             | AGGTCAACATAGTAACGCTT  | AT5G47770<br><br>AT4G17190                  | Farnesyl diphosphate synthase 1 (FPS1)<br><br>Farnesyl diphosphate synthase 2 (FPS2)                                                                                                                                            | Yes             | No                                      |

**Table 1. Identified Candidate Lines with Putative Target Genes. (continued)**

**B.**

| ID                                                        | Phenotype                                                                                                       | AmiRNA Sequence      | Target Genes ID | Target Gene Description                                       | Validated in T3 | Validated via Retransformation          |
|-----------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|----------------------|-----------------|---------------------------------------------------------------|-----------------|-----------------------------------------|
| <b>P74-1-1</b><br><br><b>and</b><br><br><b>P74-1-4</b>    | <b>Root Length;</b><br><br>More sensitive than ami-CT on -Fe media                                              | TAGACGTGATCGTTCGCCAA | AT1G27680       | ADPGLC-Ppase large subunit (APL2)                             | Yes             | In-Progress via CRISPR Retransformation |
|                                                           |                                                                                                                 |                      | AT4G39210       | Glucose-1-phosphate adenylyltransferase family protein (APL3) |                 |                                         |
|                                                           |                                                                                                                 |                      | AT2G21590       | Glucose-1-phosphate adenylyltransferase family protein (APL4) |                 |                                         |
| <b>P126-1-1</b>                                           | <b>Root Length;</b><br><br>More sensitive than ami-CT on -Fe media. Also more tolerant than ami-CT on +Fe media | TGTAATCACCTTAAGGCTG  | AT2G17130       | Isocitrate dehydrogenase subunit 2                            | Yes             | No                                      |
|                                                           |                                                                                                                 |                      | AT4G35650       | Isocitrate dehydrogenase III                                  |                 |                                         |
| <b>P134-3-5</b><br><br><b>and</b><br><br><b>P134-3-10</b> | <b>Root Length;</b><br><br>More sensitive than ami-CT on -Fe media.                                             | AAATAAGAGACTTATCC    | AT1G28230       | Purine permease 1 (PUP1)                                      | Yes             | No                                      |
|                                                           |                                                                                                                 |                      | AT2G33750       | Purine permease 2 (PUP2)                                      |                 |                                         |
| <b>P137-6-1</b><br><br><b>and</b><br><br><b>P137-6-2</b>  | <b>Root Length;</b><br><br>More sensitive than ami-CT on both +Fe and -Fe media.                                | GAAAAACGGCGTGGTACCA  | AT3G11320       | Nucleotide-sugar transporter family protein                   | Yes             | No                                      |
|                                                           |                                                                                                                 |                      | AT3G10290       | Nucleotide-sugar transporter family protein                   |                 |                                         |
|                                                           |                                                                                                                 |                      | AT5G04160       | Nucleotide-sugar transporter family protein                   |                 |                                         |
|                                                           |                                                                                                                 |                      | AT5G05820       | Nucleotide-sugar transporter family protein                   |                 |                                         |

**Table 1. Identified Candidate Lines with Putative Target Genes. (continued)**

| ID                                                       | Phenotype                                                           | AmiRNA Sequence    | Target Genes ID | Target Gene Description                                                 | Validated in T3 | Validated via Retransformation |
|----------------------------------------------------------|---------------------------------------------------------------------|--------------------|-----------------|-------------------------------------------------------------------------|-----------------|--------------------------------|
| <b>P138-1-1</b><br><br><b>and</b><br><br><b>P138-1-4</b> | <b>Root Length;</b><br><br>More sensitive than ami-CT on -Fe media. | ATACTACAAGCTCCC    | AT1G76800       | Vacuolar iron transporter (VIT) family protein (VTL2)                   | Yes             | No                             |
|                                                          |                                                                     |                    | AT3G25190       | Vacuolar iron transporter (VIT) family protein (VTL5)                   |                 |                                |
|                                                          |                                                                     |                    | AT3G43630       | Vacuolar iron transporter (VIT) family protein (nodulin-like3 protein)  |                 |                                |
|                                                          |                                                                     |                    | AT3G43660       | Vacuolar iron transporter (VIT) family protein (nodulin-like21 protein) |                 |                                |
| <b>P138-3-2</b><br><br><b>and</b><br><br><b>P138-3-5</b> | <b>Root Length;</b><br><br>More sensitive than ami-CT on -Fe media. | CTATAACAAGCTCCC    | AT2G23980       | Cyclic nucleotide-gated channel 6 (CNGC6)                               | Yes             | No                             |
|                                                          |                                                                     |                    | AT3G48010       | Cyclic nucleotide-gated channel 16 (CNGC16)                             |                 |                                |
|                                                          |                                                                     |                    | AT5G14870       | Cyclic nucleotide-gated channel 18 (CNGC18)                             |                 |                                |
| <b>P138-5-3</b><br><br><b>and</b><br><br><b>P138-5-8</b> | <b>Root Length;</b><br><br>More sensitive than ami-CT on -Fe media. | GTGATTGATTATCGCGCG | AT3G28345       | ATP-BINDING CASSETTE B15 (ABCB15)                                       | Yes             | No                             |
|                                                          |                                                                     |                    | AT3G28360       | ATP-BINDING CASSETTE B16 (ABCB16)                                       |                 |                                |
|                                                          |                                                                     |                    | AT3G28380       | ATP-BINDING CASSETTE B17 (ABCB17)                                       |                 |                                |
|                                                          |                                                                     |                    | AT3G28415       | ATP-BINDING CASSETTE B22 (ABCB22)                                       |                 |                                |

**Table 1. Identified Candidate Lines with Putative Target Genes. (continued)**

| ID                                                       | Phenotype                                                           | AmiRNA Sequence      | Target Genes ID                                            | Target Gene Description                                                                                                                                          | Validated in T3 | Validated via Retransformation |
|----------------------------------------------------------|---------------------------------------------------------------------|----------------------|------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|--------------------------------|
| <b>P139-2-4</b><br><br><b>and</b><br><br><b>P139-2-7</b> | <b>Root Length;</b><br><br>More sensitive than ami-CT on -Fe media. | AATGCGTATTCATACGGCTT | AT1G25480<br><br><br><br><br><br><br><br><br><br>AT2G17470 | Aluminum activated malate transporter family protein (ALMT4)<br><br><br><br><br><br><br><br><br><br>Aluminum activated malate transporter family protein (ALMT6) | Yes             | No                             |

### Identification, Expression, and Confirmation of the Putative Target Genes

In order to understand the effectiveness of the amiRNA on the putative target genes, the relative gene expression of the two sets of candidate genes were quantitatively analyzed. To do this, an experiment utilizing Reverse Transcriptional quantitative Polymerase Chain Reaction (RT-qPCR) was conducted to assess the relative fold-change of expression compared to ami-CT on both iron-sufficient and iron-deficient conditions (*Figure 2*).

Quantitative analysis of gene expression using RT-qPCR on P11-1-1 revealed that when comparing the ami-CT (+Fe) to the ami-CT (-Fe), the trend appears to have decreased expression of the genes under iron-deficient conditions. This potentially indicates that in *Arabidopsis* Col-0 eco-type plants, *PKP1*, *2*, and *3* have decreased expressions when the plants are iron-deprived. Meanwhile, when comparing the ami-CT (+Fe) to the ami-CT (-Fe) for P74-1-1, there is a trend of increased expression of the genes under iron-deficient conditions, potentially revealing that in *Arabidopsis* Col-0, the *APL2*, *3*, and *4* genes have increased expressions when iron-deprived.

The results from the RT-qPCR showed that in the case of P11-1-1, the average fold-change in relative expression of the *PKP1*, *PKP2*, and *PKP3* genes have significantly decreased. Additionally, the amiRNA that targeted *PKP3*, the relative expression is very minimal or not detectable at all. For P74-1-1, the expression of *APL2*, *3*, and *4* is significantly decreased when

compared to both ami-CT (+Fe) and ami-CT (-Fe). In both cases of P11-1-1 and P74-1-1, the relative expression of their respective genes are strongly decreased by the amiRNA targeting of the genes which shows that the amiRNA is effectively knocking down the putative target genes as intended.

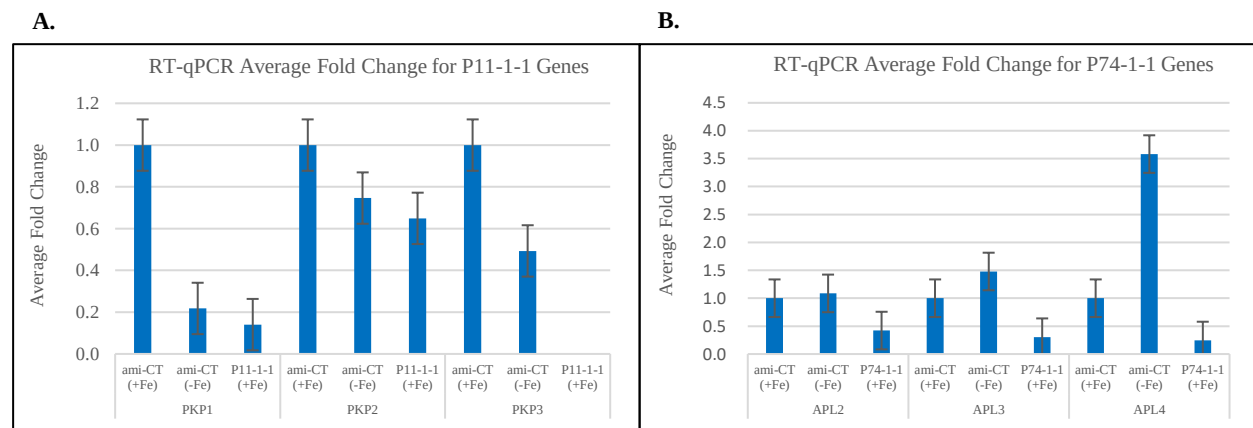


**Figure 2. Putative Target Gene Expression in P11-1-1 and P74-1-1.** Reverse transcription-quantitative Polymerase Chain Reaction (RT-qPCR) was done for genes for the two candidate lines. All lines were plated on Gruber media on either iron-sufficient (25  $\mu$ M of Na-Fe-EDTA; +Fe) or iron-deficient (-Fe) Gruber media. and grown for 11 days. Data analysis for RT-qPCR was done using the Delta-Delta Ct method. The fold change values included 3 biological samples for all lines **(A.)** The fold change for the genetic expression of *PKP1* (AT2G05060), *PKP2* (AT2G41910), and *PKP3* (AT2G42550) both ami-control (+Fe and FRZ media), and P11-1-1 on +Fe media was calculated. **(B.)** The fold change for the genetic expression of *APL2* (AT1G27680), *APL3* (AT4G39210), and *APL4* (AT2G21590) in both ami-control (+Fe and FRZ media) and P74-1-1 on +Fe media was calculated.

CRISPR-mediated deletion of these genes was done to corroborate the observed phenotypes to the effects due to single-, double-, and triple-mutations of the *PKP1/2/3* and *APL2/3/4* genes. This would also help in verifying which genes are redundant or essential for plant survival. The GoldenGate cloning method was used for CRISPR validation with the primers that were designed specifically for each gRNA for each gene set (Table 2). This CRISPR validation method is in-progress as the final CRISPR constructs (Figure 3. And Figure 4.), for both *PKP1/2/3* and *APL2/3/4* candidate gene sets were agrotransformed into Col-0 plants and T1 seeds harvested. Progression of CRISPR validation for the candidate gene sets are continuing as

both CRISPR sequencing and the T1-selection process are currently ongoing. With the subsequent T2 seeds harvested, seeds that are FAST-negative and non-fluorescent are then selected as this will prevent the Cas9 enzyme from being in subsequent generations, causing further unwanted genetic modifications of other genes. Further analysis for indels in the FAST-negative T2 seeds can then be done via CRISPR sequencing to identify sequence modifications. The FAST-negative T2 seeds would then undergo T2-selection, grown, and harvested to establish the stable T3 generation for the mutant transgenic lines.

**Table 2. Primer Sequences of gRNAs for P11-1-1 and P74-1-1.** The 20-mer forward (F) and reverse (R) sequences of the primers for each gene's gRNAs (g1, g2, g3, g4) were designed using primer3 website. Four sets of gRNAs were used for each gene. **(A.)** Primer sequences for the genes *PKP1/2/3*. **(B.)** Primer sequences for the genes *APL2/3/4*.

**A.**

|           |                          |           |                          |
|-----------|--------------------------|-----------|--------------------------|
| PKP1-g1-F | attGATCTTTCTGTTCCAAAGGT  | PKP3-g1-F | attgCGAAGGATCGTGCAATGCTA |
| PKP1-g1-R | aaacACCTTTTGAACAGAAAGAT  | PKP3-g1-R | aaacTAGCATTGCACGATCCTTCG |
| PKP1-g2-F | aatGTCGGCGTGAATTATGTTGG  | PKP3-g2-F | attGAAGAGGACTTTCGATGTCCG |
| PKP1-g2-R | aaacCCAACATAATTCACGCCGA  | PKP3-g2-R | aaacCCGACATCGAAGTCCTCTT  |
| PKP1-g3-F | attgCGTAGAGTTCACGATTCCCA | PKP3-g3-F | attGTTATGGAGTACGCAGCTGC  |
| PKP1-g3-R | aaacTGGAATCGTGAACCTCTACG | PKP3-g3-R | aaacGCAGCTGCGTACTCCATAA  |
| PKP1-g4-F | attGTTTACGCGTATGATCCTCG  | PKP3-g4-F | attgACGCCGCTACGGACTGATTC |
| PKP1-g4-R | aaacCGAGGATCATACGCGTAAA  | PKP3-g4-R | aaacGAATCAGTCCGTAGCGGCGT |
| PKP2-g1-F | attgCGGAAAGGGTTCATACGGTT |           |                          |
| PKP2-g1-R | aaacAACCGTATGAACCTTTCCG  |           |                          |
| PKP2-g2-F | attGCGGTGTAGAGAGTCGTGCG  |           |                          |
| PKP2-g2-R | aaacCGCACGACTCTCTACACCG  |           |                          |
| PKP2-g3-F | attgCTAATGGAGTACGCAGCTGG |           |                          |
| PKP2-g3-R | aaacCCAGCTGCGTACTCCATTAG |           |                          |
| PKP2-g4-F | aatGAATTGTGGCTAAGCCTTCG  |           |                          |
| PKP2-g4-R | aaacCGAAGGCTTAGCCACAATT  |           |                          |

**B.**

|           |                          |           |                           |
|-----------|--------------------------|-----------|---------------------------|
| APL2-g1-F | attGCGTCTATCATACTAGGTGG  | APL4-g1-F | attgACACAAACACCAGGGGAAGC  |
| APL2-g1-R | aaacCCACCTAGTATGATAGACG  | APL4-g1-R | aaacGCTTCCCCTGGTGTGTTGTGT |
| APL2-g2-F | attgAGGGAAGTCTGATGCTGTT  | APL4-g2-F | attgTGCTGTCAGAAAATTCCTCT  |
| APL2-g2-R | aaacAACAGCATCAGCAGTTCCT  | APL4-g2-R | aaacAGAGGAATTTTCTGACAGCA  |
| APL2-g3-F | attgACCCAAACTTCCGGAGATGC | APL4-g3-F | aatGATTTCAGGTCGACTCCTGT   |
| APL2-g3-R | aaacGCATCTCCGGAAGTTTGGGT | APL4-g3-R | aaacACAGGAGTCGACCTGAAAT   |
| APL2-g4-F | attgTGTCTGGGATCATCTCTAT  | APL4-g4-F | attgCATATTCAGAGATTACTGGG  |
| APL2-g4-R | aaacATAGAGATGATCCCCAGACA | APL4-g4-R | aaacCCCAGTAATCTCTGAATATG  |
| APL3-g1-F | aatGTTGCTGCTCGCTTGTAAG   |           |                           |
| APL3-g1-R | aaacCTTACCAAGCGAGCAGCAA  |           |                           |
| APL3-g2-F | attgCCGTGCGTCGGAGTATGGAC |           |                           |
| APL3-g2-R | aaacGTCCATACTCCGACGCACGG |           |                           |
| APL3-g3-F | attGGCTTCCCTCAATCGCCATT  |           |                           |
| APL3-g3-R | aaacAATGGCGATTGAGGGAAGC  |           |                           |
| APL3-g4-F | attgCTGGTGAAGCCGGGAAAAAG |           |                           |
| APL3-g4-R | aaacCTTTTTCCCGGCTTACCAG  |           |                           |

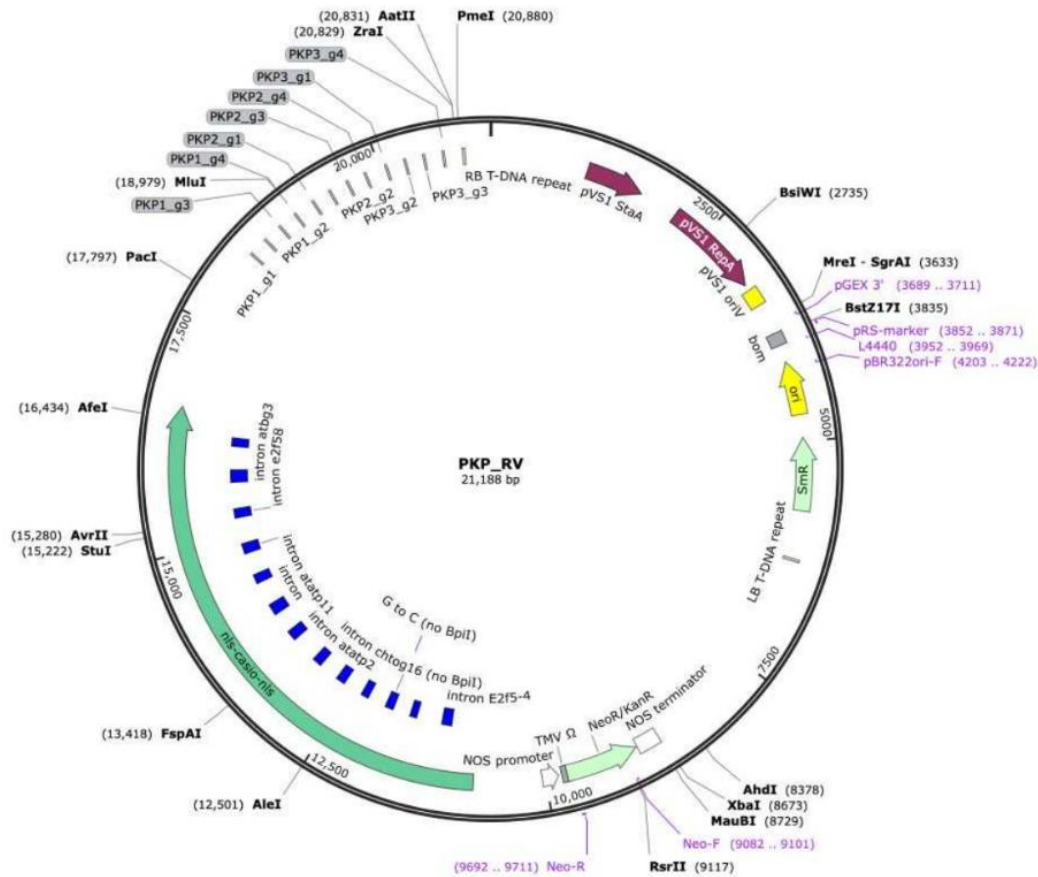


**Figure 3. Design of the CRISPR/Cas9 System for Genome Editing of the PKP Gene in P11-1-1.** Schematic overview of the CRISPR construct with gRNAs that targets PKP1, PKP2, and PKP3. Four gRNAs were designed to target each putative target gene; in total 12 gRNAs were designed.



## Mechanisms of the Putative Target Genes

Plant roots grow along three-dimensional paths and factors such as curvature, direction, and overall root structure architecture can depend on root intrinsic signals and extrinsic environmental cues. Plant intrinsic parameters can include hormones and carbohydrate metabolism that are involved in controlling cell production rate and root meristem sizes (Yazdanbakhsh et al., 2010). As such, external nutrient availability can be one of the factors that can alter a root's elongation rate.

The time-lapse of measuring the primary root growth rate was done over the course of an 8-hour time-course. As a result, root growth rates at subsequent periods of time during the later stages of root growth and development would have allowed additional information and understanding of the mechanisms that are involved with iron. Such was the case in *Figure 5A*, where seedlings grown under iron-sufficient conditions had begun to exhibit changes and trends towards the end of the 8-hour time course. For example, at the 6.5-hour mark, primary root growth of the ami-CT had begun to plateau, potentially signaling of the reduction of primary root growth rate. Meanwhile, at the 7-hour mark, the candidate line P74-1-1 is starting to appear to have an increased root growth rate compared to P11-1-1; this however was not revealed to be statistically significant at the end of the 8-hour timelapse. This should not discount the fact that it could prove to be significant at later time points, however. Therefore, longer timelapse measurements should be conducted to better understand the mechanisms of root growth rate of the candidate lines; perhaps 12- or 24-hour time courses could be recommended. In *Figure 5B*, the distinction between growth rates of the candidate lines and the control under iron-deficient conditions is clear. The growth rates of both candidates are slower compared to the ami-CT, though longer time courses could provide additional information towards later root development.

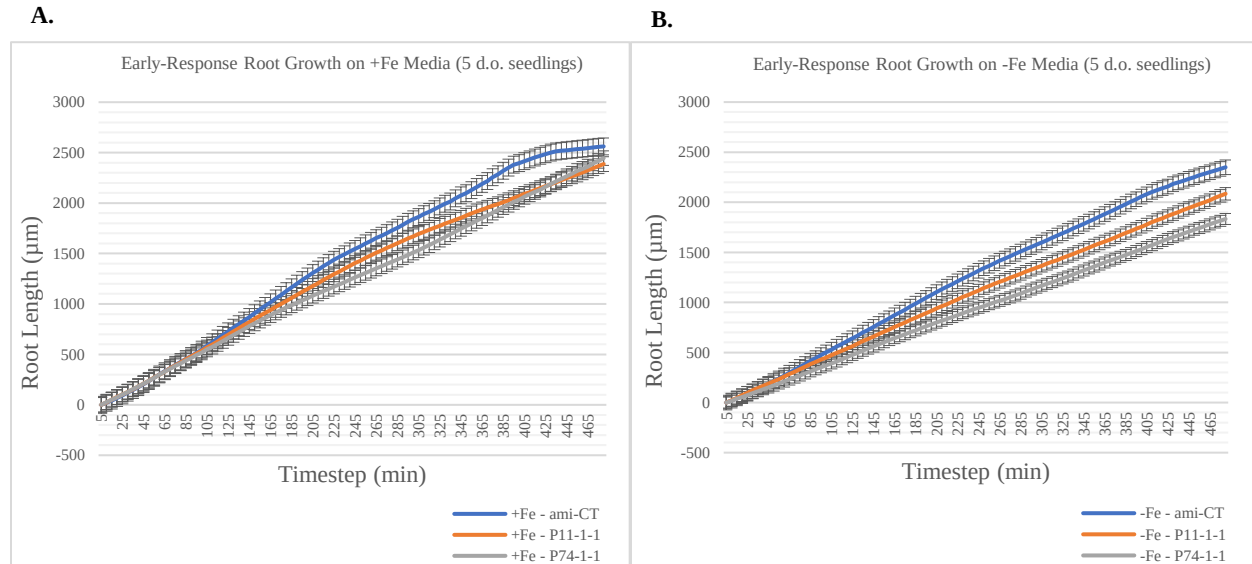


**Figure 5. Measurement of Root Growth and Elongation for P11-1-1 and P74-1-1.** Seeds were grown on either iron-sufficient (+Fe) or iron-deficient (-Fe) Gruber media for 5 days. Seedlings were then transferred onto Keyence microscope chambers with the same respective media for timelapse capture. The time lapse capture was done on a Keyence BZ-X810 microscope with BZ Nikon Objective Lens (4X). Images were captured every 5 minutes over an 8-hour period and the primary root length measurements were tracked and recorded using MatLab scripts for the set parameters. **(A.)** 5-day old seedlings' primary root lengths were imaged and recorded on +Fe media. **(B.)** 5-day old seedlings' primary root lengths were imaged and recorded on FRZ media.

Additional characterization of the mechanism for the candidate lines were done by analyzing the seed sizes in relations to iron content. In *Arabidopsis*, seed development is divided into two phases: 1.) the active proliferation and growth of the endosperm which is followed by 2.) the growth of the embryo at the expense of the endosperm (Garcia et al., 2005). Iron in *Arabidopsis thaliana* embryos is loaded solely in the vacuoles of endodermal cells within the embryo during seed maturation and is then remobilized during seed germination revealing the essential role that iron plays in embryo development (Roschztardt et al., 2020; Grillet et al., 2013).

As iron plays an important role during the development of the seed and embryo, it is important to take into consideration the size of seeds to get a glimpse of the relationship of the

iron that is present in the candidate lines. It was discovered that P11-1-1 and P74-1-1 have seed sizes that are significantly larger than both Col-0 and ami-CT. In the case of P11-1-1, this would validate the phenotype that is observed in *Figure 1A*., which the primary roots were “iron-tolerant” and were longer than the ami-CT in both iron-sufficient and iron-deficient media. The larger seed sizes could indicate that higher levels iron content are loaded in the seeds of P11-1-1, thus allowing for the seedlings to grow unhindered and unaffected by the lack of iron.

Potentially, a hypothesis can be formed to hint that as the *PKP1/2/3* genes are targeted and silenced by the amiRNA, these genes may be responsible for playing a role in regulating kinase activity that is involved in storing excess iron from the bigger seeds. Interestingly, even as the seeds of P74-1-1 were quantified to be larger than the control, the phenotype of the seedlings grown on iron-deficient media resulted in severely decreased primary root growth (*Figure 1B*). A hypothesis may be given to hint that as the *APL2/3/4* genes are a part of the starch biosynthesis pathway, even though the seeds are bigger and have higher iron content, the silencing of these genes drastically affected the seedlings’ ability to synthesize starch, which acts as glucose storage for the plant’s future use. With starch biosynthesis hindered along with iron-deficient conditions, seedlings grown under iron-deprivation appears chlorotic and have decreased primary root growth.

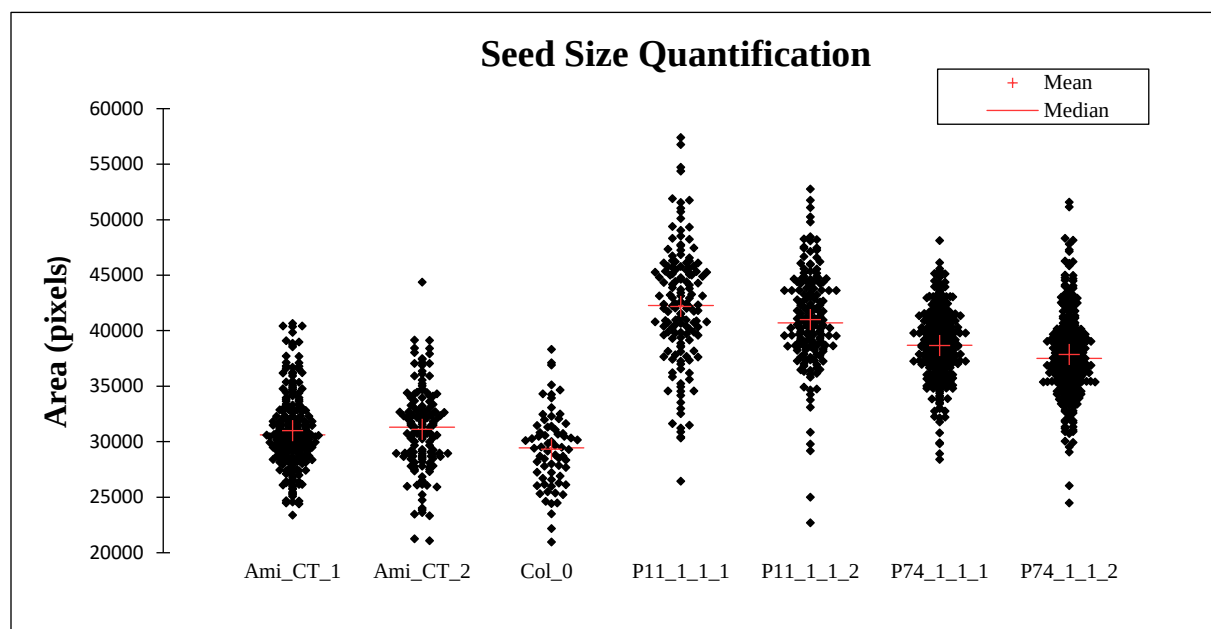


**Figure 6. Quantification of Seed Size for P11-1-1 and P74-1-1.** Seed images were captured using a Keyence BZ-X810 microscope with BZ Nikon Objective Lens (10X). Images were then processed using ImageJ FIJI, plotted, and calculated with XLSTAT. Seed sizes were quantified by measuring the area of pixels of each seed. Col-0 and 2 sets of ami-CT lines were quantified as additional replicates and references. Two replicates of P11-1-1 and P74-1-2 were quantified.

Just as iron plays an essential role within the seeds during embryo development, iron is heavily involved in mechanisms of plant root and shoot development, iron localization, distribution, and many other roles that iron plays. Under low iron availability, morphological changes including root architecture, root hair formation, and formation of transfer cells occur (Kobayashi et al., 2012). In Arabidopsis, the majority of iron in the shoot is localized in the chloroplast where photosynthetic processes occur (Shikanai et al., 2013). Additionally, iron uptake and distribution not only relies on transport activities, but also on regulatory mechanisms that controls the abundance and activity of transporters in both roots and shoots to adjust iron uptake based on the demand of the whole plant in response to iron-deficiency (Kobayashi et al., 2012; Vert et al., 2003). As such, the elucidation of iron localization of the candidate lines would

be important to understand how the amiRNA had affected the putative genes and its distribution of iron.

To further understand the effects that amiRNA had on the mechanisms of the silenced of putative target genes, quantification of the iron concentration in the roots and shoots of the seedlings was conducted. In the roots of P11-1-1, it was revealed to have a significant increase of iron content under iron-sufficient conditions. Unexpectedly, P11-1-1 had no difference in iron content under iron-deficient conditions when compared to the respective ami-CT lines. In the shoots of P11-1-1, there was no difference or iron content compared to the ami-CT under iron-sufficiency, however in iron-deficient conditions it had a significantly higher iron level. Meanwhile, the roots of P74-1-1 had no significant difference in iron-sufficient conditions but had significantly decreased iron content in iron-deficient conditions. While in the shoots, similar to that of P11-1-1, P74-1-1 had no significant difference in iron-sufficient conditions. However, in iron-deficient media it had higher iron levels than not only the ami-CT but P11-1-1 as well, interestingly.

From these results, in the case of P11-1-1, when iron levels are sufficient, it appears that higher amounts of iron content is present in the roots of the seedlings while the shoots had no difference compared to the ami-CT. When P11-1-1 is deprived of iron, there is a shift in the localization of iron content from the roots to the shoots where high iron concentrations are measured. Studies have shown that intercellular distribution of iron between the roots and shoots have given plants an edge in tolerating fluctuations of iron availability (Long et al., 2010). From this, it may be speculated that this shift in localization of iron in P11-1-1 could explain how seedlings could grow quite well even in iron-deprived conditions. By translocating iron to increase the iron concentration of the shoots, it allows more resources to become available for

photosynthetic processes. From this, more primary root growth is enabled and thus allows P11-1-1 to overcome the effects of iron deprivation. Furthermore, the results from the iron content measurements builds on the previously mentioned hypothesis. The results have shown that the amiRNA silencing of the putative genes *PKP1/2/3* could in effect have knocked down the expression of a protein in the protein kinase family that is not only responsible for storing excess iron, but also iron translocation between the roots and shoots. As a result, this allows the roots to be “iron-tolerant.”

Meanwhile, while there are no significant differences in either the roots or shoots under iron-sufficiency, P74-1-1 had shown to have significantly reduced iron content in the roots which seems to have been redistributed into the shoots under iron-deficient conditions. However, even as the iron content within the shoots were increased, the primary root growth was severely hindered. This further encapsulates the aforementioned hypothesis that by silencing the *APL2/3/4* putative genes, starch biosynthesis is interrupted, thus greatly stunting the primary root growth under iron-deficient conditions seen in *Figure 7B*. From this observed phenotype, the roots were deemed to be “iron-sensitive.”

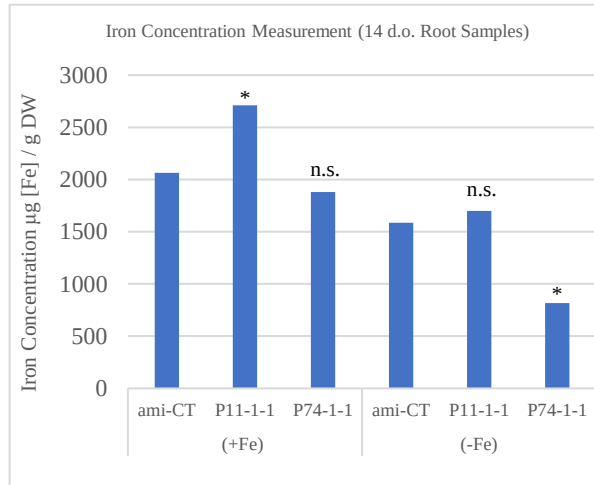
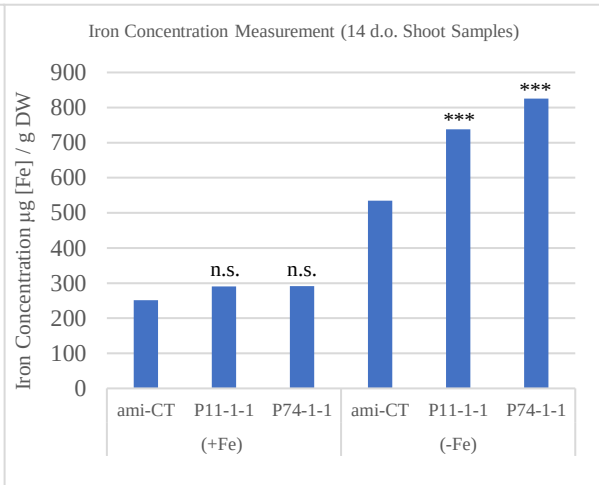
**A.****B.**

**Figure 7. Iron Concentration Measurement of Candidate Lines P11-1-1 and P74-1-1.** Seeds were grown on either iron-sufficient (25 µM of Na-Fe-EDTA; +Fe) or iron-deficient (-Fe) Gruber media. Seedlings were grown for 14 days. Sample digestion, preparation, and analysis was done as described in Gautam et al., 2022. Data represents the average iron concentration per dry weight of three replicates for each line. n.s. = not significant, \* =  $P \leq 0.05$ , and \*\*\* =  $P \leq 0.001$ . **(A.)** Iron concentration measurements of 14-day old root samples (n=30) for both candidate lines under +Fe and -Fe conditions. **(B.)** Iron concentration measurements of 14-day old shoot samples (n=30) for both candidate lines under +Fe and -Fe conditions.

As mentioned above how iron localization is important to understand how the putative genes were affected by amiRNAs, further experimentation and analysis of iron localization at the apical meristem of the roots would be an additional strategy to validate the findings. The primary root apical meristem is a region located at the most distal part of the primary root where root growth, elongation, and development occurs (Perilli et al., 2012). As root development occurs in this region of the primary root, we can visualize how iron localization is affected within the primary roots of the candidate lines under different iron conditions.

From *Figure 8.*, iron localization for P11-1-1 can be observed throughout the primary root apical meristem, elongation zone, and at the differentiation zone under iron-sufficient conditions. Meanwhile, for P74-1-1, there is a notable decrease in iron staining localized at the

primary root apical meristem. These results mirrors the results that were obtained from the iron content measurement in the roots. From the Perls/DAB staining, the hypothesis of the “iron-insensitive” phenotype for P11-1-1 and “iron-sensitive” phenotype for P74-1-1 is further strengthened.

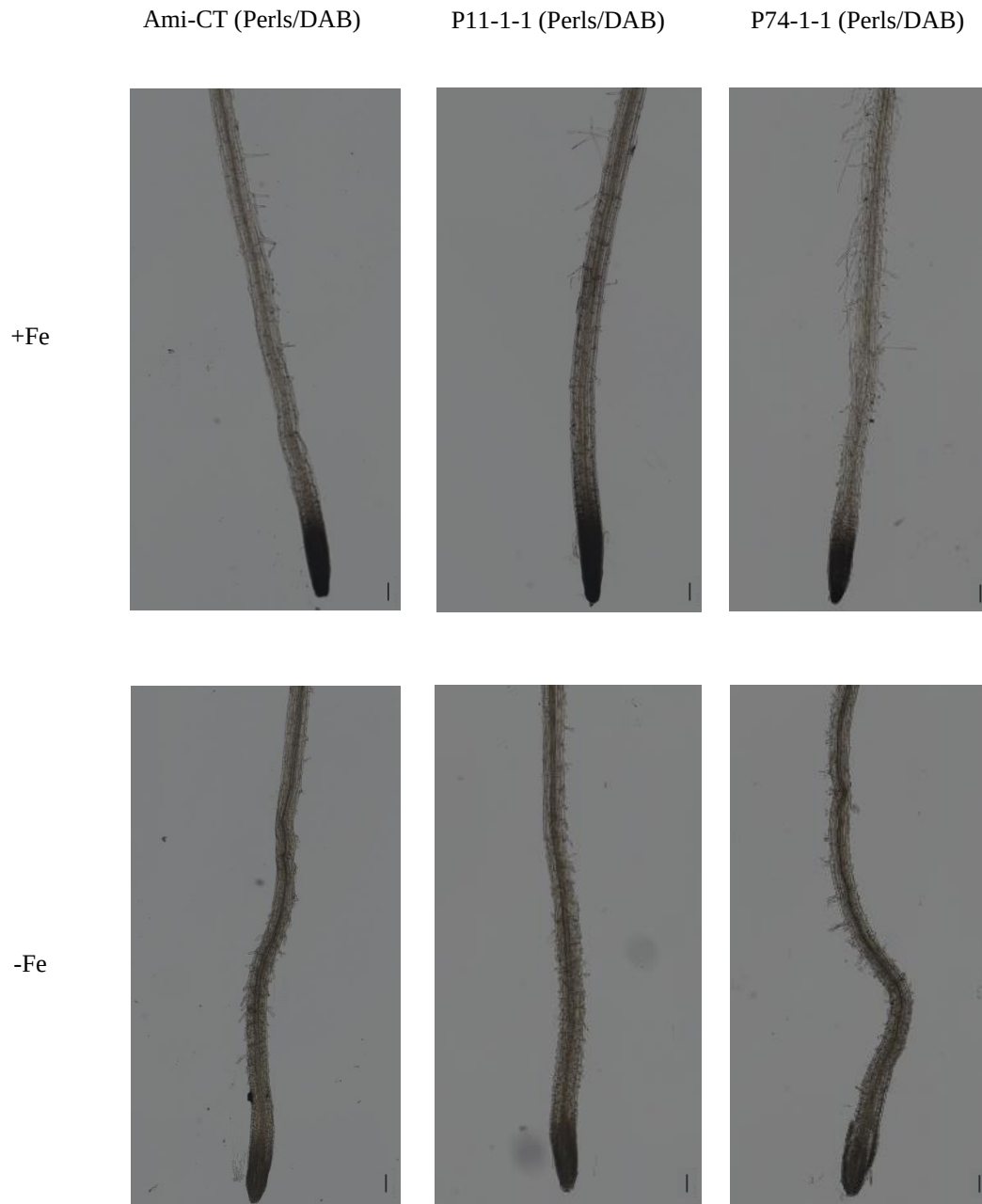


**Figure 8. Perls/DAB Staining for Iron Localization in P11-1-1 and P74-1-1.** Seeds were grown on either iron-sufficient (+Fe) or iron-insufficient (-Fe) Gruber media. 7-day old seedlings were then stained with the Perls method and the cellular level of iron detection was further intensified using 3,3'-diaminobenzidine tetrahydrochloride (DAB). Root samples were imaged with a Keyence BZ-X810 microscope with BZ Nikon Objective Lens (4X). Bar represents 100  $\mu$ m.

## Alkaline Soil Experiment

From these experiments, the mechanisms for the two candidate lines were better understood. Besides characterization, it would be beneficial to test the candidate lines in-vivo to further corroborate that the observed phenotypes and results can also be correlated and observed in the soil. The pH within the soil affects plant growth through complex interactions involving changes in physical properties and soil chemistry that impact physiological processes (Rengel, 2002). Normal soil would have a pH range of 5.0-6.0, while alkaline soil has a range of 7.0-8.0 (Satbhai et al., 2017). Higher soil pH would in effect decrease the availability and solubility of iron for plant uptake (Xu et al., 2020). With this knowledge, we conducted an alkaline soil experiment for the candidate lines to observe the growth and development of the plants under iron-deficient soil conditions.

Results from the soil experiments have shown that the sizes of 14-day old leaves and rosettes for P11-1-1 grown in the high pH alkaline soil were bigger when compared to the ami-CT. This would indicate that even as iron availability is significantly reduced, P11-1-1 growth was not only unhindered but had grown and developed better than the control. For P74-1-1, there was a significant difference in the size of the rosettes that appeared to be smaller on the alkaline soil compared to the control soil. It is also worth noting that the density of rosettes would indicate that there are less successful germination on the alkaline soil, further showing the effects of reduced iron availability in soil with high pH. Additionally, phenotypes of 8-week-old plants were recorded to better visualize the effects that iron-deficient alkaline soil had on the growth and development of P11-1-1 and P74-1-1 during the maturation phase (*Figure 10.*). The mature plants of P11-1-1 was bigger compared to the ami-CT under iron-deficiency. In the case of P74-1-1, the mature plants were smaller and less dense compared to the ami-CT under iron-

deficiency. The phenotypes at week 8 further corroborated what was observed from 14-day old seedlings.

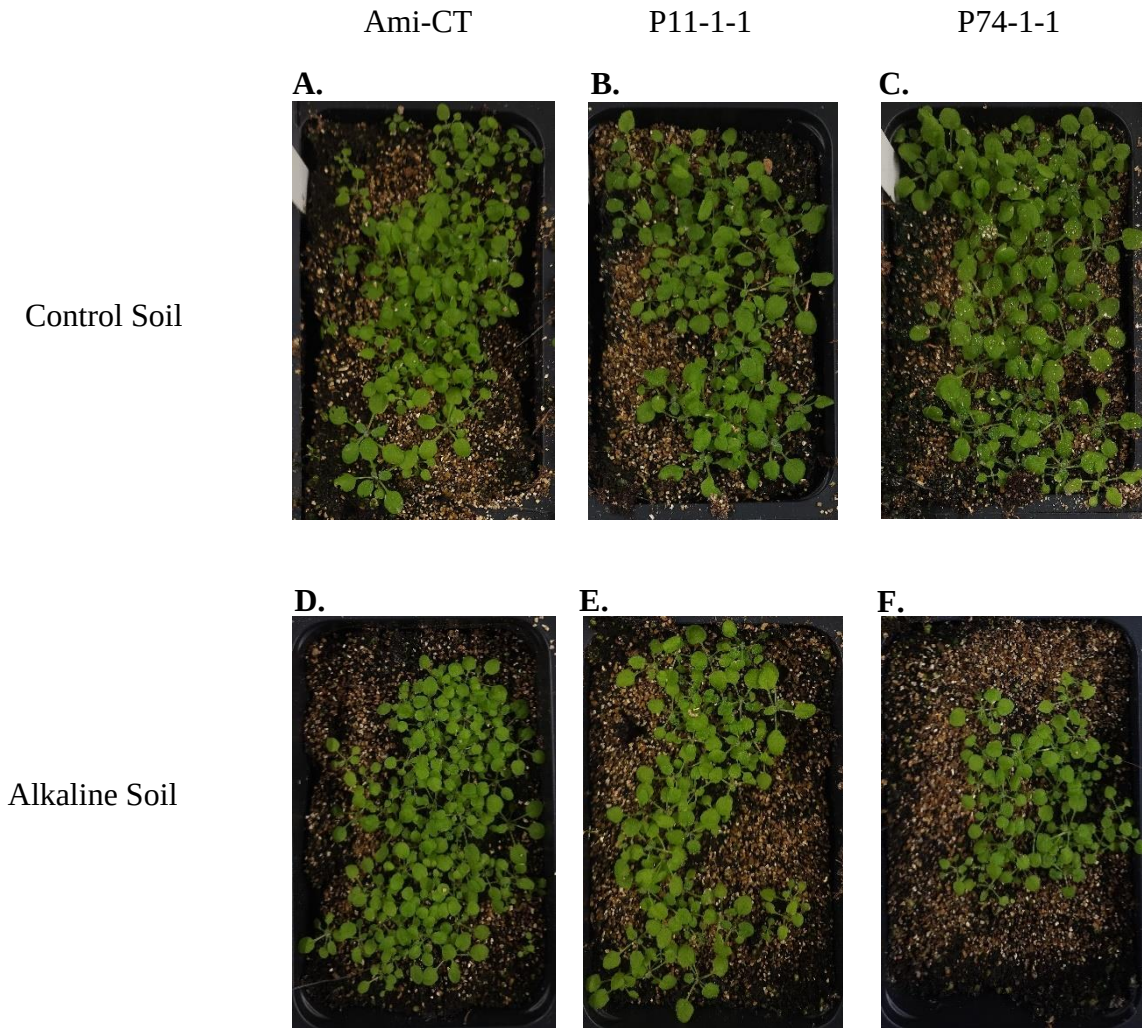


**Figure 9. Phenotypic Growth of P11-1-1 and P74-1-1 on Alkaline Soil vs. Control Soil (14 d.o.).** Seeds were sprinkled onto two types of soil conditions. The control soil was soaked with Heritage fungicide and Peters 20-20-20 General Purpose Fertilizer. Alkaline soil was prepared with the addition of 8g CaO/ kg of soil to a final soil pH of 7.0-8.0. The alkaline soil was soaked with Heritage fungicide was added and no fertilizer was added into the water used to soak the soil to avoid iron contamination. Plants were 14-day old when the images were taken. **(A.)** Ami-control line grown on control soil. **(B.)** P11-1-1 seeds grown on control soil. **(C.)** P74-1-1 seeds grown on control soil. **(D.)** Ami-control line grown on alkaline soil. **(E.)** P11-1-1 seeds grown on alkaline soil. **(F.)** P74-1-1 seeds grown on alkaline soil.

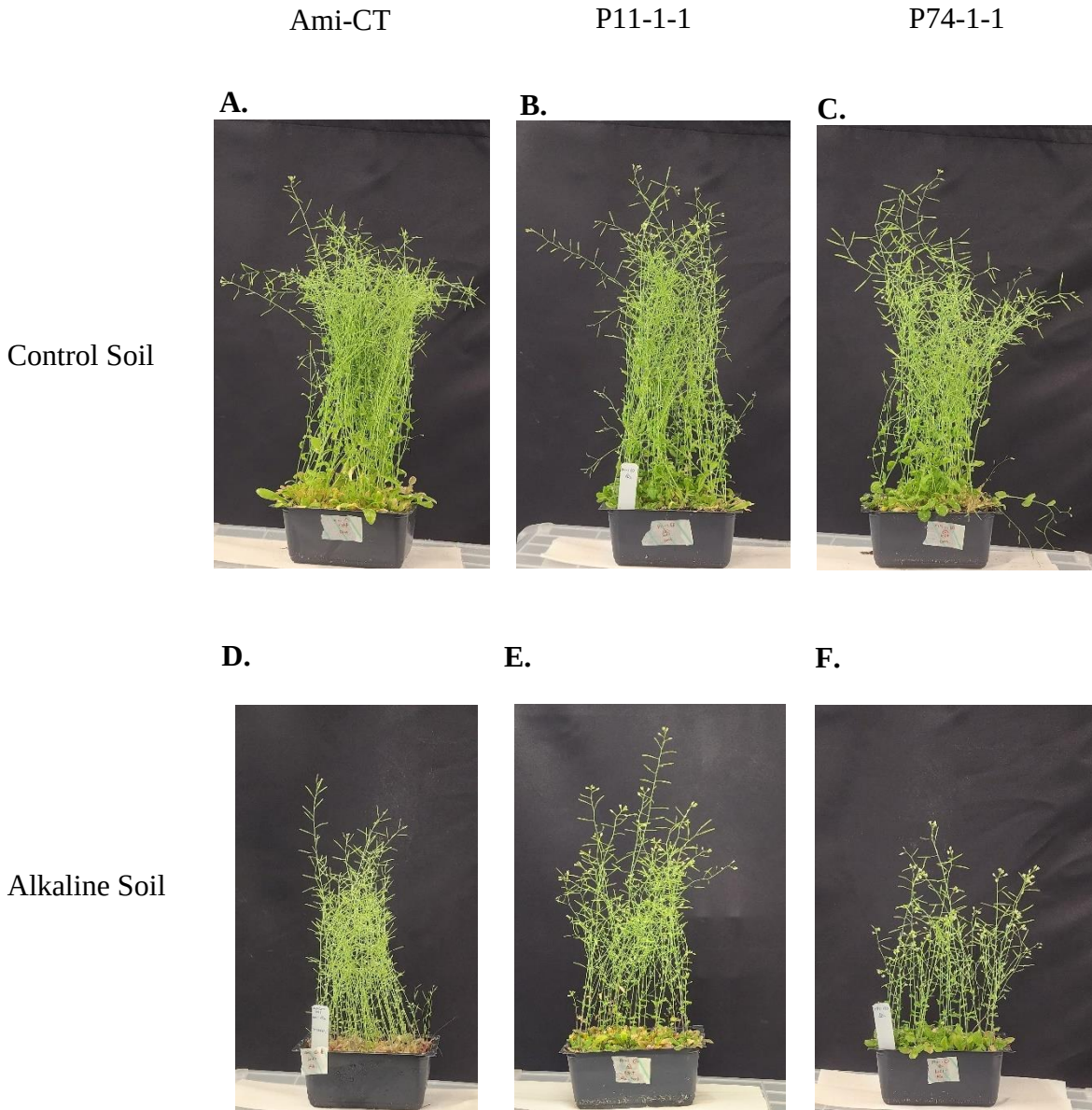


**Figure 10. Phenotypic Growth of P11-1-1 and P74-1-1 on Alkaline Soil vs. Control Soil (8-week-old).** Seeds were sprinkled onto two types of soil conditions. The control soil was soaked with Heritage fungicide and Peters 20-20 General Purpose Fertilizer. Alkaline soil was prepared with the addition of 8g CaO/ kg of soil to a final soil pH of 7.0-8.0. The alkaline soil was soaked with Heritage fungicide was added and no fertilizer was added into the water used to soak the soil to avoid iron contamination. Plants were 8-week-old when the images were taken. **(A.)** Ami-control line grown on control soil. **(B.)** P11-1-1 seeds grown on control soil. **(C.)** P74-1-1 seeds grown on control soil. **(D.)** Ami-control line grown on alkaline soil. **(E.)** P11-1-1 seeds grown on alkaline soil. **(F.)** P74-1-1 seeds grown on alkaline soil.

In plants, photosynthesis is an important biological function that synthesizes glucose which is used as an important energy source. For photosynthesis to occur, chloroplasts and chlorophylls play a crucial function that enables photosynthetic processes to occur. Chloroplasts are membrane-bound organelles in plant cells that contain chlorophyll, a pigment that absorbs blue- and red-wavelengths of light of the electromagnetic spectrum and converts it into chemical energy (Martins et al., 2023). Research has shown that there are interconnective relationships between chlorophyll and iron. As iron is involved in electron transport and chlorophyll synthesis, iron deprivation would in effect alter photosynthetic processes and promote chlorosis of the leaves (Kroh et al., 2020; Therby-Vale et al., 2022).

Knowing that there is a relationship between chlorophyll content and iron-deficiency in plants, chlorophyll measurements were taken for the candidate lines grown on both the control soil and on alkaline soil. Measurement of the chlorophyll content revealed that on high pH alkaline soil, P11-1-1 had a significantly higher chlorophyll content compared to the ami-CT even under iron-deficient conditions. This trend of higher P11-1-1 chlorophyll content matches the in-vivo observation of bigger rosettes grown on alkaline soil (*Figure 9E.*). In regard to P74-1-1, chlorophyll content was lower compared to the ami-CT under iron-deficient conditions. This also aligns with what was observed on soil in *Figure 9F.*, further corroborating the smaller rosettes and lower seedling density.

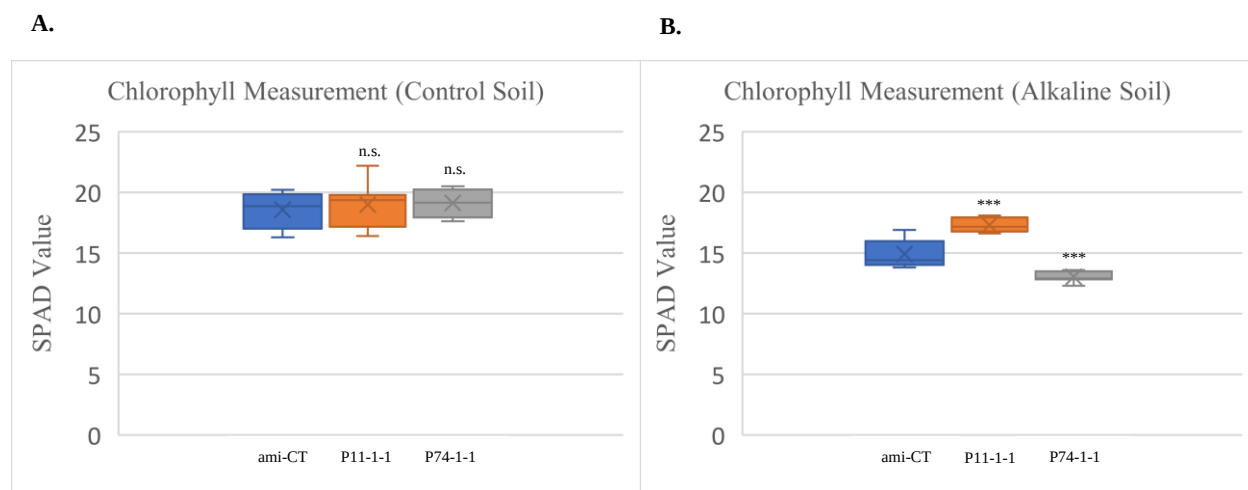


**Figure 11. Chlorophyll Content Measurement of P11-1-1 and P74-1-1.** The same samples from *Figure 9* had the chlorophyll content measured. Chlorophyll content was measured on true leaves using a Konica Minolta SPAD-502 Plus chlorophyll meter on 14-day old plants. Three technical measurement replicates were done for each true leaf. Three true leaves from separate random seedling replicates were taken and analyzed. Data represents SPAD Values which are numerical representations of the measurement of absorbance values of the leaf in the red and near-infrared regions. n.s. = not significant and \*\*\* =  $p \leq 0.001$ . **(A.)** Chlorophyll measurements of 14-day old plants grown on control soil. **(B.)** Chlorophyll measurements of 14-day old plants grown on alkaline soil.

## DISCUSSION

Utilizing the amiRNA library collection that combinatorially co-silences closely homologous gene clade members, facilitated a forward genetic screen to identify putative target genes that are related to iron-deficiency. From this library screening, 12 candidate lines that were separated into two separate categories were identified based on the observations of primary root growth under iron deficient conditions: “iron-insensitive” and “iron-sensitive.” One candidate line from each of the categories was chosen for further characterization and elucidation of the mechanisms involved related to iron-deficiency. With the two candidate lines, the putative target genes that were targeted by amiRNA were then identified. The putative target genes for the “iron-insensitive” candidate line, P11-1-1, was revealed to be the genes *PKP1*, *2*, and *3* and the “iron-sensitive” candidate line, P74-1-1, was revealed to be the genes *APL2*, *3*, and *4*.

From the RT-qPCR results, the ami-CT control lines for P11-1-1 potentially reveals that upon iron-deprivation, the genes *PKP1*, *2*, and *3* have decreased expression in *Arabidopsis* Col-0 ecotype. This would indicate that they are down-regulated to continue to grow and develop when experiencing iron-deficiency. As such, when the genes *PKP1*, *2*, and *3* were knocked down in P11-1-1, the mechanisms and physiological responses associated with the decreased expression of these genes have potentially allowed for the P11-1-1 to grow quite well on media and in soil, in fact better than the control, under iron-deficient conditions. In contrast, the ami-CT lines of P74-1-1 may reveal that under iron-deficient conditions, the genes *APL2*, *3*, and *4* have increased expression in *Arabidopsis* Col-0. This would insinuate that they are up-regulated to try to combat the negative effects that iron-deficient conditions poses on the plant. However, with *APL2*, *3*, and *4* now knock down by amiRNA, the decreased expression of the genes caused the P74-1-1 plants to grow poorly with stunted root growth when under iron-deficient conditions.

Additionally, the RT-qPCR results revealed that the effectiveness of the amiRNA targeting was corroborated as the average fold change of the gene expressions for P11-1-1 and P74-1-1 were decreased when compared to both iron-sufficient and iron-deficient conditions. Thus, the characterization of the iron mechanisms were elucidated for both candidate lines.

For candidate line P11-1-1, early root growth indicated that it had grown slower than the ami-CT under both iron-sufficient and iron-deficient conditions. It was also revealed that the seeds were bigger than the control which would indicate that more iron is stored and present within the seeds. From this, it may be speculated that P11-1-1 grows and develops well under iron-deprivation conditions due to the fact that it has more iron that would be available upon germination, enabling P11-1-1 to be “iron-insensitive” to the lack of iron in its environment. Additionally, P11-1-1 would appear to have enough iron globally from the start so iron would not prove to be a challenge during its growth and development.

Typically, under normal iron-sufficient conditions, plants store some iron in the roots, such as in the vacuoles of root cells, root epidermal cells, and root hairs to maintain iron homeostasis. Additionally, plants store the majority of iron in ferritins, which are located in the chloroplasts up in the shoots for photosynthetic processes (Briat et al., 2010). However, when intrinsic iron levels are high, iron is usually sequestered in ferritin proteins and stored in the vacuoles of the roots to avoid over-accumulation in the chloroplasts that would otherwise cause oxidative stress to chloroplast components (Long et al., 2010). Based on the results from the measurements of iron content, P11-1-1 had increased iron content in the roots under iron-sufficient conditions. This is also seen in the images from the Perls/DAB staining where there are increased iron staining in the root epidermal cells in the apical meristem, elongation zone, and the differentiation zone when grown in iron-sufficient conditions (*Figure 8*). Additionally, it had

increased iron content in the shoots under iron-deficient conditions. This may be speculated that some sort of cellular iron homeostasis or iron storage and translocation is involved. One potential explanation can be that as P11-1-1 seeds are bigger and thus contain much more iron, the excess iron that the embryo inherits from the endosperm gets stored in the roots as it develops to avoid oxidative stress on the chloroplasts. Under sufficient iron, P11-1-1 seedlings stores the excess iron within the roots. However, when P11-1-1 experiences iron-deficiency, much of the excess iron stored in the roots is rapidly translocated to the shoots. This is supported from the iron content measurements revealing that as the roots experience iron-deficiency, the excess iron stored in the epidermal cells and in vacuoles are translocated into the shoots to maintain the iron needed for photosynthesis. Even as this occurs, the amount of iron that is stored in the roots is enough maintain photosynthesis without depleting all the iron in store within the roots. This is shown by how there is no significant differences between P11-1-1 and the ami-CT under iron-deficiency in the roots and iron-sufficiency in the shoots. It is worth noting that the Perls/DAB iron staining of root epidermal cells that was observed in iron-sufficiency is notably absent in iron-deficient conditions. This would prove to further substantiate this hypothesis.

The alkaline soil experiments and chlorophyll content measurements are evidence towards this belief that P11-1-1 is involved in the facilitation of translocating iron into the shoots. P11-1-1 displays bigger leaves and rosettes on iron-deficient alkaline soil even as iron availability is reduced in soil with a high pH (7.0-8.0). Additionally, P11-1-1 has higher chlorophyll content on alkaline soil which would potentially indicate that the excess iron stored in the roots would be translocated to the chloroplasts in the shoots to maintain photosynthetic processes and offset the iron-deficiency that P11-1-1 is facing.

For candidate line P74-1-1, interestingly, early root growth rates were also indicated to have had grown slower than the ami-CT under both iron-sufficient and iron-deficient conditions. Additionally, the seed sizes of P74-1-1 were also quantified to be bigger than the control. Similar to P11-1-1, it would also be expected that with the additional iron stored in the bigger seed sizes, that P74-1-1 would also develops well under iron-deprivation conditions as more iron that would be available upon germination to enable P74-1-1 to be “iron-insensitive.” P74-1-1 would also appear to have enough iron globally from the start so iron would not be an issue during its growth and development.

However, the results from the iron concentration measurements for P74-1-1 would shatter this expectation. The iron concentration levels of P74-1-1 within the roots under iron-deficient conditions are significantly lower than the control; the complete opposite from what was observed in P11-1-1. Furthermore, P74-1-1 also had a very significantly high iron content measured in the shoots under iron-deficiency. Looking at the pattern and the trend of P74-1-1’s iron measurements, this would be a response that is similar with what would be observed when normal plants experience iron-deficiency where iron localized in the root would be translocated into the shoots for photosynthetic functions to keep the plant alive (Kobayashi et al., 2012). What is not expected is that the observed phenotypes are exacerbated, as is evident by the significantly decreased iron measurement in the roots and increased iron content in the shoots. It can be speculated that, as P74-1-1 experiences iron-deficiency and iron is being redistributed to the shoots, there may be an enhanced signal or perception by the plant that is occurring which is indicating that the shoot requires more iron than usual for cases under iron-deprivation. The Perls/DAB staining in *Figure 8.*, is evidence that this is occurring. Iron staining located at the root apical meristem in P74-1-1 is not only lower than when it was grown under iron-sufficient

conditions, but when compared to the ami-CT under iron-deprivation, it is severely decreased not only in the root epidermal cell layer, but also appearing to be decreased in the more interior root layers such as the stele within the root apical meristem. A possible explanation behind this occurrence is due to the knockdown of *APL2*, *3*, and *4*. Four isoforms of the large subunit exists (*APL1-4*), with three of them identified to be putative target genes for P74-1-1. These three genes are involved in encoding for the large subunit protein of ADP-glucose pyrophosphorylase, an enzyme that is involved in catalyzing the first, rate-limiting step in starch biosynthesis (Sokolov et al., 1998). Starch plays a crucial role in photosynthesis for plants. When glucose is produced as a product of photosynthesis, not all glucose is used by the plant for energy immediately. Some is stored up in the form of starch, acting as a reserve energy source for the plant when energy is expended more than what is produced. In this case, starch is then broken back down into glucose molecules for plant use. Starch is a polysaccharide made up of glucose molecules that is linked together in a complex structure and stored in storage organelles called amyloplasts that are found in plant roots, seeds, tubers, and other storage organs. Starch is compact and insoluble, making it a great long-term storage of energy in plant cells. The large subunits (ApL) are the regulatory isoforms, while the small subunit proteins (ApS) are the catalytic isoforms (Crevillén et al., 2005). Additionally, mutational analysis has suggested that *APL1* and *2* can compensate for the loss of *APS1* catalytic activity, suggesting that both have both catalytic and regulatory functions (Ventriglia et al., 2008). *APL3* is found to be a major large subunit isoform present in inflorescences, fruits, and roots of the plant (Villand et al., 1993). As such, the knockdown of *APL2*, *3*, and *4* would elucidate the observations that were previously mentioned under iron-deficient conditions. As the plant is experiencing iron-deficient stress, the plant naturally diverts iron from the roots to the chloroplasts in the shoots to maintain

photosynthesis. However, knowing that *APL2*, *3*, and *4* are involved in the regulatory role of starch biosynthesis, knockdown of these genes would cause the plants to lose its essential energy storage reserve. Additionally, when the seedlings are grown under iron-deficiency, the lack of stored energy in the form of starch, and the lack of iron for plant photosynthesis and other biological functions, P74-1-1 seedlings grow extremely poorly under iron-deprivation.

The alkaline soil experiments for P74-1-1 would solidify such hypothesis as the smaller shoots and lower seed density observed under iron-deficiency would prove that P74-1-1 cannot germinate well and would experience the effects of iron-deficiency more severely. Chlorophyll content measurements of P74-1-1 rosettes under iron-deficiency would also show that due to the *APL2*, *3*, and *4* gene knockdowns, there was significant decrease of photosynthetic processes. Consequently, without adequate photosynthesis, glucose production would be greatly affected. With the knowledge that *APL2*, *3*, and *4* gene knockdowns leads to hindered starch biosynthesis and with the additional stress that iron-deficiency poses on root growth, P74-1-1 seedlings experienced chlorosis and severely stunted primary root growth.

## MATERIALS AND METHOD

### Generation of the AmiRNA Library

In the amiRNA library collection that was used in this research, the 10 sublibraries in which the collection is categorized in is covered by more than 95% of the designed 22,000 individual amiRNA designs (Hauser et al., 2013). The amiRNA library was first transformed into *Agrobacterium tumefaciens* and then further transformed into the *Arabidopsis* Col-0 ecotype (Hauser et al., 2019). Based on the article by Hauser, the T1 seeds were then harvested, selected on herbicide-resistant plate or soil-based selection methods, and grown to harvest the T2 seeds. From these T2 seeds, a phenotypic screening was done to establish and identify lines with interesting phenotypes to characterize and study.

### Phenotypic Screening of the Artificial microRNA Library

Seeds of the amiRNA control (ami-CT) line, amiRNA-HsMYO which targets human *MYOSIN2* gene that has no target gene in *Arabidopsis*, along with seeds from the amiRNA library of individually isolated T3 lines from the amiRNA libraries were chlorine-gas sterilized for 1.5 hours. The seeds were imbibed in water and stratified at 4°C for 72 hours in the dark and germinated under photoperiods of 16-hours light/ 8-hours dark at 21°C.

Root phenotype assays were done by sowing seeds onto the surface of iron-sufficient Gruber media (+Fe) with 25 µM Na-Fe-EDTA and no Ferrozine or on iron-deficient Gruber media (-Fe) with no Na-Fe-EDTA and 75 µM Ferrozine, a strong iron chelator [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazinesulfonate, Sigma-Aldrich], as described in Platre et al. The Gruber agar plates consists of 5% (w/v) sucrose and 0.8% (w/v) agar (Phytotech Micropropagation Agar) adjusted to pH 5.7. In the Gruber media, it 750 µM of MgSO<sub>4</sub>·7H<sub>2</sub>O, 625 µM of KH<sub>2</sub>PO<sub>4</sub>, 1000 µM of NH<sub>4</sub>NO<sub>3</sub>, 9400 µM of KNO<sub>3</sub>, 1500 µM of CaCl<sub>2</sub>·2H<sub>2</sub>O,

0.055  $\mu\text{M}$  of  $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$ , 0.053  $\mu\text{M}$  of  $\text{CuCl}_2\cdot 2\text{H}_2\text{O}$ , 50  $\mu\text{M}$  of  $\text{H}_3\text{BO}_3$ , 2.5  $\mu\text{M}$  of KI, 50  $\mu\text{M}$  of  $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$ , 0.52  $\mu\text{M}$  of  $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$ , 15  $\mu\text{M}$  of  $\text{ZnCl}_2$ , 25  $\mu\text{M}$  of Na-Fe-EDTA (iron-sufficient media) or 0  $\mu\text{M}$  of Na-Fe-EDTA (iron-deficient media), 1000  $\mu\text{M}$  of MES adjusted to pH 5.5 with KOH. The seedlings were grown on Gruber plates for 11-days, with the plates scanned using Epson Perfection V600 photo scanners (1200 dots per inch), and the phenotypes were recorded (Slovak et al., 2014). The seedlings were then transplanted to soil, grown, and then harvested. This cycle was repeated for a total of three generations.

### **Root Length Measurements of Candidate Lines**

Seedlings that were grown on +Fe and -Fe Gruber media plates for 11-days were scanned using Epson Perfection V600 photo scanners. The images were then manually measured using the ImageJ FIJI image processing software and analyzed using Excel.

### **Identification of Putative Target Genes**

Genomic DNA for candidate amiRNA lines were extracted (Kang, Cho, Yoon, & Eun, 1998) and was used as the template DNA. OneTaq PCR was done to amplify the fragment which the amiRNA would target putative genes using the primers QQ474 and QQ475 (*Table 3*; listed in Material & Method). The primer PHA2804F (*Table 3*; listed in Material & Method) was used to amplify the PCR product. The purified PCR samples were sent to be sequenced to identify the amiRNA that is present in the candidate lines (Hauser et al., 2019). The amiRNA targeted genes were putatively identified by using the “Target Search” function on the WMD3 website (Ossowski et al., 2008). Afterwards, the genes were cross-referenced to the PHANTOM library

database to accurately determine the putative target genes (Hauser et al., 2013; Hauser et al., 2019).

**Table 3. Primers Used for OneTaq PCR Sequencing and DNA Sequencing.** The primers QQ474 and QQ475 were used in OneTaq PCR to amplify the fragment where the amiRNA would target putative genes and the fragments were checked on a 1% agarose gel. Primer PHA2804F was used to determine the DNA sequence which would identify the putative target genes that were affected by amiRNA gene silencing. Note, primer PHA2804F is a primer from Hauser et al., 2019.

| Primer Name | Primer Sequence      |
|-------------|----------------------|
| QQ474       | CGCACAATCCCACTATCCTT |
| QQ475       | CAACGTGCACAACAGAATTG |
| PHA2804F    | AGAGAACACGGGGGACGAG  |

**Table 4. miRNA319a Precursor Sequences.** The forward and reverse sequences of the miR319a backbone (Schwab, 2005; Schwab et al., 2006) were used to determine the 21-nucleotide base pairs that is targeted by the amiRNA.

| Sequence Name   | Sequence             |
|-----------------|----------------------|
| Forward - miRNA | TTGGACTGAAGGGAGCTCCC |
| Reverse - miRNA | GAGAGCTTCCTTGAGTCCAT |

### RT-qPCR and temperature and time, volume, primers used

Seeds from the candidate lines were plated and grown for 11-days on the surface of Fe-sufficient Gruber media with 25  $\mu$ M Na-Fe-EDTA (+Fe) and no Ferrozine or on Fe-free Gruber media (-Fe) with no Na-Fe-EDTA and 75  $\mu$ M Ferrozine. Afterwards, the 11-day-old plant seedlings were collected and frozen in liquid nitrogen in microfuge tubes. The plant tissues were kept liquid nitrogen and ground manually by hand. Total RNA was isolated by using the Sigma-Aldrich Spectrum Total RNA Kit, following the manufacturer's protocol. The RNA samples were eluted using the elution buffer provided by the kit. The concentrations of the RNA samples were measured by using a nanodrop spectrophotometer. cDNA synthesis was done using the

Maxima H-Minus cDNA Synthesis Master Mix kit by ThermoScientific, following the manufacturer's protocol. A reverse-transcription reaction for cDNA synthesis was done in a thermal cycler with the following steps and temperatures: incubated at 25 °C for 10 minutes, incubated at 50 °C for 15 minutes, reaction terminated at 85 °C for 5 minutes. When running the qPCR, each well contain 5 µL of New England Biolabs' Luna Universal qPCR Master Mix, 0.25 µL of forward primer of the putative target gene, 0.25 µL of reverse primer of the putative target gene, 1 µL of 1:5 diluted cDNA (with nuclease-free water), and 3.5 µL of nuclease-free water. A Bio-Rad CFX Opus 384 Real-Time PCR System was used for the PCR thermal gradients and qPCR. The temperatures and steps used for the protocol was as follows: Initial denaturation at 95 °C for a minute for one cycle, denaturation at 95 °C for 15 seconds and extension at 60 °C for 30 seconds for 40-45 cycles, and the melt-curve of 60-95 °C in increments of 0.5 °C over 5 seconds for one cycle. Calculations for data analysis was done using the Delta-Delta Ct method. The housekeeping gene *AT2G37620 (ACT1)* was used to normalize the calculations for the Delta-Delta Ct method. The RT-qPCR experiments consists of 3 biological samples for all the candidate lines.

### **Ami-RNA CRISPR Construct, Knockout and Validation**

GoldenGate Protocol is as follows:

- 1. Level I - Loading oligos into shuttle vectors**
  - 1.1. Design relevant ~20bp primers for targeting (refer to Table 2.)
  - 1.2. Make oligonucleotide stock at a concentration of 100uM
  - 1.3. Mix respective oligos and H2O
    - 1.3.1. In a labeled PCR tube, combine 40ul H2O + 5ul of each oligo (forward and reverse) for a total volume of 50ul.
  - 1.4. Denature oligos by heating in thermocycler
    - 1.4.1. *5 minutes @ 98C → Hold ∞ @ 10C*
  - 1.5. Let tubes sit at room temperature for ~2 minutes before proceeding.

- 1.6. Dilute hybridized oligos
  - 1.6.1. As a 1:200 dilution, in newly labeled PCR tubes for each rep, add 1ul of respective hybridized oligos and 199ul of H<sub>2</sub>O.
- 1.7. Prepare golden gate 1 (GG1) master mix. \*Following formula is for one tube. Multiply accordingly (recommended n+1 so that there is some left over)
  - 1.7.1. 5.4ul H<sub>2</sub>O
  - 1.7.2. 0.3ul T4 DNA Ligase
  - 1.7.3. 0.3ul BbsI
  - 1.7.4. 1ul 10x BSA
  - 1.7.5. 1ul 10x Ligation buffer
    - 1.7.5.1. Total of 8ul per tube.
- 1.8. In newly labeled PCR tubes for each rep, add 8ul of GG1 master mix, 1ul of respective shuttle vectors (refer to Table 5.), and 1ul of respective diluted hybridized oligos.
- 1.9. Put tubes into thermocycler with GoldenGate1 configuration (GG\_1\_STU)
  - 1.9.1. (2 minutes @ 37C → 5 minutes @ 16C)x30 → 10 minutes @ 50C → 10 minutes @ 80C → Hold ∞ @ 4C
- 1.10. Transform onto LB plates + relevant antibiotic (Ampicillin)
  - 1.10.1. Mix 2ul of reaction into thawed ThermoFisher OneShot™ Top10 Competent E. Coli cells on ice.
  - 1.10.2. Ice for 15 minutes
  - 1.10.3. Heat block @ 42C for 30 seconds
  - 1.10.4. Ice for 1 minute
  - 1.10.5. Add 250ul SOC medium.
  - 1.10.6. Shake in incubator for 1 hour @ 37C
  - 1.10.7. Plate 50ul onto LB plates + relevant antibiotic (Ampicillin)
  - 1.10.8. Incubate overnight @ 37C
- 1.11. Pick colonies and add to liquid cultures
  - 1.11.1. Make liquid LB cultures for as many reaction tubes you have with relevant antibiotic (Ampicillin) in a 1:1000 ratio of media to antibiotic
    - 1.11.1.1. E.G. Make 5ml tubes of LB with 5ul of antibiotic.
  - 1.11.2. Incubate overnight @ 37C
- 1.12. Use QIAprep Spin Miniprep Kit™ for miniprepping and use relevant primers (refer to Table 5.) to send for sequencing with Eton Biosciences. Keep remaining miniprep.

## **2. Level II - Loading shuttle vectors into multi-multi vectors**

- 2.1. Get concentrations of all miniprep shuttle vectors and make diluted aliquots to get all shuttle vectors to 40ng/uL. Make sure multi-multi vectors (refer to Table 5.) are concentrated to 50ng/uL.
- 2.2. Prepare multi-multi golden gate 1 (GG1) master mix. \*Following formula is for one tube. Multiply accordingly (recommended n+1 so that there is some left over)
  - 2.2.1. 2ul 10x Ligation Buffer
  - 2.2.2. 2ul 10x BSA
  - 2.2.3. 1ul BsaI
  - 2.2.4. 1ul T4 DNA Ligase
  - 2.2.5. 1ul multi-multi vector
  - 2.2.6. 1ul gRNA of each shuttle vector (from step 1.12)
  - 2.2.7. Add remaining water to 20ul
- 2.3. Vortex mix briefly, ~1 second, and spin down.
- 2.4. (2 minutes @ 37C → 5 minutes @ 16C)x30 → 10 minutes @ 50C → 10 minutes @ 80C → Hold ∞ @ 4C
- 2.5. Transform onto LB plates + relevant antibiotic (Kanamycin)
  - 2.5.1. Mix 2ul of reaction into thawed ThermoFisher OneShot™ Top10 Competent E. Coli cells on ice.
  - 2.5.2. Ice for 15 minutes
  - 2.5.3. Heat block @ 42C for 30 seconds
  - 2.5.4. Ice for 1 minute
  - 2.5.5. Add 250ul SOC medium.
  - 2.5.6. Shake in incubator for 1 hour @ 37C
  - 2.5.7. Plate 50ul onto LB plates + relevant antibiotic (Kanamycin)
  - 2.5.8. Incubate overnight @ 37C
- 2.6. Pick colonies and add to liquid cultures
  - 2.6.1. Make liquid LB cultures for as many reaction tubes you have with relevant antibiotic (Kanamycin) in a 1:1000 ratio of media to antibiotic
    - 2.6.1.1. E.G. Make 5ml tubes of LB with 5ul of antibiotic.
  - 2.6.2. Incubate overnight @ 37C
- 2.7. Use QIAprep Spin Miniprep Kit™ for miniprepping, get concentration values, and use relevant primers (refer to Table 5.) to send for sequencing with Primordium services. Keep remaining miniprep.

### **3. Level III - Loading into recipient vector**

- 3.1. Dilute multi-multi vectors (from step 2.7) to 50ng
- 3.2. Prepare golden gate 2 (GG2) master mix. \*Following formula is for one tube.  
Multiply accordingly (recommended n+1 so that there is some left over)
  - 3.2.1. 2ul 10x Ligation Buffer
  - 3.2.2. 2ul 10x BSA
  - 3.2.3. 1ul of recipient vector [at 300ng] (refer to Table 5.)
  - 3.2.4. 1ul of end linked [at 30ng] (refer to Table 5.)
  - 3.2.5. 0.7ul BsaI
  - 3.2.6. 0.7ul BbsI
  - 3.2.7. 0.7ul T4 DNA Ligase
  - 3.2.8. 1ul of shuttle/multi-multi vectors (from step 3.1)
  - 3.2.9. Add water to 20ul
- 3.3. Vortex mix briefly, ~1 second, and spin down.
- 3.4. (2 minutes @ 37C → 5 minutes @ 16C)x50 → 10 minutes @ 50C → 10 minutes @ 80C → Hold ∞ @ 4C
- 3.5. Transform onto LB plates + relevant antibiotic (Spectinomycin)
  - 3.5.1. Mix 2ul of reaction into thawed ThermoFisher OneShot™ Top10 Competent E. Coli cells on ice.
  - 3.5.2. Ice for 15 minutes
  - 3.5.3. Heat block @ 42C for 30 seconds
  - 3.5.4. Ice for 1 minute
  - 3.5.5. Add 250ul SOC medium.
  - 3.5.6. Shake in incubator for 1 hour @ 37C
  - 3.5.7. Plate 50ul onto LB plates + relevant antibiotic (Spectinomycin)
  - 3.5.8. Incubate overnight @ 37C
- 3.6. Pick colonies and add to liquid cultures
  - 3.6.1. Make liquid LB cultures for as many reaction tubes you have with relevant antibiotic (Spectinomycin) in a 1:1000 ratio of media to antibiotic
    - 3.6.1.1. E.G. Make 5ml tubes of LB with 5ul of antibiotic.
  - 3.6.2. Incubate overnight @ 37C
- 3.7. Use QIAprep Spin Miniprep Kit™ for miniprepping, get concentration values, and use relevant primers (refer to Table 5.) to send for sequencing with Primordium services. Keep remaining miniprep.

To do this, GoldenGate cloning was utilized to efficiently assemble multiple DNA fragments into a plasmid vector and be used to create the CRISPR construct. To start, relevant ~20bp primers were designed using the Primer3 software (Koressaar et al., 2007; Koressaar et al., 2018; Untergasser et al., 2012). Four sets of primers were designed for each of the three genes from the PKP and APL gene sets (*Table 2.*). With the primers, they were used for the GoldenGate cloning method to create the CRISPR construct (GoldenGate Protocol; In Material and Methods). The plasmids used for each level of the GoldenGate cloning and the sequencing primers used are listed in *Table 5*.

Plant transformation was done by transforming the sequenced plasmids into *Agrobacterium tumefaciens* strain GV3101. *Arabidopsis thaliana* ecotype, Col-0, was transformed as described using the floral dip method (Bent, 2006). The T0 seeds were collected and used for subsequent selections. T1 selection was done on ½ MS plates (1/2 MS, 1% sucrose, 0.8% phytigel, adjusted with KOH to a pH of ~5.7) with Kanamycin (working concentration of 50 µg/mL) added into the media. Surviving seedlings were transplanted to soil, grown, and T2 seeds were harvested. T2 seeds will be selected for non-fluorescent seeds (i.e., seeds without the FAST marker) through a direct observation with a stereomicroscope. The T2 non-fluorescent seeds will be grown on ½ MS plates + Kanamycin (50 µg/mL) and the surviving seedlings will be transplanted to soil. T2 leaf samples will be cut to conduct a CRISPR sequencing to validate the knockout of the putative target genes in the T2 lines by confirming existence of indels. Primers for the putative genes have not been designed for CRISPR sequencing but will be done in the near future. T3 seeds will then be harvested and will be used as the established lines with knocked out putative genes.

**Table 5. Plasmids, Sequencing Primers, and End-linkers for GoldenGate Cloning.** The plasmid vectors, sequencing primers, and end-linkers that were used in the different stages of the GoldenGate protocol are listed. Plasmid information can be searched on AddGene. Additional information on the shuttle vectors, multi-multi, recipient, and end-linker plasmid vectors can be found in Stuttmann et al. sgRNA shuttle vectors with *Arabidopsis thaliana* U6 promoter; guide sequence overhangs ATTG-GTTT. In Level 2, both the PKP1/2/3 and APL2/3/4 gene sets used two multi-multi vectors: pDGE 503 and pDGE 504. Both were needed to hold the 12 shuttle vectors from Level 1. Each multi-multi vector can only hold up to 8 shuttle vectors. In Level 3, both PKP1/2/3 and APL2/3/4 gene sets used one recipient vector, pDGE 652 which can hold both multi-multi vectors from Level 2.

|                | Plasmid Vectors                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | Sequencing Primers                                        | End-linker |
|----------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------|------------|
| <b>Level 1</b> | <p><b>PKP Shuttle Vectors (Module Type):</b></p> <p>PKP1_gRNA1: pDGE 332 (M1)<br/> PKP1_gRNA2: pDGE 333 (M2)<br/> PKP1_gRNA3: pDGE 335 (M3)<br/> PKP1_gRNA4: pDGE 336 (M4)</p> <p>PKP2_gRNA1: pDGE 495 (M5)<br/> PKP2_gRNA2: pDGE 496 (M6)<br/> PKP2_gRNA3: pDGE 341 (M7)<br/> PKP2_gRNA4: pDGE 342 (M8E)</p> <p>PKP3_gRNA1: pDGE 332 (M1)<br/> PKP3_gRNA2: pDGE 333 (M2)<br/> PKP3_gRNA3: pDGE 335 (M3)<br/> PKP3_gRNA4: pDGE 337 (M4E)</p> <hr/> <p><b>APL Shuttle Vectors (Module Type):</b></p> <p>APL2_gRNA1: pDGE 332 (M1)<br/> APL2_gRNA2: pDGE 333 (M2)<br/> APL2_gRNA3: pDGE 335 (M3)<br/> APL2_gRNA4: pDGE 336 (M4)</p> <p>APL3_gRNA1: pDGE 495 (M5)<br/> APL3_gRNA2: pDGE 496 (M6)<br/> APL3_gRNA3: pDGE 341 (M7)<br/> APL3_gRNA4: pDGE 342 (M8E)</p> <p>APL4_gRNA1: pDGE 332 (M1)<br/> APL4_gRNA2: pDGE 333 (M2)<br/> APL4_gRNA3: pDGE 335 (M3)<br/> APL4_gRNA4: pDGE 337 (M4E)</p> | <p><b>Eton Universal Primer:</b><br/> M13F - 21</p>       | N/A        |
| <b>Level 2</b> | <p><b>PKP and APL Multi-Multi Vectors:</b></p> <p>pDGE 503 (holds 8 shuttle vectors)<br/> pDGE 504 (holds 4 shuttle vectors)</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 615 - Forward                                             | N/A        |
| <b>Level 3</b> | <p><b>PKP and APL Recipient Vectors:</b></p> <p>pDGE 652 (holds 2 multi-multi vectors)</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | <p>N/A;<br/> Primordium Whole<br/> plasmid sequencing</p> | pDGE 508   |

### **Quantifying Root Growth Rate Utilizing the Keyence Microscope**

Seeds were chlorine gas sterilized, imbibed in water, and stratified for 2-3 days. Seedlings were grown on Gruber media in +Fe and FRZ media treatments on plates in the growth chambers in long day photoperiods (16 hrs in light) for 5 days. On the day of Keyence time-lapse capture, 6 mL of +Fe and FRZ Gruber media were poured into Keyence microscope chambers. Seedlings were then transferred to their respective media treatments. The time lapse capture was done on a Keyence BZ-X810 microscope with BZ Nikon Objective Lens (4X) Plan-Apo Lambda in brightfield/phase channels. Images of root growth was captured every 5 minutes over an 8-hour period. Root growth tracking and measurements were done on MatLab using a specifically scripted code for the set parameters and settings.

### **Seed Size Quantification**

Seeds were sprinkled onto microscope slides and still-images were captured with a Keyence BZ-X810 microscope with BZ Nikon Objective Lens (10X) Plan-Apo Lambda in brightfield/phase channels. The images were then processed and analyzed using the ImageJ FIJI image processing software. Once the seed sizes had been calculated, XLSTAT was used to compute complex calculations to determine the distribution of the seed sizes and plot a scattergram of all the seed sizes for all the lines.

### **Iron Concentration Measurement**

Arabidopsis seeds were chlorine gas sterilized, imbibed in water, and stratified in the dark for 3 days in the dark at 4°C. The seeds were then sown onto Gruber media plates and transferred into a growth chamber to grow under a 16-hours light/ 8-hours dark photoperiod for

14 days. A total of 30 seedlings were separated into shoots and roots and used for iron concentration measurements. Root samples may contain residual media, which would contain Fe, as a result, residual media was absorbed with Kimwipes and washed twice with MilliQ water and gently dried with Kimwipes. Roots and shoots were then dried in 2.0 mL Eppendorf tubes and dried in an oven for at least two days at 60-65°C. Dry weight of the samples were measured and then transferred into 15mL conical tubes. Sample digestion, preparation, spectroscopic readings, and analysis were done as described in Gautam et al. Spectroscopic readings were done using the Tecan Safire 2 Plate Reader set to measure absorbance values at a wavelength of 535 nm. Data analysis and calculations were done on Excel.

### **Perls/DAB Staining**

Seeds were sown onto Gruber iron-sufficient and iron-deficient media and were grown in a growth chamber under 16 hours light/ 8 hours dark photoperiod for 7 days. 7-day old seedlings were then stained with the Perls method and the cellular level of iron detection was further intensified using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, catalog number: 32750) as described in Brumbarova et al. The stained samples were imaged with a Keyence BZ-X810 microscope with BZ Nikon Objective Lens (4X) Plan-Apo Lambda in brightfield/phase channels.

### **Phenotypic Growth on Alkaline Soil**

Alkaline soil was prepared with the addition of 8g CaO/ kg of soil to a final soil pH of 7.0-8.0 (Satbhai et al., 2017). When soaking the soil, a solution was made by adding 30 mL of Heritage fungicide (prepared by adding 6.75 g of the fungicide into 1L of water), no fertilizer,

and 2.5 L of water was used to avoid iron contamination in the experiment. The control soil was soaked with a solution containing 30mL Heritage fungicide (prepared by adding 6.75 g of the fungicide into 1 L of water), 10 mL of Peters 20-20-20 General Purpose Fertilizer (prepared by adding 250 g of fertilizer into 1L of water) and 2.5L of water. Seeds were sprinkled onto both soil-type flats and grown under a 16-hour light/ 8-hour dark photoperiod at 21°C. Two replicate pots were grown for each genotype. Plants were grown for 14-days and the initial phenotypes of the rosettes were observed and recorded. Additionally, at week 8 the plants' phenotype at senescence was observed and recorded.

### **Chlorophyll Measurement**

Chlorophyll content was measured on true leaves using the same plants that were grown for the phenotypic analysis on alkaline soil. Measurements were taken using a Konica Minolta SPAD-502 Plus chlorophyll meter on 14-days old plants. From each pot of each genotype, the true leaves of 14-days old plants were taken and the chlorophyll measurements were recorded. Three technical measurement replicates were done for each true leaf. Three true leaves from separate random seedling replicates were taken and analyzed.

Chlorophyll has absorbance peaks in two wavelength regions: the blue (400-500 nm) and red (600-700 nm) regions, with no absorbance in the near-infrared region. Using this characteristic of chlorophyll, the Konica Minolta SPAD-502 Plus chlorophyll meter measures the absorbance of the leaf in the red and near-infrared regions. Using these two absorbance values, the meter calculates a numerical SPAD value which is proportional to the amount of chlorophyll content that is present within the leaf.

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