Characterization of an unknown gene and identification of its role in Iron deficiency response in *Arabidopsis thaliana*

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Certificate of Examination

This is to certify that the dissertation titled "Characterization of an unknown gene and identification of its role in iron deficiency response in *Arabidopsis thaliana*" submitted by Mr. Bitra Jyothi Srinivas (Reg. No. MS16103) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Shashi Bhushan Pandit Dr. Ram Kishor Yadav Dr. Santosh Satbhai

(Supervisor)

Dated: 23 April, 2021

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Santosh Satbhai at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Bitra Jyothi Srinivas
(Candidate)
Dated: 23 April, 2021

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Santosh Satbhai
(Supervisor)
Dated: 23 April, 2021

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Notations

CTAB : Cetyltrimethylammonium bromide

EtBr : Ethidium Bromide

LB : Luria Broth

MS : Murashige Skoog

OD : Optical density

PCR : Polymerase Chain Reaction

TAE : Tri Acetate EDTA

Y2H : Yeast-2-Hybrid

MCT: Micro Centrifuge Tube

CRISPR: Clustered regularly interspaced short palindromic repeats

TF: Transcription Factor

Ox: Over Expression Line

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Abstract

Iron is an essential micronutrient for plants, many enzymes involved in hormone biosynthesis have iron as a critical component. It is also required for chlorophyll biosynthesis, respiration, pathogen defense, nitrogen assimilation, and photosynthesis. Plants maintain iron homeostasis because both excess and deficiency of iron affect plant growth and development. Iron homeostasis is not entirely understood, and many genes involved are yet to be discovered. In this project, I tried to characterize an unknown gene, which might be involved in iron homeostasis. I found that the unknown gene overexpression improves root growth under iron deficiency conditions. Consequently, it might play a redundant role in the iron deficiency signaling pathway with a close homologue of an unknown gene.

Chapter 1

Introduction

1.1 Background

All living organisms require iron for their growth and development. In plants, it plays an essential role in several cellular functions like respiration, photosynthesis, chlorophyll and hormone biosynthesis [1]. In animals, it is essential for the biosynthesis of hemoglobin, myoglobin, and other iron-containing enzymes involved in electron transfer and oxidation-reductions [2]. Iron deficiency in humans leads to anaemia, lower immunity and impairs cognitive development. The animals obtain iron from plants either directly or indirectly. Therefore, it is vital to understand the iron homeostasis in plants.

1.2 Iron uptake strategies

Plants obtain the iron from the soil through roots. Although it is the fourth most abundant element in the earth crust, its bioavailability is still limited. Because at basic and neutral pH, in the presence of oxygen Fe (III) gets oxidized and forms insoluble crystals of ferric oxide, which the plants cannot take up. Therefore, plants have evolved different strategies for obtaining iron from the soil [3].

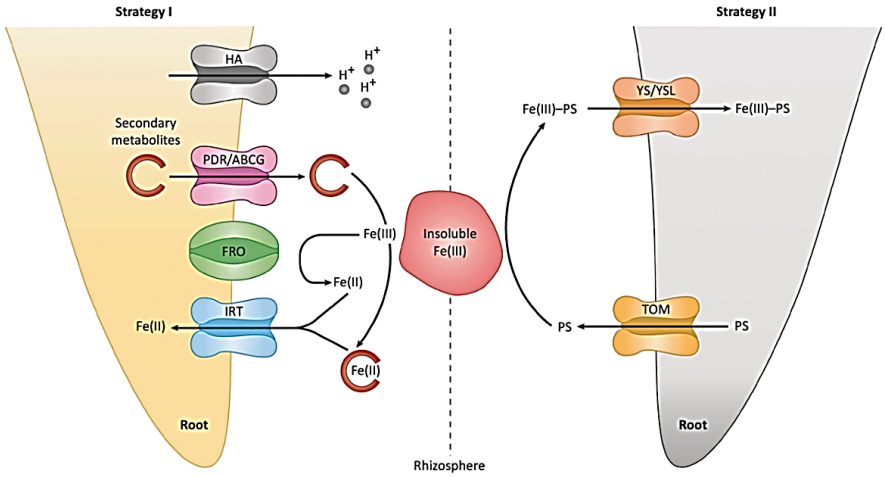


Figure 1: Iron uptake strategy I in dicotyledons and non-graminaceious monocotyledons and strategy II in graminaceous plants [4] .

The strategy I, also known as reduction-based strategy, is adopted by dicotyledons and monocotyledon plants except for graminaceous plants (Fig.1). In this strategy, proton pump (H+-ATPase) releases protons into the rhizosphere, leading to lower soil pH, converting insoluble iron to a soluble form. The soluble iron is chelated by the secondary metabolites released by the pleiotropic drug resistance 9 (PDR9) into the rhizosphere [5]. The chelated Fe^{3+} is reduced to Fe^{2+} by the ferric reductase oxidase (FRO2), and Fe^{2+} is transported into the root epidermal cell by the iron-regulated transporter 1 (IRT1) [6].

Strategy II, also known as the chelation strategy, is adopted by graminaceous plants (Fig.1). In this strategy, phytosiderophores (PS) are secreted into the rhizosphere, which binds to Fe^{3+} forming Fe(III)-PS complexes which are transported into the root by the yellow stripe 1 (YS1) transporter [7] .

1.3 Genetic regulation of Strategy I

In Arabidopsis, several transcription factors are involved in the regulation of Fe uptake. FER-like iron deficiency-induced transcription factor (FIT) plays an important role in controlling the expression of iron uptake genes [8]. Under iron deficiency, it interacts with subgroup Ib bHLH (TFs) transcription factors (bHLH38, bHLH39, bHLH100, and bHLH101) to form heterodimers and induce the expression of iron uptake genes FRO2 and IRT1 (Fig.2). The expression of subgroup Ib bHLH transcription factors is also activated under iron deficiency, and their expression is controlled by the subgroup IVc bHLH transcription factors (bHLH34, bHLH104, ILR3 bHLH105 and bHLH115) along with UR1 (bHLH121). The subgroup IVc bHLH TFs are regulated at the protein level by an E3 ligase BTS which degrades them under iron sufficient conditions to activate subgroup Ib genes only when the iron is limiting [8].

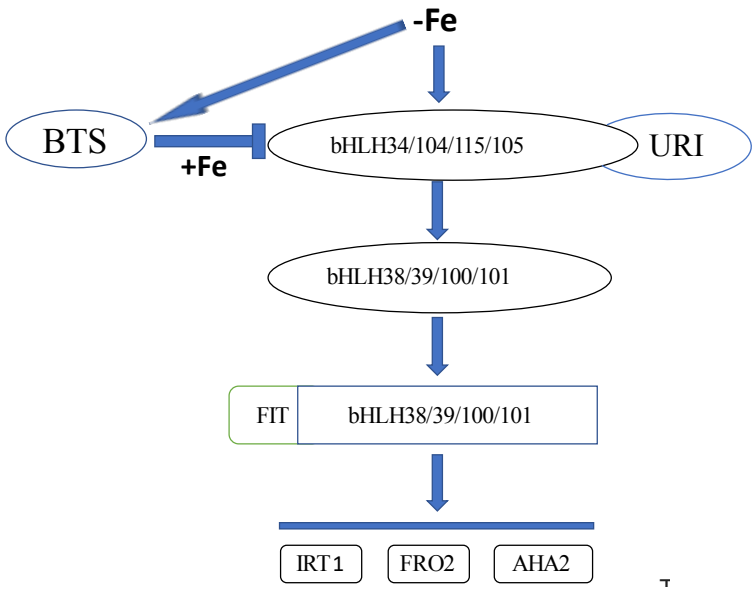


Figure 2: Iron deficiency response signaling pathway in *Arabidopsis thaliana* (adapted from [9]).

The iron homeostasis is not completely understood, and there are many genes involved in iron homeostasis which are still uncharacterized and their role is not clearly known. In this project, I aim to find out and characterize unknown genes involved in the network. To find out new genes that play an important role in iron homeostasis, I started a literature search and found that Dinneny et al. (2008) performed time-course microarray analysis to characterize the transcriptional response to iron deprivation in *Arabidopsis* [10]. I analyzed their expression data and found that the expression level of *AT1G12030* increases up to 98-fold under iron deficiency as compared to control condition, suggesting that this gene might be involved in iron homeostasis [10]. Thus, to find out whether this gene has any role in iron homeostasis or not, I generated a knockout mutant of this gene using CRISPR-Cas9 and used an overexpression line of this gene and compared the phenotype of mutant and overexpression plants with the wild type plants under both iron-sufficient as well as iron-deficient conditions. Next, I performed various qualitative and quantitative analyses of the phenotype to derive information on its chlorophyll content, root ferric chelate reductase activity and iron content. To further understand this gene's function, I performed Yeast–two-hybrid to identify the protein interaction with Transcription Factors (bHLH34, bHLH38, bHLH39,

bHLH100, bHLH101, bHLH104, bHLH105, PYE, FIT and BTS) that are known to be involved in the iron deficiency responsive signalling pathway.

Chapter 2

Materials and Methods

2.1 Plant Materials and Growth conditions.

In this study, *Arabidopsis thaliana* ecotype Columbia (Col-0) was used as wild type and *AT1G12030* mutant was generated by CRISPR-Cas9 in Col-0. In addition, the overexpression lines of *AT1G12030* was also used which were available in the lab. The plants were grown using autoclaved soil composed of solarite, perlite and compost in 3:1:1 ratio in pots or on half-strength Murashige and Skoog medium (½ MS) containing 1% (w/v) Sucrose and 1% (w/v) agar in plant chambers having controlled temperature 22^{o}C, 60% humidity, 110 µmol m-2 white light with 16 hours day and 8 hours night.

Seed Sterilization and Stratification

In a 1.5 ml MCT, seeds were taken and washed with 1ml 70 % (v/v) ethanol for 2 minutes. The ethanol was discarded and then seeds were washed with 0.1% sodium hypochlorite for 2 minutes. The seeds were finally washed with autoclaved water 3-4 times and kept in MCT with 1ml autoclaved water. MCT containing sterilized seeds with 1ml autoclaved water was kept in 4°C for four days and then seeds were transferred to MS plates.

2.2 Solutions and Media Composition

dNTPs stock preparation

A 2 ml MCT was kept on ice and 1500 µl of double distilled water was added to it. 500 µl of 10mM dNTPs was added to it and mixed properly. 50 µl aliquots were made in 1.5 ml MCTs.

Antibiotics stock preparation

Rifampicin (stock- 25 mg/ml)

250 mg Rifampicin was taken and dissolved in 10 ml DMSO and filter-sterilized. 1 ml aliquots were made and stored at -20^{o}C. In 100 ml media, 200 µl antibiotic should be used.

Ampicillin (stock- 100 mg/ml)

1 g of Ampicillin was added to 10 ml of millipore water, mixed properly and filter-sterilized. 1 ml aliquot were made in MCTs. In 100 ml media, 100 µl antibiotic should be used.

Tetracycline (stock- 12.5 mg/ml)

125 mg tetracycline was dissolved in 10 ml 100% (v/v) ethanol and filter-sterilized. 1ml aliquots were made and stored in -20^{o}C. In 100 ml media, 40µl antibiotic should be used.

Kanamycin and Gentamycin (stock- 50 mg/ml)

1g of Kanamycin and Gentamycin were dissolved in 20 ml millipore water and filter-sterilized.

1ml aliquots were made and stored in -20^{o}C. In 100 ml media, 100µl antibiotic should be used.

Hygromycin (stock- 50 mg/ml)

1g of Hygromycin was taken and dissolved in 20 ml millipore water and filter-sterilized.

1ml aliquots were made and stored in -20^{o}C. In 100 ml media, 100µl antibiotic should be used.

Cefotaxime (stock- 100 mg/ml)

1 g of Cefotaxime was added to 10 ml of millipore water, mixed properly and filter-sterilized.

1 ml aliquots were made in MCTs. In 100 ml media, 100 µl antibiotic should be used.

½ MS media (1 litre)

MS 2.15g

Sucrose 10g

pH was adjusted to 5.7 after adding water

Agar 10g

½ MS -Fe media (1 litre)

-Fe 2.135

Sucrose 10g

pH was adjusted to 5.7 after adding water

Agar 10g

Ferrozine media preparation

492mg FerroZine (Fz)was dissolved in 10 ml water and filter sterilized to prepare 0.1M Fz stock solution. It was added to ½ -Fe media to make 100µM or 300 µM Fz media.

Selection media preparation

To the 1000 ml ½ MS media, 1000 µl of both cefotaxime and hygromycin was added respectively, to make hygromycin selection media.

Y2H Dropout Media (250ml)

Yeast Nitrogen Base (YNB) - 0.467g

Ammonium sulphate – 1.25g

Dropout - 0.5g

Glucose - 5g

Agar - 5g

YNB, Ammonium sulphate and dropout were dissolved in 50ml water, glucose was also dissolved in 50ml water in another flask and agar was dissolved in 150ml water and autoclaved.

LB media (1 litre)

Sodium chloride	10g
Tryptone	10g
Yeast Extract	5g

LB Agar (1 litre)

Yeast Extract	5g
Tryptone	10g
Sodium chloride	10g
Agar	20g

Bacterial stocks

Added 500 µl autoclaved glycerol and 500 µl bacterial culture into a cryovial and stored in -80^{o}C.

Yeast stocks

Added equal amounts of 30% glycerol and yeast culture into a cryovial and stored in -80^{o}C.

SOB media (100 ml)

Tryptone 2g

Sodium chloride 0.05g

Yeast extract 0.5g

Transformation Buffer 1 (TB1) 200ml

Chemical	Stock concentration	Working concentration	Volume in ml
MOPS	1M (pH 6.5 with KOH)	10mM	2
Potassium Chloride	1M	1000mM	20
Manganese Chloride	1M	45mM	9
Calcium Chloride	1M	10mM	2
Potassium Acetate	1M (pH 7.5 with HCl)	10mM	1
Water			166

Transformation Buffer 2 (TB2) 200ml

Chemical	Stock concentration	Working concentration	Volume in ml
MOPS	1M (pH 6.5 with KOH)	10mM	2
Potassium Chloride	1M	1000mM	20
Manganese Chloride	1M	45mM	9
Calcium Chloride	1M	10mM	2
Potassium Acetate	1M (pH 7.5 with HCl)	10mM	1
Glycerol	80% (v/v)		12.5
Water			153.5

Transformation Buffer 3 (TB3) 200ml

Chemical	Stock concentration	Working concentration	Volume in ml
Calicum Chloride	1M	100mM	20
Magnesuim Chloride	1M	50mM	10
Water			170

Sorbitol (200ml)

Chemical	Working concentration	Quantity in grams	Volume in ml
Lithium Acetate	100mM	2.04	
Sorbitol	1M	36.4	
EDTA	1mM		0.4
Tris Chloride	10mM		2
Distilled Water			197.6

The mixture was filter sterilized

40% Polyethylene glycol (PEG) 50ml

Chemical	Working concentration	Quantity in grams	Volume in ml
PEG	40% w/v	20	
Lithium Acetate	100mM	0.51	
Tris Chloride	10mM		0.5
EDTA	1mM (pH 8)		0.1
Distilled water			49.4

The resultant mixture is then filter sterilized

3- Amino triazole Preparation

5 mM -0.105 gm in 250 ml

10 mM -0.210g in 250 ml

20 mM -0.420g in 250 ml

40 mM -0.840g in 250 ml

60 mM -1.680g in 250 ml

Ethidium bromide

0.5g of EtBr was dissolved in 50ml of distilled water to make a stock of 0.01g/ml. The final concentration of EtBr used was 0.5µg/ml while preparing the agarose gel.

TAE (50X)

Chemical	Volume in ml	Quantity in grams
Tris base		121
Glacial Acetic acid	28.55	
EDTA (pH 8, 0.5M)	50	
Distilled water	421.45	

Agarose gel

1. 1X TAE buffer (20ml 50X TAE and 980ml of distilled water) and 1% (w/v) Quick dissolve Agarose were mixed in a glass bottle.
2. The solution was heated in a microwave oven for 2 minutes and 20 seconds.
3. The solution was left aside for few minutes for cooling. 0.5µg/ml of Ethidium bromide was added to the solution and mixed properly.
4. The tray and the combs were arranged according to the requirement and the agarose solution is transferred to the tray.

CTAB Buffer (100 ml)

Chemical	Stock Concentration	Working concentration	Quantity in grams/ ml
CTAB		2% w/v	2 grams
NaCl	5M	1.4M	28ml
EDTA	0.5M	20mM	4ml
Tris Chloride	1M	100mM (pH 8)	10ml
Distilled water			58ml

2.3 Methods:

DNA isolation:

1. The leaf samples were collected in a 1.5 ml Eppendorf tube.
2. 600 µl of CTAB buffer (0.6 µl of beta-mercaptoethanol was added to 559.4 µl of CTAB) was added to the tube.
3. The sample tubes were heated at 65^0C in a thermomixer for 25 minutes.
4. The sample was then crushed using an autoclaved pestle till a clear green colour solution was obtained.
5. Tubes were transferred again to a thermomixer at 600rpm for 15 minutes at 65^0C.
6. 600 µl PCI (Phenol: Chloroform: Isoamyl alcohol is in 25:24:1 ratio) was added to the sample tube and centrifuged at 13000 rpm for 15 minutes.
7. The sample got separated into different layers; the topmost layer was transferred into another 1.5 ml tube.
8. 600 µl of CI (Chloroform: isoamyl alcohol was in 24:1 ratio) was added to the tube with the solution. The mixture was centrifuged at 13000rpm for 15 minutes.
9. The topmost clear layer was transferred to a new 1.5ml tube, and a 0.7x volume of isopropanol was also added to the tube.
10. The resultant mixture was then stored at -20 ^{0}C overnight.
11. The sample tube was centrifuged at 13,000 rpm for 5 minutes.

12. The supernatant was discarded carefully without disturbing the pellet.
13. 70% (v/v) Ethanol was added to the tube and centrifuged at 13,000 rpm for 5 mins.
14. The supernatant was again discarded without disturbing the pellet.
15. The pellet was driedand 50 µl Nuclease free water was then added to the tube such that the pellet was dissolved.
16. The DNA extracted was stored at -20 ^{0}C for further use.

Table 1: Primer Sequences

Primer Name	Primer Sequence
AT1G12030 BSF1	ATTGGGACGATTTATCAGAACAG
AT1G12030 BSR1	AAACCTGTTCTGATAAATCGTCC
AT1G12030 Forward Primer	CACCACTAGTATGGTAGAGATAGGAGGACGA
AT1G12030 Reverse Primer with Stop Codon	CTAAAATATTTGCAACCCCAC
Cas9 Forward Primer	GATCGGCCTCGATATTGGAC
Cas9 Reverse Primer	ATCCCCCTCGATCAGGAAATG
qRT AT1G12030 Forward Primer	TTTGATGCAGGTCTCTGCAAG
qRT AT1G12030 Reverse Primer	CACTTGAGCTAAGACGGAGAG
qRT AT1G62420 Forward Primer	CGTTCGTCGGAGATATTAGTTC
qRT AT1G62420 Forward Primer	ACTTGGCTTAGTATTGAGAGG

Polymerase Chain reaction (50µl)

PCR was performed to amplify a gene of interest and later processed the amplified DNA fragment into different vectors.

Table 2: PCR reaction mixture

Component	Volume in µl
5x High Fidelity Phusion Buffer	10.0
dNTPs	4.0
Forward Primer	2.5
Reverse Primer	2.5
Template DNA (100ng/µl)	2
High Fidelity Phusion Polymerase	0.5
Nuclease Free water	29.5
Total	50 µl

Table 3: PCR reaction condition:

Reason	Temperature	Duration	Number of cycles
Initial Denaturation	98 ^{o}C	3/5 minutes	1
Denaturation	98 ^{o}C	30 seconds	35
Annealing	56 ^{o}C	30 seconds	
Extension	72 ^{o}C	60 seconds	
Final Extension	72 ^{o}C	7 minutes	1
	10 ^{o}C	hold	

The PCR product was later run on 1% agarose gel to verify the amplification

Gel Purification:

1. The agarose gel was viewed under an UV torch and the band of interest was cut using a scalpel blade and taken into a 1.5ml MCT.
2. The weight of the gel was measured using weighing machine and QG buffer was added 3x the mass of the gel (ex: 1g = 3ml).
3. The solution was then heated in a thermomixer at 60 ^{o}C and 800rpm until the gel got dissolved into the QG buffer.
4. The resultant solution was completely transferred to the Gel Spin column and centrifuged for 1 minute at 13000 rpm.
5. The flow through was discarded and 750 µl of PE buffer (Wash Buffer + Ethanol) was added to the column and centrifuged for 1minute at 13,000rpm.
6. The flowthrough was discarded and the column was centrifuged for 1 minute at 13,000 rpm to dry the column.
7. The column was then placed in a new 1.5ml MCT and 50 µl of 65 ^{o}C Nuclease free water was added at the center of the column and incubated for 5 minutes.
8. The column + MCT was centrifuged at 13,000 rpm for 1 minute. The product was flow-through into the MCT and the gel product concentration was checked with the help of spectrophotometer.

PCR purification:

1. PCR product volume was measured and taken into a MCT and PB buffer was added 5x times the volume of PCR product.
2. The solution was mixed by using pipette and was completely transferred to the Gel Spin column and centrifuged for 1 minute at 13000 rpm.
3. The flowthrough was discarded and 750 µl of PE buffer (Wash Buffer + Ethanol) was added to the column and centrifuged for 1minute at 13,000rpm.
4. The flowthrough was discarded and the column was centrifuged for 1 minute at 13,000 rpm to dry the column.
5. The column was then placed in a new 1.5ml MCT and 20 µl of 65 ^{0}C Nuclease free water was pipetted out at the center of the column and incubated for 5 minutes.

6. The column + MCT was centrifuged at 13,000 rpm for 1 minute. The PCR product was flow-through into the MCT and it's concentration was checked with the help of a spectrophotometer.

pENTR/D-TOPO reaction

Table 4: Reaction Mixture

Component	Volume in µl
Insert - gene of interest (Gel of PCR purified product) (50ng)	1
pENTR/D-TOPO (20ng/µl)	0.3
Salt Solution	0.5
Nuclease Free water	1.2

The reaction mixture was incubated at 25^{o}C for 2-3 hours and was used for bacteria transformation.

Bacterial competent cells preparation

1. DH5α bacteria was streaked on a LB agar plate. The plate was incubated at 37^{o}C overnight.
2. A single colony of DH5α was picked from the plate and inoculated in 5ml LB solution (primary culture) kept in 37 ^{o}C at 220 rpm overnight.
3. 500 µl of primary culture was used to inoculate 50ml S.O.B and the solution was kept in 37 ^{o}C at 220rpm till the $O.D_{600}$ reached 0.5.
4. The culture was then kept on ice for 10 minutes and was transferred to 50ml eppendorf centrifuge tubes.
5. The tubes were centrifuged at 4 ^{o}C and 3,500 rpm for 10 minutes
6. The resultant supernatant was discarded and the pellet was dissolved in 25ml TB1 buffer.
7. The mixture was kept on ice for 10 minutes.
8. The pellet was dissolved in 4ml of TB2 buffer and 140 µl DMSO was added to the mixture.
9. The tube was kept on ice for 15 minutes and 140 µl DMSO was added once again to the mixture.

10. 50 µl of DH5α competent cells were aliquoted into 1.5ml vials and stored in -80 ºC for future use.

Bacterial Transformation:

1. 50 µl of DH5 α competent cells were taken in a 1.5ml MCT and placed on ice.
2. 50 µl of TB3 buffer and 100-200ng plasmid DNA or reaction mixture was added to the MCT.
3. The resultant mixture was placed in ice for 15 minutes.
4. Heat shock was given in a water bath at 42ºC for 1 minute and the MCT was immediately transferred back to ice for 5 minutes.
5. 1ml of LB solution was added to the mixture and then it was incubated for 1 hour at 37 ºC at 220rpm.
6. 200 µl of the culture from the MCT mixture was taken and spread on the selection LB agar plate.
7. The plate was incubated overnight at 37 ºC.

Colony Polymerase Chain Reaction

The colonies grown on the bacteria plate were numbered. The PCR tubes were labelled accordingly and 10µl Nuclease Free water (NFH_20) was added in it. The colonies were picked using autoclaved toothpicks and dissolved in this NFH_20 in the laminar. The reaction master mix was prepared and 5 µl of it was added to all PCR tubes.

Table 5: Colony PCR mixture

Component	Volume in µl
Dream Green Taq Master mix	3
Vector specific Forward Primer	0.75
Gene of interest specific Reverse Primer	0.75
Total	5 µl

Table 6: Colony PCR Reaction condition:

Reason	Temperature	Duration	Number of cycles
Initial Denaturation	95 °C	3 minutes	1
Denaturation	95 °C	30 seconds	25
Annealing	55 °C (primer specific)	30 seconds	
Extension	72 °C	30 to 150 seconds (gene specific)	
Final Extension	72 °C	7 minutes	1
	10 °C	hold	

Plasmid Isolation:

Plasmid extraction was done with the help of QIAGEN Plasmid extraction kit

1. 5ml of LB was taken in an inoculation tube and selection antibiotic was also added to the LB.
2. The colony of interest was picked using a microtip and then colony was inoculated into the LB media and the bacterial culture was grown for 14 hours in 37 °C at 220rpm.
3. 2ml of the grown bacterial culture was taken in 2ml MCT and then centrifuged for 1min at 13,000 rpm.
4. The supernatant was discarded and 2ml of culture was again taken into the same MCT and centrifuged for 1min at 13,000 rpm.
5. 250 µl of P1 resuspension buffer was added to the MCT and vortexed such that the pellet formed earlier was dissolved into the buffer.
6. 250 µl of P2 lysis buffer was added to the solution and the MCT was inverted slowly back and forth for 5-7 times such that the added buffer is mixed properly.
7. 350 µl of N3 neutralization buffer was added to the lysed solution and the MCT was inverted slowly back and forth for 5-7 times.
8. The MCT was then centrifuged for 10 minutes at 13,000 rpm.
9. The clear supernatant was transferred to a spin column provided in the QIAGEN kit.
10. The column was centrifuged for 1 minute at 13,000 rpm and the flowthrough was discarded.

11. 750 µl of PE buffer (Wash Buffer + Ethanol) was added to the column and centrifuged for 1minute at 13,000 rpm.
12. The flowthrough was discarded and the column was centrifuged for 1 minute at 13,000 rpm to dry the column.
13. The column was then placed in a new 1.5ml MCT and 50 µl of Nuclease free water which was kept in 65 ^{o}C was pipetted at the center of the column and incubated for 5 minutes.
14. The column + MCT was centrifuged at 13,000 rpm for 1 minute. The plasmid was flow-through into the MCT and the plasmid concentration was checked with the help of a spectrophotometer.

Table 7: Digestion reaction

Component	Volume in µl
Plasmid (200ng/ µl)	1
Enzyme	0.2
10x Cutsmart Buffer	1
Nuclease Free water	7.8
Total	10 µl

*for each extra enzyme add 0.2 µl of that enzyme and reduce the volume of enzyme from the Nuclease Free water volume

The reaction mixture was prepared in a 1.5ml MCT and labelled properly. The MCT was kept for incubation in a water bath at 37 ^{o}C for 1 hour.

Table 8: LR reaction mixture

Component	
LR Clonase II enzyme	0.3
TE Buffer	0.3
Entry Vector (100ng/µl)	2
Destination Vector (200ng/µl)	0.3
Total	3µl

The reaction mixture was incubated at 25 ^{o}C for 2-3 hours and was used for bacterial transformation.

Agrobacterium electro competent cell preparation

1. Agarobacterium GV3101 strain was streaked on Rifampicin, Gentamicin, Tetracycline (RGT) selection LB agar plate and incubated at 28 ^{o}C for 2 days.
2. A single colony was picked and inoculated in 5ml LB solution (primary culture) with RGT (Rifampicin - 10µl, Gentamicin - 5µl, Tetracycline - 2µl) as selection antibiotics for 36 hours at 28 ^{o}C and 220 rpm.
3. 500 µl of primary culture was added to the 500ml of LB solution (1% w/v Tryptone, 0.5% Yeast Extract, 0.1% NaCl) and before this 1 ml of the LB solution was taken aside for setting blank.
4. The culture was kept on a shaker at 28 ^{o}C and 220rpm till the $O.D_{600}$ reached 0.5-0.6.
5. 500 ml mixture was divided in two flat bottom bottles (250ml each) and centrifuged at 4^{o}C and 3,500 rpm for 10 minutes.
6. The supernatant was discarded and chilled 200 ml 10% (v/v) Glycerol was added in each of the bottle and centrifuged at 4 ^{o}C at 3,500 rpm for 20 minutes.
7. The supernatant was discarded and chilled 100ml of 10% (v/v) glycerol was added and the pellet was washed in the same procedure as in the above step.
8. Chilled 50ml of 10% (v/v) glycerol was added and the same above procedure was followed.
9. The supernatant was discarded and chilled 50ml of 10% (v/v) glycerol was added and the pellet was washed in the same procedure as in the above step.
10. The supernatant was discarded and chilled 25ml of 10% (v/v) glycerol was added and the same above procedure was followed.
11. The supernatant was discarded and 4ml of 10% (v/v) glycerol was added and 100 µl cells were aliquoted in vials and stored in -80 ^{o}C.

Agrobacterium transformation

1. 100 µl of electrocompetent cells were taken in a 1.5 ml MCT and placed on ice. 100ng of plasmid was added to the cells.
2. The mixture was taken into a cuvette (which was washed with ethanol and later dried).
3. Electric pulse was given to the cells using Pulser X cell Electroporation system, 200 µl of LB solution was added to the cuvette with cell mixture.
4. The cells and the LB solution were mixed properly and the resultant was added to a new 1.5ml MCT containing 800 µl LB solution.
8. The culture was incubated for 90 minutes in 30^{o}C at 220 rpm.
9. 200 µl of the bacteria from the culture was taken and spread on the selection LB agar plate. The plate was incubated for 2 days at 28 ^{o}C

Floral Dipping

1. A single colony of agrobacterium was inoculated in 5ml of LB with appropriate antibiotics and incubated in 28 ^{o}C at 220 rpm for 48 hours.
2. 250 µl of primary culture was added to 250 ml LB media with antibiotics for the secondary culture.
3. The secondary culture was incubated for 18 hours in28 ^{o}C at 220 rpm.
4. The culture was then taken into flat bottom bottles and centrifuged at 3,500 rpm for 25 minutes.
5. The supernatant was discarded and the pellet was dissolved in a solution of 25g (w/v) 5% sucrose in 500ml double distilled water.
6. 100µl (v/v 0.02%) silwet was added to the solution.
7. The grown plants whose siliques were already cut were inverted and dipped in the solution for 10-15 seconds.
8. The plants were kept for drying in dark by laying them horizontally for 12 hours.

CRISPR-Cas9

Crispr/cas9 technique was used to make knock out mutant of our gene of interest, amplified sgRNA fragment was cloned into a Cas9 expressing vector pHEE401E using golden gate cloning. The

vector was transformed into DH5α *(E. coli)* bacteria and confirmed the clone through digestion and sequencing. The plasmid was transformed into agrobacterium. The WT (col-0) plants were infected with agrobacterium to introduce the cas9 expressing vector into the plants. The T0 generation seeds were collected and transformants were selected on MS + selection media. T1 seedlings were then transferred from selection plates to soil (solarite + perlite + compost - 3:1:1 respectively) and Cas9 +ve plants were selected through genotyping and identified the plants with frame shift/ non-silent mutation through sequencing of the DNA. The T2 generation plants were grown and genotyping was performed to identify Cas9 –ve plants and their seeds were collected. Later the desired mutation was verified by sequencing the DNA fragment of the gene [11].

Designing Primer for single target CRISPR

1. Target guide RNA sequence was first identified using CHOP CHOP website (https://chopchop.cbu.uib.no/), where gene ID was entered with *Arabidopsis thaliana* as organism name. “Using CRISPR/Cas9” was selected and “for knock out” option was chosen.
2. “Find Target sites” was clicked to initiate the search.
3. The target sequence with the highest efficiency was selected.
4. The reverse complement of the target was obtained from the bioinformatics website (https://www.bioinformatics.org/sms/rev_comp.html) as the target sequence was pasted and submitted.
5. The forward primer was designed by adding ATTG at 5’ end of Target sequence.
6. The reverse primer was designed by adding AAAC was added to the 5’ end of the Reverse complement of the Target sequence.

Amplification of sgRNA

Oligomerization of sgRNA for single target mutant:

Table 9: sgRNA oligomerization Reaction mixture

Component	Volume in µl
Forward Primer (100µM)	1.0
Reverse Primer (100µM)	1.0
Annealing Buffer (10x)	5
Nuclease Free water	43
Total	50

Reaction Condition

The PCR machine was set to 95 ^{o}C and then the temperature was ramped down to 25 ^{o}C at 5 ^{o}C/min.

10x Annealing Buffer

Chemical	Concentration	10x Concentration
Tris Chloride (pH 8)	10mM	100mM
Nacl	50mM	500mM
EDTA	1mM	10mM

Golden Gate reaction

Golden gate reaction was performed to integrate the annealed sgRNA into cas9 expressing pHEE401E vector.

Table 10: Golden gate reaction composition

Reaction Components	Volume in µl
Annealed sgRNA	1.5
pHEE401E vector	1.5
Cutsmart Buffer (NEB) 10x	1.5
Bsa1 Enzyme	1.0
T4 Ligase (NEB)	0.75
T4 Ligase Buffer 10x	1.5
Nuclease Free water	7.25
Total	15 µl

Table 11: Golden gate reaction condition

Temperature	Duration	Number of Cycles
37 ^{o}C	180 minutes	1
50 ^{o}C	5 minutes	1
80 ^{o}C	10 minutes	1

Genotyping for checking Cas9

To identify the presence of Cas9 in the transformants through genotyping, the DNA extracted from the plants was used as template, Cas9 specific forward and reverse primers were used for PCR. The presence and absence of Cas9 was checked through running the PCR product on the 1% agarose gel, if there was a band observed then it was Cas9 +ve otherwise it was Cas –ve.

Table 12: Genotypying PCR reaction composition

Component	Volume in µl
Dream Green Taq Master mix	3
Template DNA (extracted from plants) 100ng	2
Cas9 Forward Primer	0.75
Cas9 Reverse Primer	0.75
Nuclease Free water	8.5
Total	15 µl

Table 13: Genotypying PCR reaction condition

Stage	Temperature	Duration	Number of cycles
Initial Denaturation	95 ^{o}C	5 minutes	1
Denaturation	95 ^{o}C	30 seconds	25
Annealing	55 ^{o}C	30 seconds	
Extension	72 ^{o}C	30 seconds	
Final Extension	72 ^{o}C	7 minutes	1
	10 ^{o}C	hold	

RNA isolation

The Plant RNeasy kit (Qiagen) was used to extract total RNA using roots of *Arabidopsis thaliana* grown on iron sufficient media for seven days and then transferred to iron sufficient and iron deficient media for three days.

1) The RNA was isolated by collecting around 100 mg of plant roots in 1.5ml MCT and crushing properly using liquid nitrogen.
2) To this 100 mg crushed tissue, 450 µl of buffer RLT was added and vortexed.
3) The lysate was transferred to a QIAshredder spin column and centrifuged at 13,000rpm for two minutes and the supernatant of the flow-through was transferred to a new microcentrifuge tube.
4) Added 0.5 volume ethanol to the supernatant and mixed by pipetting.

5) The sample was transferred to an RNeasy Mini spin column (pink), centrifuged for 15 seconds at 13,000 rpm and flow-through was discarded.
6) Added 700 µl buffer RW1 to the column, centrifuged for 15 seconds at 13,000 rpm and flow-through was discarded.
7) Added 500 µl buffer RPE to the column, centrifuged for 15 seconds at 13,000 rpm and flow-through was discarded.
8) Added 500 µl buffer RPE to the column, centrifuged for 2 minutes at 13,000 rpm and flow-through was discarded.
9) The column was placed in a new 2ml collection tube and centrifuged for 1 minute for drying the membrane.
10) The column was placed in a new 1.5 ml tube. Added 30 µl RNase-free water to the spin column membrane and centrifuged at 13,000 rpm to elute the RNA.

Removal of genomic DNA

1) To remove genomic DNA from the RNA the following components were added to an RNase-free tube:

RNA	1
10X Reaction Buffer with $MgCl_2$	1 µl
DNase I, RNase-free	1µl
Water, nuclease-free	to 10 µl

2) Incubated for 30 minutes at 37 ^{o}C.
3) Added 1 µl 50 mM EDTA and incubated for 10 minutes at 65^{o}C.
4) The treated RNA sample was used as a template for cDNA synthesis.

cDNA synthesis

1) The template RNA (0.1ng – 5 µg) was added to a sterile, nuclease-free tube on ice.
2) 1 µl of Random Hexamer primer was added.
3) Nuclease-free water was added to this reaction mixture such that final volume becomes 12 µl.
4) The sample was incubated for 5 minutes at 65°C and then placed on ice.
5) To the sample following components were added in the mentioned order:

5X Reaction Buffer	4 µl
RiboLock RNase Inhibitor	1 µl
10mM dNTP Mix	2 µl
RevertAid M-MuLV RT	1 µl
Total volume	20 µl

6) The sample was then mixed gently, centrifuged briefly, incubated at 25°C for 5 min and then finally incubated at 42°C for 60 min.
7) The reaction was terminated by heating for 5 minutes at 70°C.

Quantitative Real Time PCR (qRT-PCR)

qRT-PCR reaction was performed using a LightCycler 480 II (Roche) and KAPA SYBR FAST UNI (FROM SIGMA). β-Tubulin was used as a reference gene. Expression levels were calculated using the comparative threshold cycle method.

qRT-PCR reaction components:

Template (50ng/ µl) - 2 µl

Syber green - 10 µl

Forward primer - 1 µl

Reverse primer - 1 µl

Nuclease-free water - 6 µl

Total - 20 µl

Table 14: **qRT-PCR reaction conditions:**

Stage	Temperature	Duration	Number of cycles
Initialization	95 °C	15 minutes	1
Amplification	95 °C	15 seconds	45
	55 °C	30 seconds	
	72 °C	30 seconds	
	80 °C	15 seconds	
Melting curve	95 °C	20 seconds	1
	55 °C	1 minute	
	95 °C		

Yeast Two-Hybrid (Y2H)

For performing Y2H, the coding sequence of AT1G12030 was amplified, cloned into pENTR/D TOPO and sequenced. Then, LR cloning was used to fuse AT1G12030 pENTR/D TOPO to the pDEST/pGBKT7 BD to generate BD-AT1G12030. The BD-AT1G12030 construct was then co-transformed with each AD construct of bHLH 34, 38, 39, 100, 101, 104, 105, PYE and FIT (were already available in the lab) in PJ697a and plated on -2 (-Leu, -Trp) selection media to get co-tranformed colonies. After 2 to 3 days, colonies were obtained which were dissolved in 100µl autoclaved millipore water. $O.D._{600}$ was measured, adjusted to 2.0 and 5.0 in a 96-well plate and spotting was done on -3(-Leu, -Trp, -His) and -4(-Leu, -Trp, -His, -Ade) selection media to identify interacting proteins.

Preparation of yeast competent cells (PJ697a)

1) The PJ697a yeast strain was streaked on a YPAD plate and allowed to grow for 2 days in 30 ^{o}C.
2) A single colony was inoculated in 20 ml YAPD media and kept for 12 hours in 30 ^{o}C with shaking.
3) The secondary culture was set up by adding a certain volume of primary culture so that starting O.D.$_{600}$ of secondary culture becomes 0.200.
4) The secondary culture was incubated at 30 C until the O.D.$_{600}$ reached 0.6-1.0.
5) The cells were harvested by centrifugation of the secondary culture at 4000rpm for 5 minutes.
6) The pellet obtained was washed with 50ml autoclaved Millipore water and centrifuged at 4000 rpm for 5 minutes.
7) The pellet was resuspended in 1/10th volume SORB and centrifuged again at 4000 rpm for 5 minutes.
8) The pellet was dissolved in 360µl/50ml culture SORB + 40µl salmon sperm DNA.
9) The 20µl cells were aliquoted in 1.5 ml tubes and stored at -80^{o}C.

Yeast transformation

1) A 1.5 ml MCT was taken and to this 1µl of plasmid to be transformed was added.
2) An aliquot of 20µl yeast competent cells was taken out from -80^{o}C, thawed on ice and added to the MCT.
3) To this, six times volume 40% polyethylene glycol (PEG) was added and mixed well.
4) It was kept for incubation at 30^{o}C for 30 minutes.
5) Heat shock was given by keeping the MCT at 42^{o}C for 30 minutes.
6) The MCT was kept in ice for 10 minutes and then centrifuged at 4000 rpm for 5 minutes.
7) The supernatant was discarded and pellet was dissolved in 1ml sterile water and plated on selection plate.

2.4 Plant phenotyping

Root length measurement

WT (Col-0), mutant and overexpression lines of AT1G12030 were grown on ½ MS and ½ -Fe (Fz-100µM) plates for 14 days. After 14 days, plates were scanned using Epson Perfection V600 with 1200dpi resolution and root length was measured using ImageJ 1.52a software (National Institutes of Health).

Chlorophyll Content Measurement

For chlorophyll content measurement shoot part of five seedlings grown on Fe- sufficient and Fe-deficient media for 14 days was collected, weighed and placed in 1ml 80% acetone for 24 hours respectively. After 24 hours, the absorbance of A645 and A663 was measured with the help of a spectrophotometer and chlorophyll content was calculated by using the formula:

$$(mg/g) = \frac{(20.3 \times A_{645} + 8.04 \times A_{663}) \times V}{W \times 10^3}$$

In this formula, V is the volume in ml and W denotes the weight of shoot part in gram [12].

Perl's Staining for iron content

For iron staining, Perl's staining solution (equal volume of 1% (v/v) HCl and 1% K-ferrocyanide) was prepared and kept in 37^{o}C. 5-day old seedlings grown on MS media were taken and vacuum infiltration was done with Perl's staining solution for 5 minutes. Seedlings were then washed with water three times, observed and photographed with ECLIPSE Ni U (Adapted from [13]).

Root ferric-chelate reductase (FCR) activity

The root FCR activity was determined by using whole roots from seedlings grown on iron-sufficient media for 7 days and transferred to iron-sufficient and iron-deficient medium for 3 days. The roots were collected from 10 seedlings and put in 700 µl assay solution comprising of 0.1 mM Fe (III)-EDTA and 0.3 mM ferrozine in distilled water. After 30 minutes of incubation in dark, O.D. of the samples is measured using spectrophotometer at 562 nm. The FCR activity is determined using the formula:

$$\mu M\ Fe(II)/g\ root\ FW/hr = \frac{(A/28.6) \times V}{Root\ FW \times 2}$$

In this formula, V is volume in µl and Root FW is the root fresh weight in gram [14].

Chapter 3

Results:

3.1 *AT1G12030* is Coexpressed with an Iron deficiency Responsive Transcription Factor, *bHLH101*

To know about *AT1G12030,* we used ATTED-II Network Drawer which on the basis of coexpressed genes determined from microarray analyses and predicted *cis*-elements, predicts regulatory networks [15]. From this analysis, we found that *AT1G12030* is tightly coexpressed with bHLH101 which is known to be involved in iron-deficiency responsive signaling pathway (Figure 3.). This suggests that *AT1G12030* might also be involved in iron deficiency responsive signalling pathway.

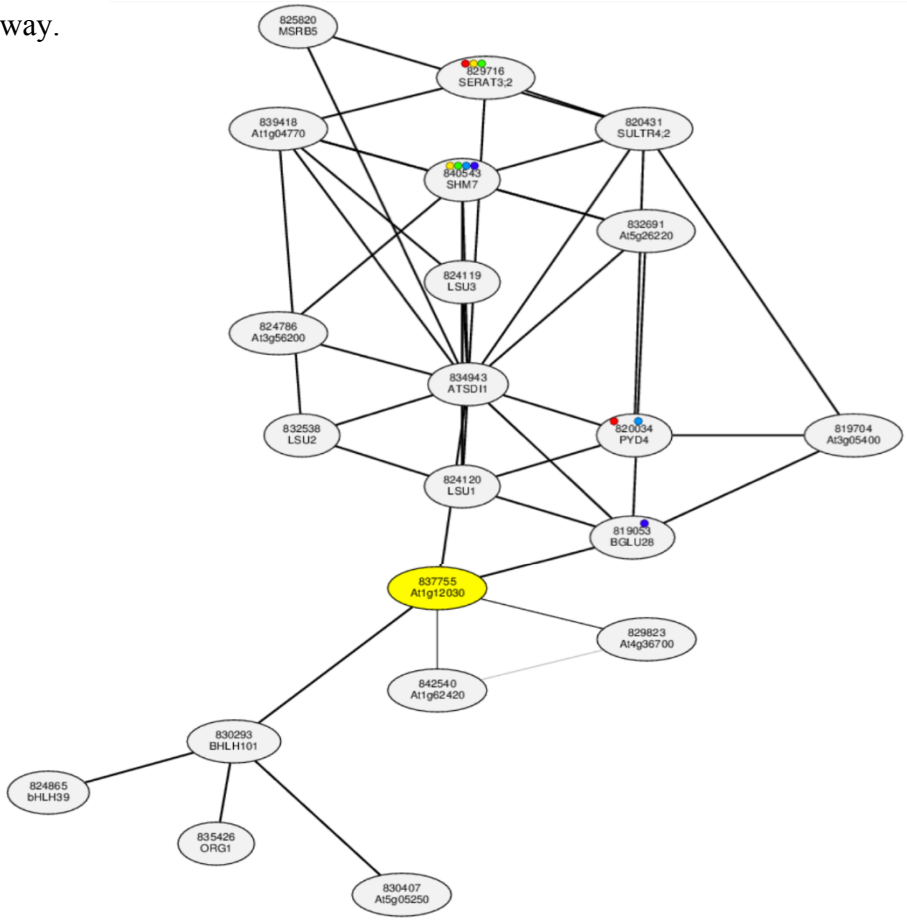


Figure 3: Coexpression network analysis representing the genes co-expressing with *AT1G12030*

3.2 *AT1G12030* expression is transcriptionally induced under iron deficiency

As the publicly available microarray data suggests that *AT1G12030* expression is induced under iron deficiency, we also confirmed that it's expression is induced under iron deficiency by performing qRT-PCR. The roots from WT (Col-0) seedlings grown on iron sufficient media (+Fe) for seven days and then transferred to both +Fe and -Fe (300µM Fz) for three days are used for extracting RNA and making cDNA for the qRT-PCR experiment. The qRT-PCR results showed that *AT1G12030* is strongly (134.4-fold) upregulated under deficiency (Fig 4) suggesting its potential role in iron homeostasis pathway.

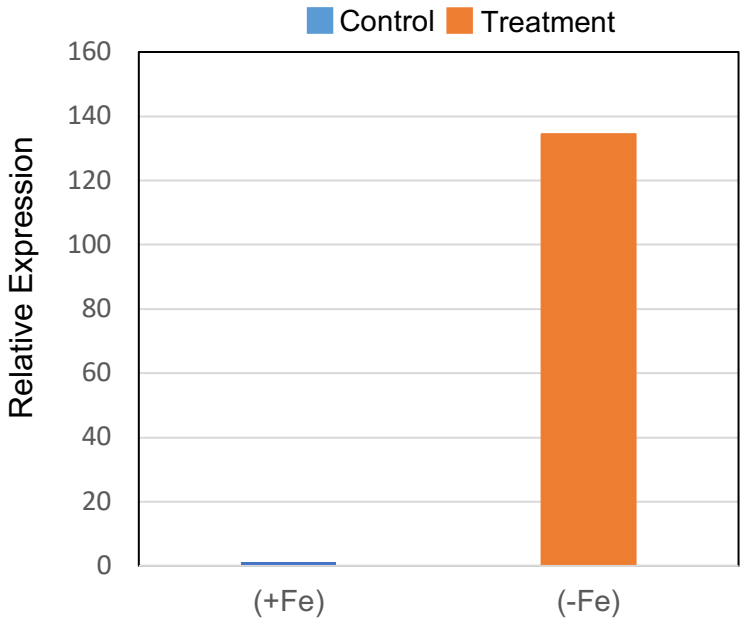


Figure 4: qRT PCR of AT1G12030 under +Fe and –Fe, WT (Col-0) plants were grown on MS for 7 days and transferred to MS and –Fe (300µM Fz) for 72 hours.

3.3 ATIG12030 knockout mutant generated using CRISPR/Cas9.

The knockout mutant of *AT1G12030* was made using CRISPR/Cas9 gene editing method, where the first exon of the *AT1G12030* was targeted and then mutated. The DNA of the mutated plant was extracted and nucleotide sequence was obtained with the help of sanger sequencing. There

was a nucleotide (Adenine) base addition in the mutant plant. This resulted in a frame shit and leading to two stop codons (Figure 5.c) in the exon region.

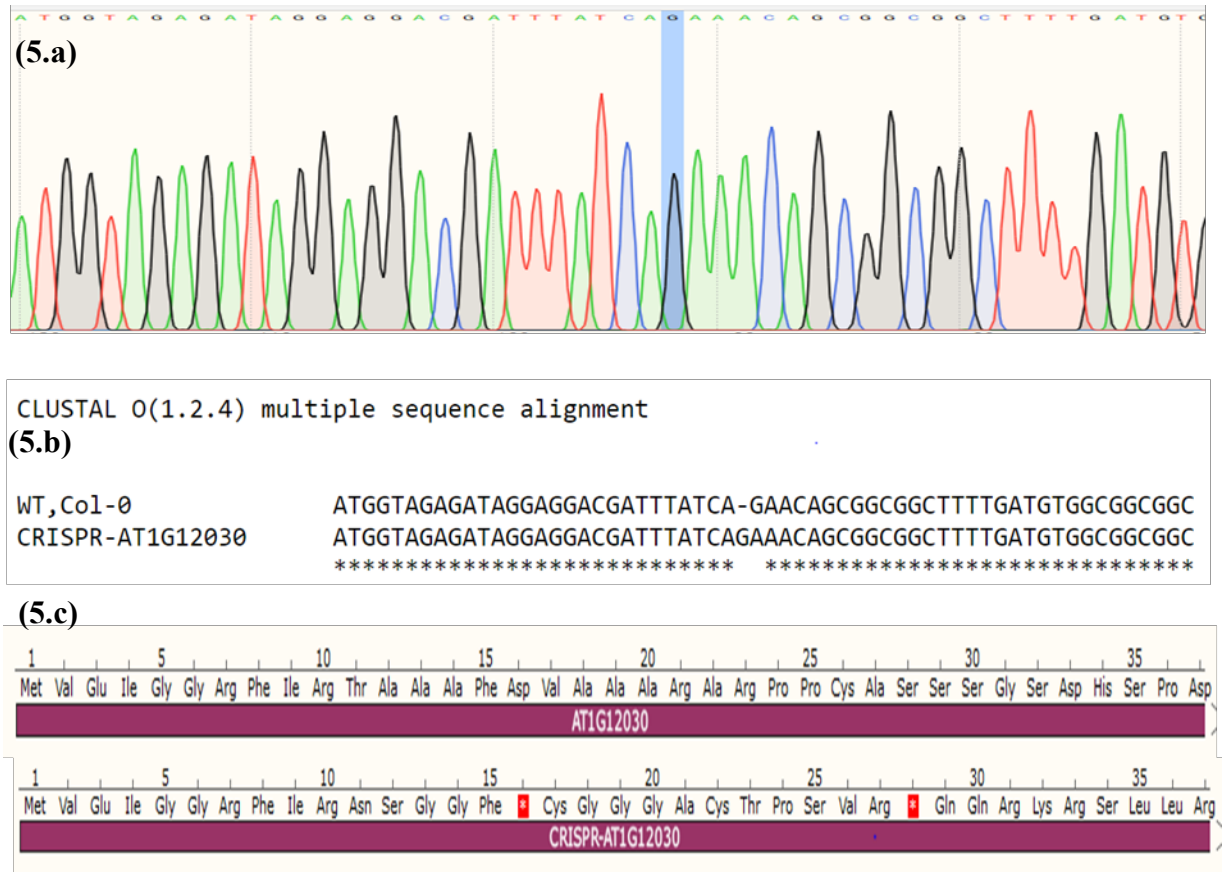
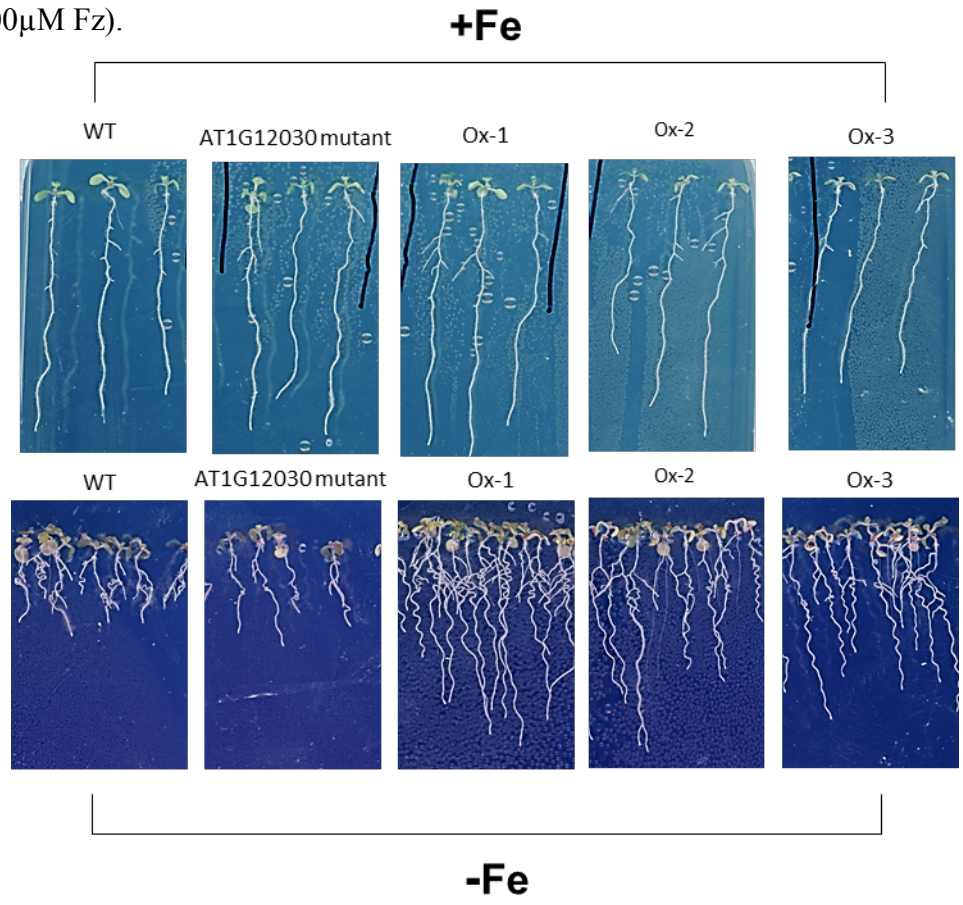


Figure 5: a) Sanger sequencing of selected region of CRISPR-AT1G12030 b) sequence alignment of AT1G12030 and CRISPR-AT1G12030 c) pictorial representation of amino acids indicating two stop codons.

3.4 Overexpression of *AT1G12030* gene shows increased root length under Iron deficiency conditions*

The WT (Col-0), Crispr-AT1G12030, AT1G12030 Ox-1, AT1G12030 Ox-2, AT1G12030 Ox-3 (over expression seeds were already available in the lab which were made by Jagannath Swain) were grown in the ½ MS and 100µM FerroZine plates. The root length measured after 7 days of seeds growth in ½ MS and 14 days after seeds growth in 100µM Ferrozine plates. In the control

conditions of plants grown on MS plates we saw no significant difference in the root length. Interestingly we found no significant root length difference between WT (Col-0) and Crispr-AT1G12030 in absence of iron but we saw a clear increase in the root length of the over-expression plants compared to Wt (col-0) and Crispr-AT1G12030 in the iron deficiency condition (100µM Fz).



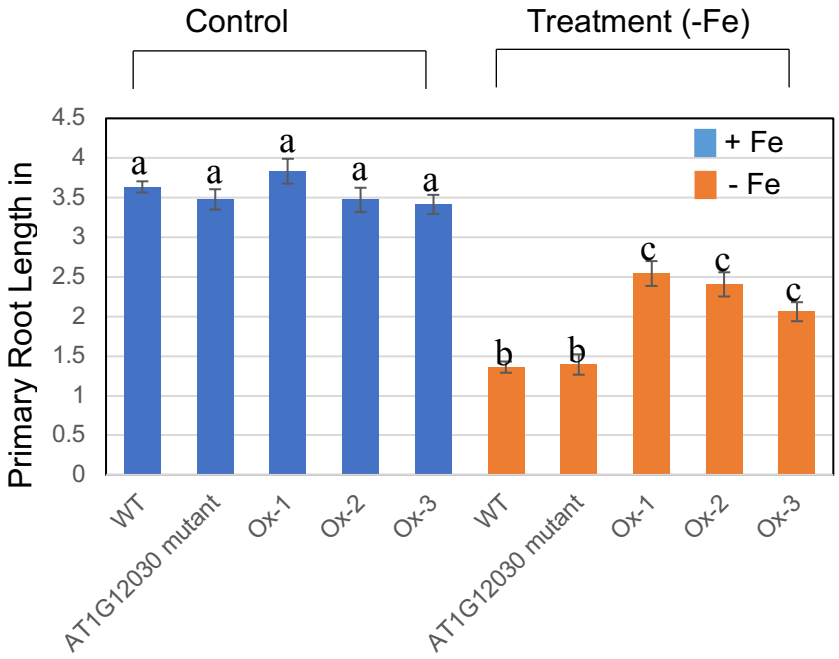


Figure 6: a) Phenotype of WT (col-0), mutant and overexpression of *AT1G12030* on iron sufficient and deficient condition. b) Primary root length analysis with means plotted on bar graph with error bars showing ±SE (n>15 seedlings of each type), same letter indicates that they are not significantly different from each other and vice versa, according to one-way ANOVA followed by post hoc Turkey Test, P<0.01. * Preliminary Data

3.5 Perls' Blue staining for Fe content measurement in roots of WT, *AT1G12030* mutant and overexpression lines

As the overexpression lines showed improved root growth compared to the WT plants under iron deficiency conditions. To see whether it also affects the iron content or not, we checked the iron content of 4 days old WT, *AT1G12030* mutant and *AT1G12030* overexpression lines grown on iron-sufficient media by performing perl's blue staining and we found that all three *AT1G12030* overexpression lines (Ox-1, Ox-2 and Ox-3) have more iron content as compared to the wild type plants while *AT1G12030* mutant showed similar iron content levels compared to the WT plants.

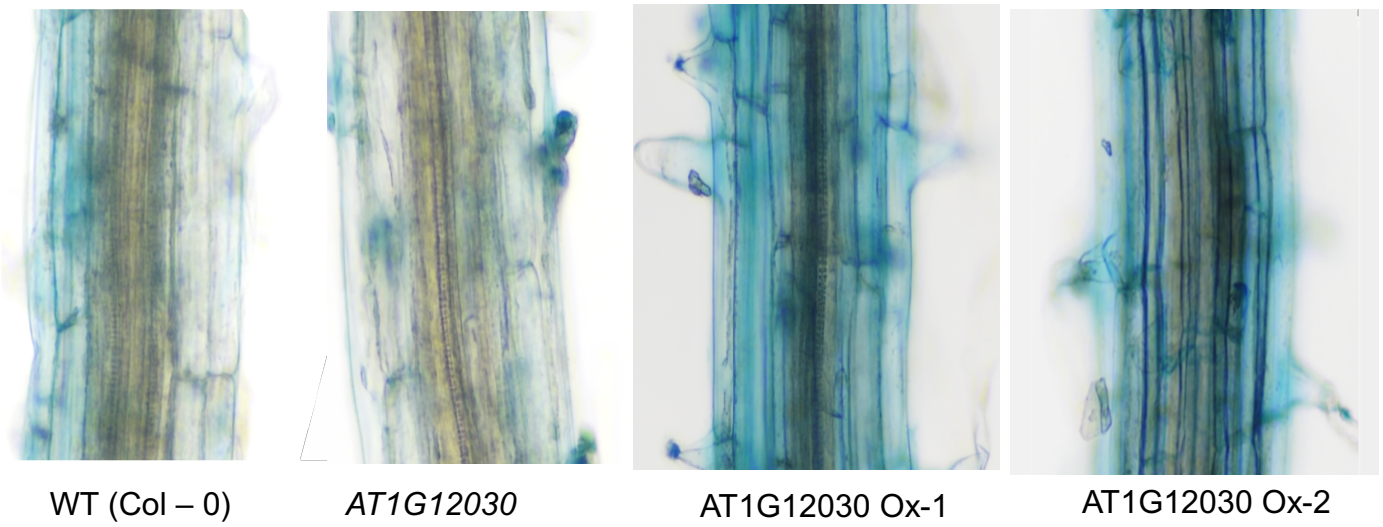


Figure 7: Iron detection by Perls' staining in WT (Col-0), mutant and overexpression plants.

3.6 Chlorophyll content measurement of WT, *AT1G12030* mutant and overexpression lines

As iron is required for chlorophyll biosynthesis and we observed that *AT1G12030* overexpression lines have more iron content than WT plants as by Perl's staining so we wanted to check whether it has any effect on chlorophyll content. For this, the chlorophyll content of WT, *AT1G12030* mutant and overexpression lines grown on MS and -Fe (100 µM Fz) grown for 14 days was measured and it was found that in case of WT (Col-0), the chlorophyll content decreases when grown on -Fe (100 µM Fz) media as compared to MS media which is a known phenotype, similar trend was observed for *AT1G12030* mutant and overexpression lines. There was no significant difference observed between the chlorophyll content of WT, *AT1G12030* mutant and overexpression lines under both +Fe and -Fe (100 µM Fz) conditions.

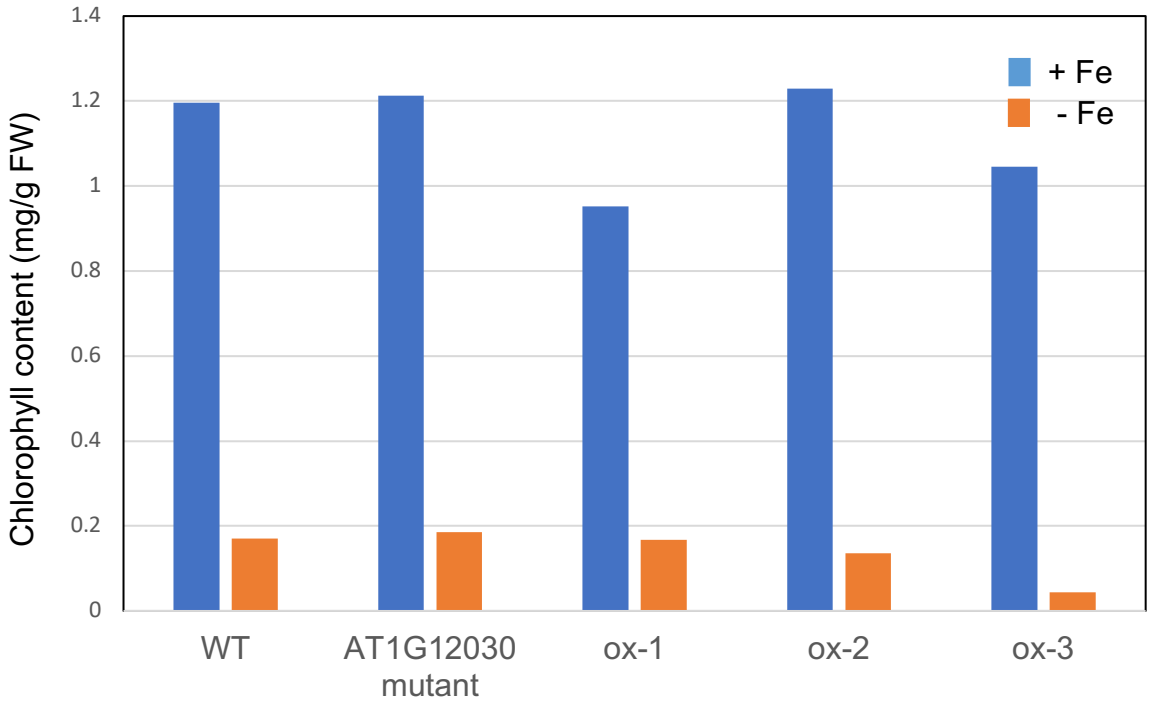


Figure 8: Chlorophyll content of the wild type (WT), mutant and overexpression of AT1G12030

3.7 Ferric-chelate reductase activity measurement of WT, *AT1G12030* mutant and overexpression lines.

To determine whether *AT1G12030* overexpression has any impact on iron deficiency-induced iron reductase activity, we grew WT, *AT1G12030* mutant and overexpression seedlings on ½ MS media for seven days and then transferred to ½ MS and ½ -Fe for 3 days and measured FCR activity. It was observed that in case of WT (Col-0) there is decrease in FCR activity when transferred from ½ MS to ½ -Fe (300 µM Fz) media, similar pattern was observed for *AT1G12030* mutant and overexpression lines and there was no significant difference seen between WT, *AT1G12030* mutant and overexpression lines.

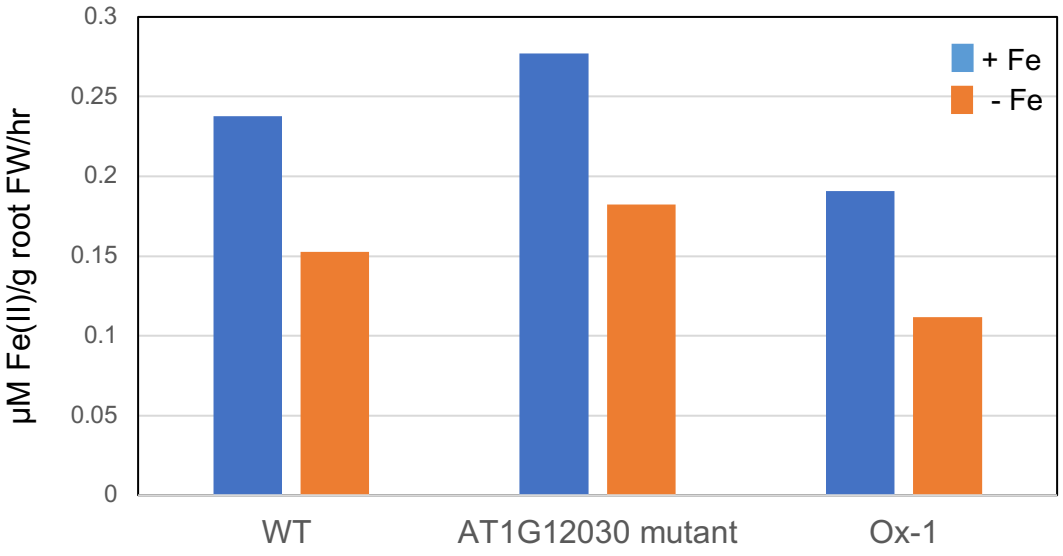


Figure 9: Ferric-chelate reductase activity of the wild type (WT Col-0), *AT1G12030* and AT1G12030-Ox-1 were grown for 1 week under control conditions and transferred to Fe-sufficient or Fe-deficient medium for 3 d. Roots were then collected for FCR activity measurement.

3.8 *AT1G62420* shares a similar sequence with *AT1G12030*.

We did not observe significant differences between the root length of the knockout mutant and WT (Col-0) but there was an increase in root length in the overexpression lines. Similarly, Perls

staining showed the roots of WT (Col-0) and the AT1G12030 mutant has similar iron content whereas the over expression plants showed significantly higher iron content indicating that AT1G12030 is involved in iron homeostasis. Since the knockout mutant did not show any phenotype we hypothesized that there might be another gene which might play a similar role as AT1G12030. We performed protein blast using TAIR blast database (https://www.arabidopsis.org/Blast/) and found out that AT1G62420 shares significantly similarity with AT1G12030 protein sequence suggesting that AT1G62420 might play a similar function in iron homeostasis.

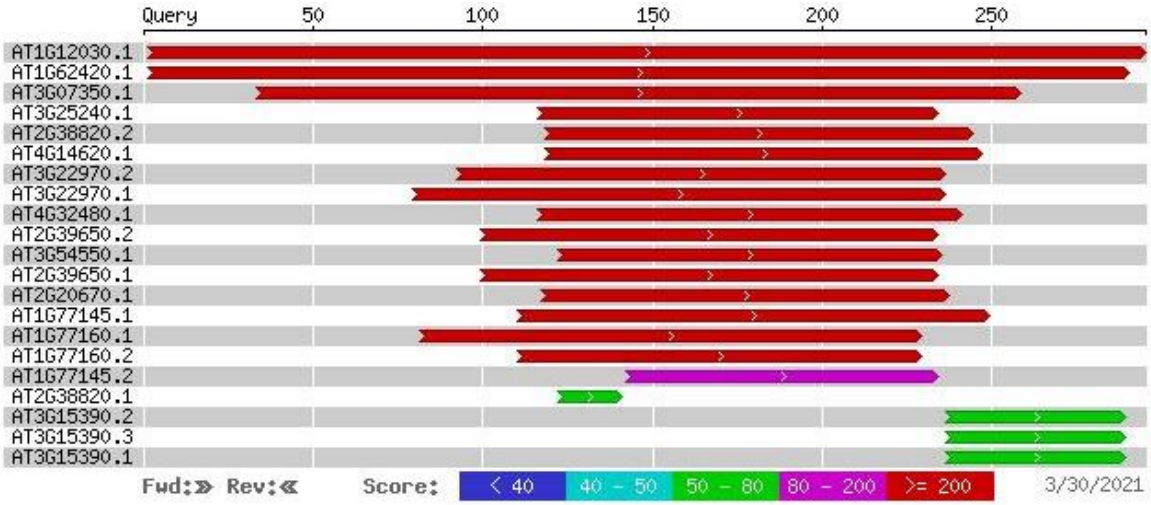


Figure 10: A graphical representation of BLASTP search results for *AT1G12030* .

3.9 *AT1G62420* expression is transcriptionally induced under iron deficiency

To further check if the gene *AT1G62420* also gets upregulated under iron deficiency condition we performed qRT PCR and found that that *AT1G62420* expression was increased by 151.299 fold under –Fe condition. This data suggests that *AT1G62420* might also be involved in iron homeostatis.

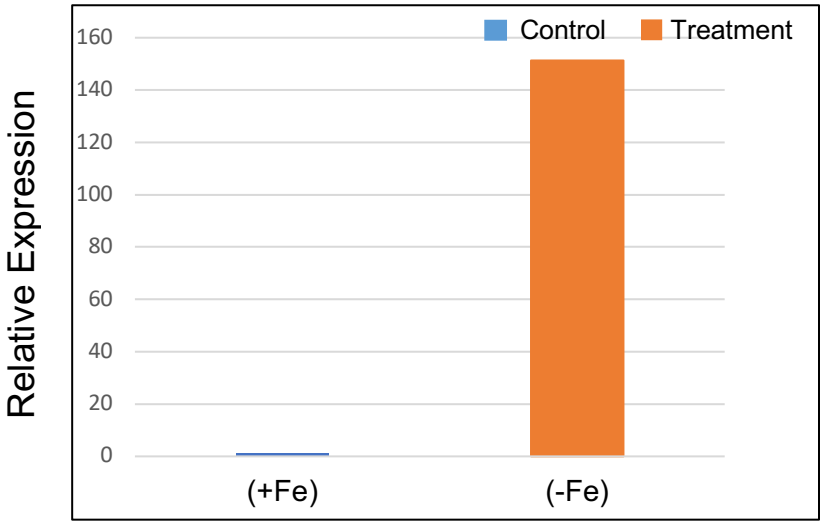


Figure 11: qRT PCR of *AT1G62420* under +Fe and –Fe, WT (Col-0) plants were grown on MS for 7 days and transferred to MS and –Fe (300µM Fz) for 72 hours.

3.10 *AT1G12030* interacts with other transcription factors involved in iron homeostasis

As we showed that bHLH101 and bHLH39 are coregulated with AT1G12030. Therefore, it is possible that AT1G12030 might interact with bHLH101, bHLH39 or other TFs involved in iron homeostasis. So, in order to check this, we performed Y2H using AT1G12030 as bait and other bHLH TFs as prey. Empty AD cotransformed with AT1G12030 BD was used as a negative control and HY5 AD cotransformed with HY5 BD was used as a positive control because HY5 is known to form homodimer. We found that AT1G12030 interacts with bHLH34, bHLH38, bHLH100, PYE as well as with itself.

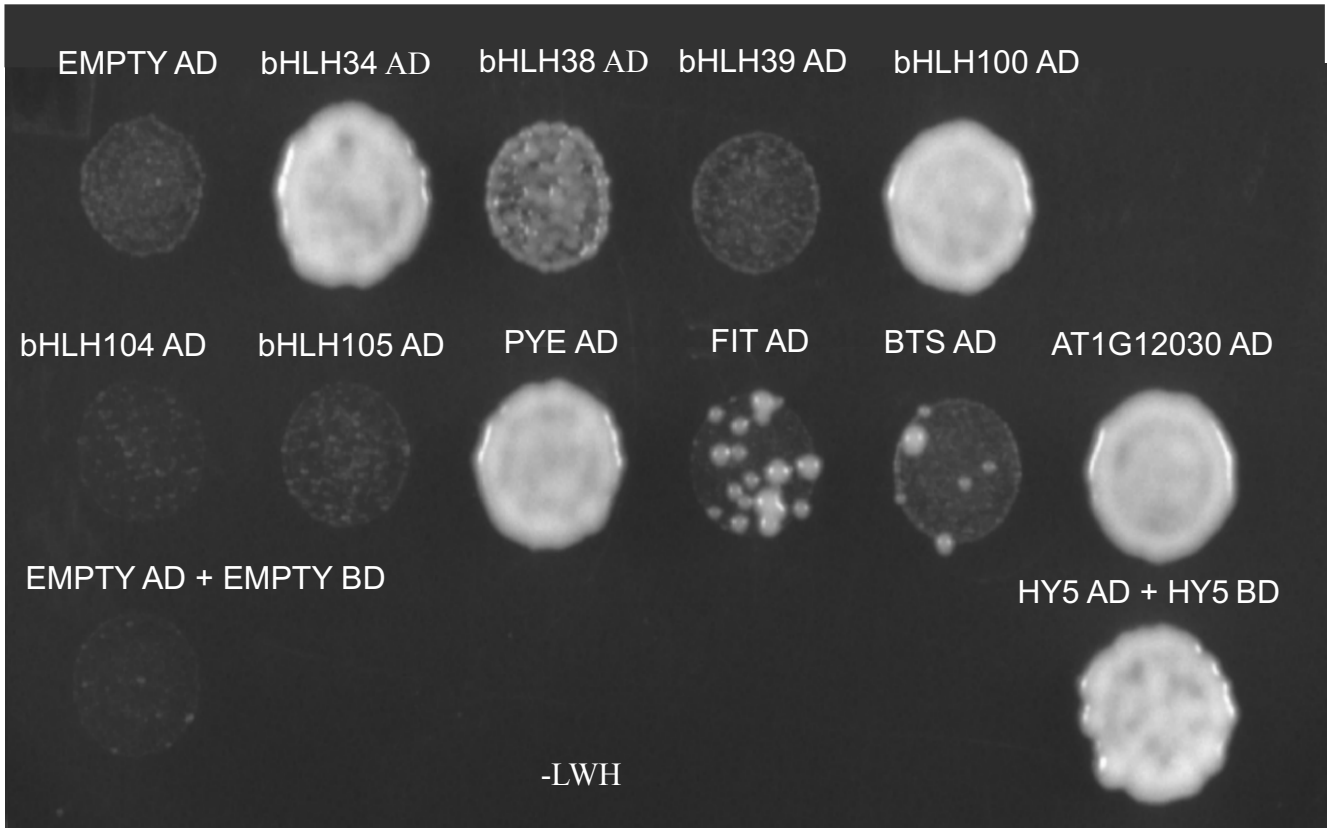


Figure 12: Yeast two Hybrid interaction between AT1G12030 BD and other AD fused iron deficiency response transcription factors on Leucine, Histidine and Tryptophan (-LWH) drop out media.

Chapter 4

Discussion

We first inferred from the time-course microarray analysis in iron deficiency condition data available from Dinneny et al. (2008), that AT1G12030 gene upregulates by 98.3 fold. Then we performed qRT-PCR of AT1G12030 of WT (col-0) under –Fe condition and found an upregulation of AT1G12030 by 134.424 times. We also inferred from ATTED-II that AT1G12030 shares a co-expression network with other known Transcription factors involved in iron deficiency signalling pathway. Since we observed that indeed there is an increase in fold change of gene expression, we silenced the gene using CRISPR/Cas9 method. Later when we checked the phenotype of the mutant in iron deficiency condition and compared it with WT (Col-0) and overexpression lines, we observed that the mutant did not show a significant difference in root length where as AT1G12030 overexpression plants showed significant root length difference. Later we also performed perls' staining to identify the iron content in the plant and found a similar result overexpression has more iron content compared to mutant and WT (Col-0) and there was no significant iron difference between mutant and wild type. We then measured chlorophyll content and FCR activity but we did not observe any significant difference between the plants, since this experiment was only measured once, we need to repeat the experiment to confirm the data.

Since we did not see any phenotype in the mutant we calculated that there might be another gene which plays a similar role like AT1G12030, we ran protein blast and found that AT1G62420 shares similar sequence. Further, to strengthen our hypothesis we performed qRT-PCR and found that AT1G62420 expression is induced by 151.299 times under iron deficiency condition, suggesting that AT1G12030 and AT1G62420 genes have a redundant function in iron homeostasis pathway. To test this hypothesis, we could make double mutant of AT1G12030 and AT1G62420 and carry out the detailed phenotypic analysis such as root length measurement and chlorophyll content, iron content measurement using Perls staining, FCR activity and Rhizosphere acidification. Since in

ATTED-II we observed that both these genes are directly involved in the co-expression network we can also check if they directly interact with each other with the help of Yeast-two-hybrid.

Chapter 5

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