Elucidating the Role of ELONGATED HYPOCOTYL5 (HY5) in Maintaining Iron Homeostasis in *Arabidopsis thaliana*.

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CERTIFICATE

The work presented in this thesis has been carried out by me under the supervision of Dr. Santosh B. Satbhai, at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or full for a degree, a diploma, or a fellowship to any other university or institute. Wherever contributions of other people have been involved, every effort has been made to indicate this clearly, with due acknowledgment 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.

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

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Dr. Santosh B. Satbhai

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Chapter 1

1.1 Background

Iron (Fe) is an essential element for all organisms. The chemical characteristics of Fe make it a good candidate for redox reactions and predispose it to easily produce reactive oxygen species. In plants, Fe is essential for chlorophyll biosynthesis, respiration, photosynthesis, immune responses and acts as a cofactor of many enzymes (Hänsch and Mendel, 2009a; Balk and Schaedler, 2014). In humans, Fe is required for the formation of myoglobin, DNA replication and repair, regulation of gene expression, cell proliferation and differentiation. Humans can obtain non-heme iron by eating nuts, cereals, and leafy, dark green vegetable. Heme iron, on the other hand, can be obtained by eating animal products (Piskin *et al.*, 2022). Fe deficiency is the most prevalent micronutrient deficiency, which is the main cause of anaemia, a serious health risk particularly for children and pregnant women. The ultimate source of Fe in human diet are plants either directly or indirectly. Therefore, the complete understanding of how plants sense and respond to iron availability is crucial for improving the Fe content of crops.

1.2 Fe uptake strategies

Even though, Fe is the fourth most abundant element in earth's crust but majority of it is present in insoluble form as it tends to form insoluble Fe^{3+} oxide or hydroxide precipitates in presence of oxygen at neutral or basic pH (Thomine and Lanquar, 2011). Fe deficiency in plants growing on low-Fe soils leads to reduction in yield because of decrease in photosynthetic capability of plants and sometimes leads to complete inhibition of growth in case of severe iron deficiency.

1.2.1 Strategy I

To make this precipitated Fe available for absorption, plants have adapted different strategies. One is reduction-based strategy (Strategy I) which is utilized by *Arabidopsis thaliana* (Arabidopsis) and non-graminaceous monocots (Kobayashi and Nishizawa, 2012a; Brumbarova *et al.*, 2015). In this strategy, first protons are released into the rhizosphere by the PLASMA MEMBRANE PROTON ATPase *(AHA2)* (Santi and Schmidt, 2009a). The release of the protons in the rhizosphere leads to acidification of the rhizosphere which enhances the solubility of Fe. In addition to this, the ABC transporter *PDR9* which is also known as *ABCG37*, releases coumarin family phenolics into the rhizosphere (Fourcroy *et al.*, 2014). This leads to chelation and mobilization of Fe^{3+}. The solubilized Fe^{3+} is reduced to Fe^{2+} with the help of FERRIC REDUCTASE OXIDASE *(FRO2)* enzyme (Ying Yi and Mary Lou Guerinot, 1996; Nigel j. Robinson *et al.*, 1999). The reduced Fe^{2+} is taken inside the root epidermal cell with

the help of IRON REGULATED TRANSPORTER 1 *(IRT1)* transporter (Eide *et al.*, 1996; Vert *et al.*, 2002) (Figure 1.1).

1.2.2 Strategy II

The other one is the chelation-based strategy which is adapted by graminaceous monocots such as rice, maize, and barley etc. In this strategy, mugineic acids (MAs) are secreted into the rhizosphere by the TRANSPORTER OF MA 1 *(TOM1)* PS efflux transporter present in the root. The secreted MAs in the rhizosphere then bind with Fe^{3+} which leads to the formation of Fe-MA complexes. The Fe-MA complexes are taken inside the root with the help of YELLOW STRIPE *(YS1)* and YS1-like *(YS1-LIKE)* transporters (Curie *et al.*, 2001; Yen *et al.*, 2001; Koike *et al.*, 2004; Schaaf *et al.*, 2004). It is interesting to note that graminaceous species like rice also takes up Fe^{2+} with the help of *OsIRT1, OsIRT2, NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN (OsNRAMP1)* and *(OsNRAMP5)* (Ishimaru *et al.*, 2006; Cheng *et al.*, 2007) (Figure 1.1).

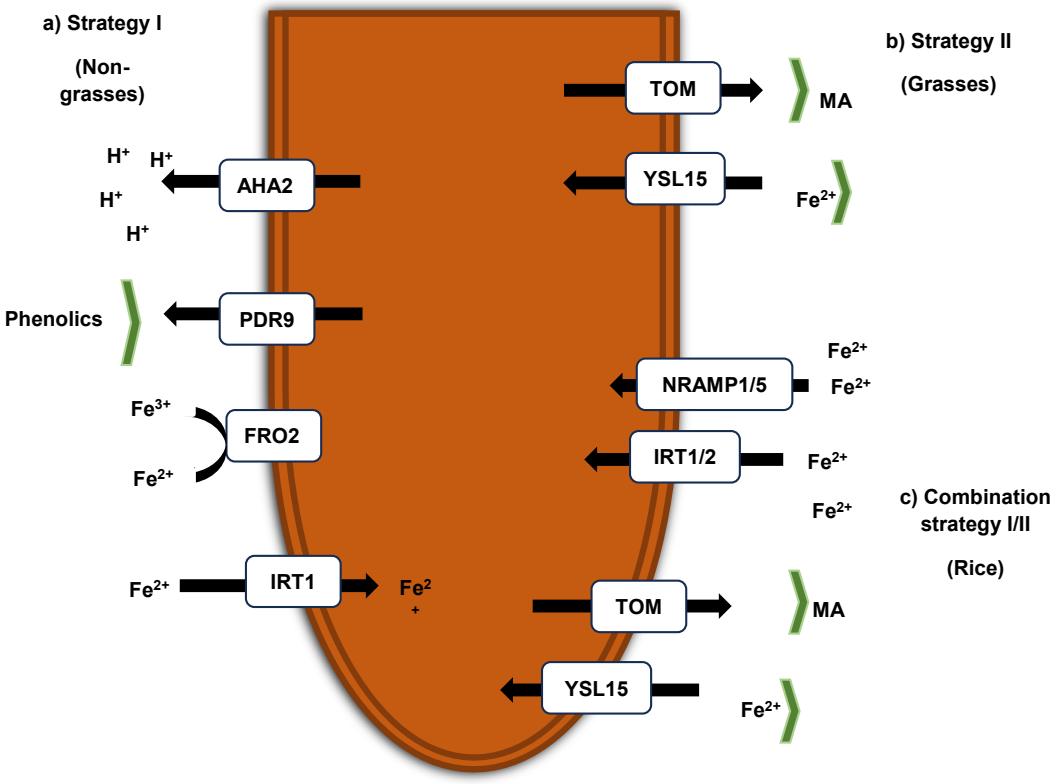

Figure 1.1. Iron uptake strategies: a) Strategy I (left side) is found in dicotyledons and non-graminaceous monocotyledons. The AHA2 releases the protons into the rhizosphere because of which it becomes more acidic. Furthermore, the coumarin family phenolics are released into the rhizosphere through the ABC transporter PDR9. Fe^{3+} is mobilized and chelated because of acidic pH and coumarins. With the help of FRO2 enzyme, the solubilized Fe^{3+} is reduced to Fe^{2+}. The Fe^{2+} enters the root cell with the help of IRT1 transporter. b) Strategy II (right side) is found in graminaceous monocots. In this, the TOM transporter secretes mugineic acids (MAs) into the rhizosphere and the Fe-MA complexes are formed in the rhizosphere when bond with Fe^{3+}. The YS1 and YS1-LIKE transporters move the Fe-MA complexes into the root and c) Rice uses a combination strategy in which Fe^{2+} can also be taken inside the root cell by the IRT1/2 and NRAMP15, in addition to TOM and YSL transporters. Modified from (Rai *et al.*, 2021).

1.3 Fe transport and storage

Once inside the root epidermal cells, Fe must be transported and remobilized to various organs and compartmentalized in cellular organelles as iron homeostasis plays a crucial function at both the inter-and intracellular levels. Fe is transported from root to shoot and different parts of the plants through vascular system. From epidermis, Fe is transported to cortex, endodermis and pericycle through apoplastic or symplastic pathways. From pericycle, Fe is proposed to be loaded into the xylem through FERROPORTINS (FPN) (Morrissey and Guerinot, 2009). The iron is transported to shoots through xylem in the form of Fe^{3+}-citrate complex. FERRIC REDUCTASE DEFECTIVE 3 (FRD3) mediates the transport of citrate into the xylem (Durrett *et al.*, 2007). In addition to this, NITRATE AND PEPTIDE TRANSPORTER FAMILY (NPF) members including NPF5.8 and NPF5.9 are also known to regulate long distance transport of Fe and homeostasis (Chen *et al.*, 2021). The transport of Fe to sink tissues and redistribution to the roots is mediated by the OLIGOPEPTIDE TRANSPORTER 3 (OPT3). It has been reported that null mutation of *opt3* leads to embryonic lethality (Stacey *et al.*, 2008; Stacey *et al.*, 2002). The main chelator aiding Fe transport in the phloem of Arabidopsis is nicotianamine (NA) and abnormalities in sink organ development and Fe homeostasis occur from either loss of function of NA synthases or Fe-NA transporters. The YELLOW STRIPE-LIKE (YSL) family of Fe (II)-nicotianamine transporters has been suggested as important mediators of Fe uptake vascular tissues. YSL1 and YSL3 are responsible for Fe redistribution from senescent leaves into developing seeds (Waters *et al.*, 2006). YSL4 and YSL6 are located to internal cellular membranes, and they have an impact on the subcellular transfer of Fe or Fe-NA (Conte

et al., 2013). YSL2 is involved in the movement of Fe from the vascular system to neighbouring cells (DiDonato *et al.*, 2004). Fe storing mechanism are induced after seed loading of Fe. Ferritins in plants are also believed to store Fe, preventing oxidative stress and acting as a buffer against excess Fe. Of the four ferritin-encoding genes in Arabidopsis, *FER1* and *FER3* are the ones that are most frequently produced in response to Fe overload treatments helping to maintain Fe homeostasis (Petit *et al.*, 2001). During seed germination and seed establishment, the vacuole plays a crucial function in the storage and mobilization of Fe. The vacuole makes a considerable contribution to the storage and sequestration of Fe, which is made possible by the Fe efflux proteins, to prevent toxicity (Jiang *et al.*, 2001). The loading of Fe into the vacuole is mainly done VACULOR IRON TRANSPORTER 1 (VIT1) and Fe is transported out of the vacuole by NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN 3 (NRAMP3) and by NRAMP4 (Kim *et al.*, 2006; Lanquar *et al.*, 2005) (Figure 1.2).

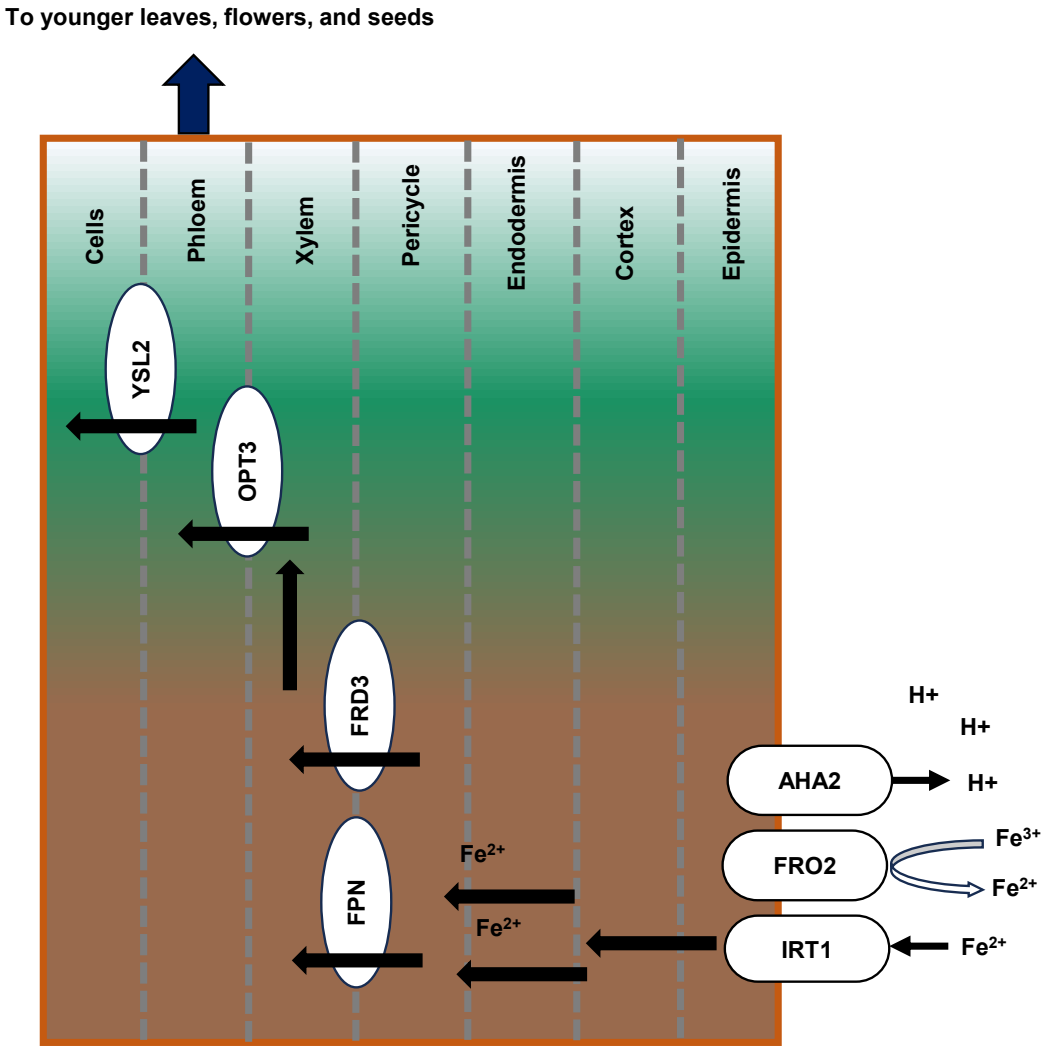

Figure 1.2. Overview of Fe uptake and Fe transport. Modified from (Kroh and Pilon, 2019; DiDonato *et al.*, 2004; Durrett *et al.*, 2007)**.** Fe is taken inside the root epidermal cell with the activity of the *AHA2, FRO2* and *IRT1*. Once inside the epidermal cell, Fe is transported from epidermal cell to the pericycle through the symplastic and apoplastic connections. From the pericycle, Fe is proposed to be transported into the xylem with the help of *FPN* and *FRD3*. *OPT3* is involved in the transport of Fe from xylem to the phloem and from phloem to the younger leaves, flowers, and seeds. *YSL2* is involved in the Fe transport from phloem to the neighbouring cells.

1.4 Transcriptional and post-transcriptional regulation of iron homeostasis in plants

The transcription factors (TFs) which regulate Fe homeostasis in plants mainly belong to the bHLH family of transcription factors (TFs). Under Fe deficiency, group IVc bHLHs (bHLH34, bHLH105/ILR3, bHLH104, and bHLH115) TFs interact with bHLH121 to induce the expression of group Ib (*bHLH38, bHLH39, bHLH100, and bHLH101*) and group IVb *(PYE)* (Li *et al.*, 2016; Liang *et al.*, 2017; Kim *et al.*, 2019; Gao, Robe and Dubos, 2020) . The group Ib bHLH TFs heterodimerize with FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR (FIT) and induce the expression of major iron uptake genes (*FRO2, IRT1* and *AHA2*) (Liang *et al.*, 2017; Wang *et al.*, 2013; Yuan *et al.*, 2008). The bHLH TF PYE interacts with ILR3 to repress the expression of Fe transport (*NAS4*), Fe assimilation (*NEET*) and Fe storage (*FER1, FER3, FER4* and *VTL2*) genes (Tissot *et al.*, 2019). The group IVc bHLH TFs are regulated at protein level by BRUTUS (BTS) which is a E3 ubiquitin ligase. It is involved in the degradation of group IVc TFs which ultimately leads to downregulation of Fe-uptake to avoid Fe overload under Fe-sufficient conditions (Selote *et al.*, 2015). IRON MAN peptides (IMAs) which is a class of small peptides are induced under -Fe and they are involved in inducing Fe-deficiency responses by interacting with BTS and inhibiting interaction and degradation of bHLH105 and bHLH115 which further leads to induction of Fe uptake genes (Grillet *et al.*, 2018a; Li *et al.*, 2021). FIT is regulated at protein level by the BRUTUS LIKE1 (BTSL1) and BTSL2 which are two RING E3 ubiquitin ligases. BTSL1 and BTSL2 protect the plant from Fe overload by targeting FIT for degradation (Rodríguez-Celma *et al.*, 2019). FIT activity is post-translationally regulated by CALCINEURIN B-LIKE-INTERACTING SER/THR-PROTEIN KINASE11 (CIPK11)-dependent phosphorylation which activates it to interact with group Ib TFs (Gratz *et al.*, 2019). Similarly, FRO2 and IRT1 proteins are regulated by CIPK23 (Tian *et al.*, 2016; Dubeaux *et al.*, 2018). The AHA2 activity is also regulated by phosphorylation (Fuglsang *et al.*, 2014; Fuglsang *et al.*, 2007). In the past

few years, a number of studies have shown that the secretion of coumarins is important for Fe uptake. The coumarin production is also induced at transcriptional level under -Fe. FIT together with group Ib bHLH TFs induce the expression of *SCOPOLETIN 8-HYDROXYLASE (S8H),* a cytochrome P450 enzyme, *CYP82C4* and *MYB72* (Schmid *et al.*, 2014; Tsai and Schmidt, 2017a; Tsai *et al.*, 2018). *S8H* and *CYP82C4* are involved in biosynthesis of fraxetin and sideretin respectively. Fraxetin biosynthesis is induced under alkaline pH and sideretin is synthesized at acidic pH for Fe mobilization in the soil (Siwinska *et al.*, 2018; Rajniak *et al.*, 2018; Murgia *et al.*, 2011). MYB72 is known to regulate the expression of *BETA GLUCOSIDASE 42 (BGLU42)* and *PDR9* (Palmer *et al.*, 2013; Tsai and Schmidt, 2017a). *BGLU42* is responsible for deglycosylation of coumarins before their secretion and *PDR9* is involved in secretion of coumarins into the rhizosphere (Fourcroy *et al.*, 2014; Sisó-Terraza *et al.*, 2016; Zamioudis *et al.*, 2014). In addition to MYB72, MYB15 is also known to be regulate the expression to regulate the expression of *FERULOYL-CoA 6'-HYDROXYLASE 1 (F6'H1)* which catalyses the first step of coumarin biosynthesis (Rodríguez-Celma *et al.*, 2013; Schmid *et al.*, 2014; Chezem *et al.*, 2017). *MYB10* is also proposed to be essential for coumarin biosynthesis (Palmer *et al.*, 2013) (Figure 1.3).

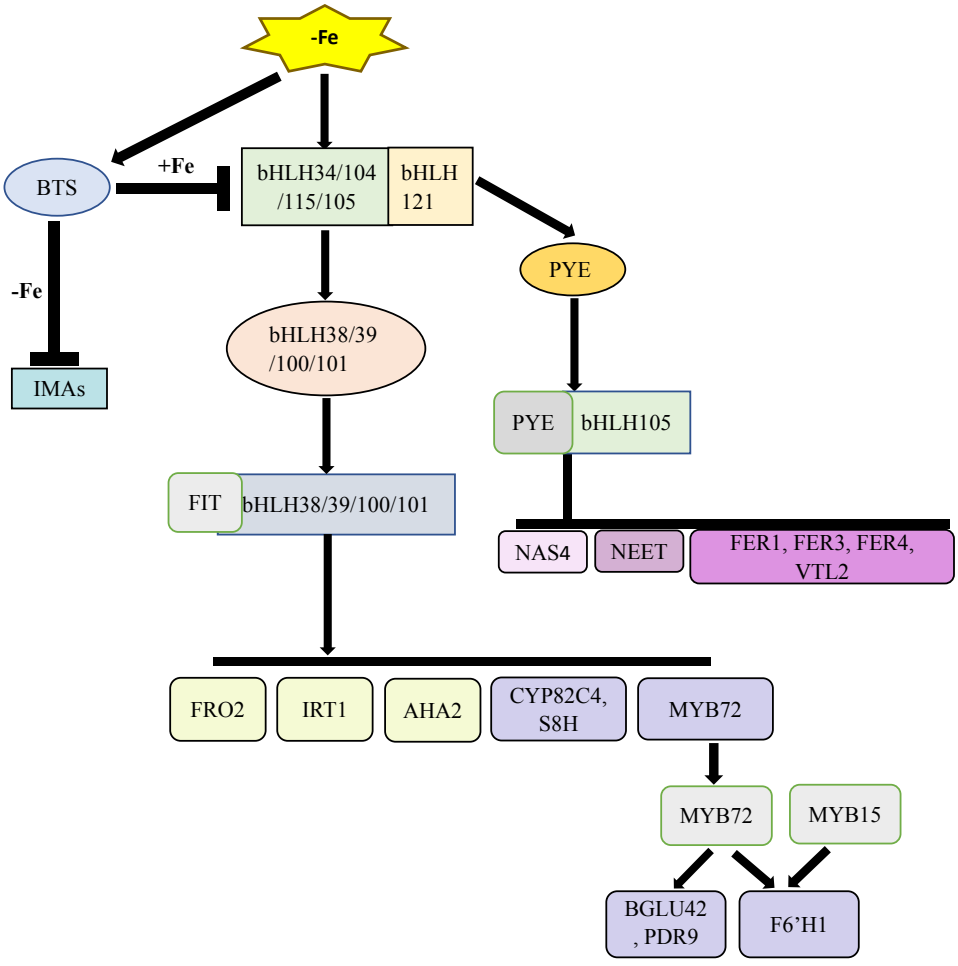

Figure 1.3. Transcriptional regulation of Fe deficiency responsive genes. Modified from (Tissot *et al.*, 2019; Robe *et al.*, 2020)**.** Under -Fe conditions, bHLH34/104/115/105 are stabilized and they heterodimerize with bHLH121 to induce the expression of *bHLH38/39/100/101* and *PYE*. The bHLH38/39/100/101 heterodimerize with FIT to induce the expression of Fe uptake related genes which include *FRO2, IRT1, AHA2, CYP82C4* and *MYB72.* MYB72 is involved in the activation of *BGLU42, PDR9* and *F6'H1* (involved in coumarin biosynthesis and secretion) expression. MYB15 is also reported to be involved in inducing the expression of *F6'H1* expression. PYE acts as a transcriptional repressor. It interacts with bHLH105 to negatively regulate the expression of *NAS4* (involved in Fe transport), *NEET* (involved in Fe assimilation) and *FER1, FER3, FER4,* and *VTL2* (involved in Fe storage). Under +Fe conditions, BTS degrades bHLH34/104/115/105 and acts as a negative regulator of Fe uptake and under -Fe conditions IMAs are induced, and they are degraded by BTS which leads to stabilization of bHLH34/104/115/105. Straight arrows represent activation and blunt arrows represent inhibition.

1.5 *ELONGATED HYPOCOTYL 5 (HY5)*: A Central Regulator of Photomorphogenesis

ELONGATED HYPOCOTYL 5 (HY5) is a transcription factor belonging to basic leucine zipper (bZIP) family. It is a central regulator of photomorphogenesis. Photomorphogenesis refers to various aspects of seedling development which include inhibition of hypocotyl growth, chlorophyll accumulation and stimulation of cotyledon expansion that are strongly regulated by light. In addition to its role in photomorphogenesis and regulating the expression of light-responsive genes, it has also been shown that HY5 regulates the expression of many genes linked to several hormone and metabolic pathways.

HY5 is usually found in two isoforms, phosphorylated and unphosphorylated. The unphosphorylated form is the most active and has a higher affinity for target promoters (Hardtke *et al.*, 2000). In the dark conditions, unphosphorylated HY5 is directly targeted for polyubiquitination and degradation by the CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1)-SUPPRESSOR OF PHYTOCHROME A-105 (COP1-SPA) E3 ligase complex (Osterlund *et al.*, 2000). Therefore, unphosphorylated HY5 is present in low levels, and phosphorylated HY5 is present in high levels under dark conditions. The COP1-SPA activity is significantly reduced in response to light irradiation through several regulatory mechanisms (Podolec and Ulm, 2018). There are at least five classes of photoreceptors, including phytochromes (phyA-phyE), cryptochromes (CRY1 and CRY2), phototropins (PHOT1 and PHOT2), F-box containing Flavin binding proteins (ZTL, FKF1, and LKP2), and UV-B

RESISTANCE LOCUS 8 (UVR8), which are responsible for the perception of the various light signals (Paik and Huq, 2019). The photoreceptors phyA, phyB, CRY1 and CRY2 are known to disrupt the formation of the COP1-SPA complex by directly associating with the COP1-SPA (Podolec and Ulm, 2018). In addition, UVR8, CRY1 and CRY2 also get activated by light and compete with HY5 for COP1 binding (Lau *et al.*, 2019; Ponnu *et al.*, 2019). Under continuous light illumination, COP1 migrates to the cytoplasm from the nucleus. Collectively, these mechanisms promote the accumulation of HY5 in the light. The HY5 accumulated in the light, directly or indirectly regulate the transcription of over 3000 genes, regulating diverse physiological and biological processes (Lee *et al.*, 2007; Burko *et al.*, 2020).

Studies have shown that HY5 interacts with a group of B-box proteins (BBXs) to regulate several downstream targets involved in various physiological and biological processes. HY5 is known to act as transcriptional activator by interacting with BBX20, BBX21, BBX22 and BBX23 (Zhang *et al.*, 2017; Bursch *et al.*, 2020). On the other hand, BBX24, BBX25, BBX28, and BBX29 interact with HY5 and repress the transcriptional activity of HY5 (Gangappa and Kumar, 2017; Lin *et al.*, 2018; Song *et al.*, 2020). Together, BBX-HY5 module regulates photomorphogenesis.

1.6 HY5 as a regulator of nutrient homeostasis

The proper growth and development of plants is dependent on nutrient acquisition and utilization. Light is a key environmental factor on which the uptake of nutrients by roots and their utilization in the aerial plant parts is dependent. Light signals are perceived by various photoreceptors which leads to activation of downstream transcription factors. Among these TFs, HY5 stands out as a key regulator of light-mediated nutrient absorption and utilisation. The role of HY5 has been studied in the regulation of carbon (C), nitrogen (N), sulfur (S), phosphorus (P), and copper (Cu) homeostasis.

1.6.1 Carbon

HY5 plays an essential role in carbon fixation and the transport of fixed carbon. It regulates the photosynthetic capacity by regulating the expression of genes involved in biosynthesis of chlorophyll, stomatal development, and photosynthesis (Andronis *et al.*, 2008; Catalá *et al.*, 2011; Toledo-Ortiz *et al.*, 2014; Zhang *et al.*, 2014; Chen *et al.*, 2016; Burko *et al.*, 2020; Wang *et al.*, 2021). The transport of fixed carbon from source to sink tissues is also regulated by HY5. It is involved in positively regulating the expression of *TREHALOSE-6-PHOSPHATE SYNTHASE 1 (TPS1)* which is known to be involved in regulation of utilization of carbohydrate

and *SUCROSE TRANSPORTER (SWEET11 and SWEET12)* genes which are involved in sucrose transport (Chen *et al.*, 2012; Chen *et al.*, 2016; Blázquez *et al.*, 1998; Schluepmann *et al.*, 2003).

1.6.1 Nitrogen

HY5 plays an important role in nitrogen homeostasis by regulating the expression of several genes involved in nitrogen uptake and assimilation. HY5 directly binds to the promoter of *NITRATE REDUCTASE 2 (NIA2)*, and *NITRITE REDUCTASE 1 (NIR1)* to positively regulate their expression (Jonassen *et al.*, 2008; Jonassen *et al.*, 2009; Huang *et al.*, 2015). *NIA2* converts nitrate to nitrite in cytosol and *NIR1* converts nitrite into ammonium. HY5 regulates the expression of the nitrate uptake genes both positively and negatively. It negatively regulates the expression of *NITRATE TRANSPORTER 1.1 (NRT1.1)* and *AMMONIUM TRANSPORTER1.2 (AMT1.2)* which are responsible for nitrogen uptake (Huang *et al.*, 2015; Jonassen *et al.*, 2008). HY5 has been demonstrated to positively regulate nitrogen uptake by regulating the expression of *NRT2.1,* while in shoots it controls carbon assimilation and translocation (Chen *et al.*, 2016). In cotton (*Gossypium hirsutum*), a recent study has demonstrated that light-mediated activation of *GhHY5* in the shoot results in the activation of *GhHY5* in the root. In the root, *GhHY5* positively regulates the expression of *GhNRT1.1* which promotes nitrogen uptake (Wang *et al.*, 2023) (Figure 1.4).

1.6.2 Sulphur

HY5 regulates sulfur assimilation by directly binding and regulating the expression of *ADENOSINE 5'-PHOSPHOSULFATE REDUCTASE 1 (APR1)* and *APR2* involved in sulfur assimilation (Lee *et al.*, 2011). *APR1* and *APR2* are involved in conversion of activated sulfate to sulfite, which is an essential step in the sulfate assimilation pathway. In addition to this, *HY5* also positively controls sulfur uptake by acting as a positive regulator of the expression of *SULFATE TRANSPORTER 1;2 (SULTR 1;2)* under conditions of low nitrogen. It has been shown that the absorption and assimilation of sulfate is affected by nitrogen deficiency in the wild type but not in the *hy5* mutant which suggests the involvement of *HY5* in coordination of nitrogen and sulphur assimilation (Lee *et al.*, 2011; Lee *et al.*, 2007) (Figure 1.4).

1.6.3 Phosphorus

The phyB-HY5 signalling module activated by red light has been found to regulate phosphate uptake and accumulation. HY5 has been shown to directly bind on the promoter of Pi

transporter 1 *(PHT1.1)* and regulate its expression positively (Sakuraba *et al.*, 2018). *PHT1.1* is mainly involved in phosphorus uptake from the soil. In tomato, red light perception by phyB activates HY5 which promotes P uptake under phosphorus deficient conditions by promoting arbuscular mycorrhizal symbioses (AMS) (Ge *et al.*, 2022). The inhibition of root growth under P deficient conditions is favourably regulated by blue light. This inhibition is thought to be necessary for improving Pi uptake from the topsoil. Blue light perception through CRY1/CRY2 in the shoot activates *HY5*. The shoot HY5 migrates to the root and activate the expression of root *HY5*. The root HY5 promotes iron accumulation in the meristematic and elongation zones by triggering the expression of *LPR1* gene which ultimately leads to ROS production, followed by the inhibition of primary root growth under phosphorus deficient conditions (Gao *et al.*, 2021) (Figure 1.4).

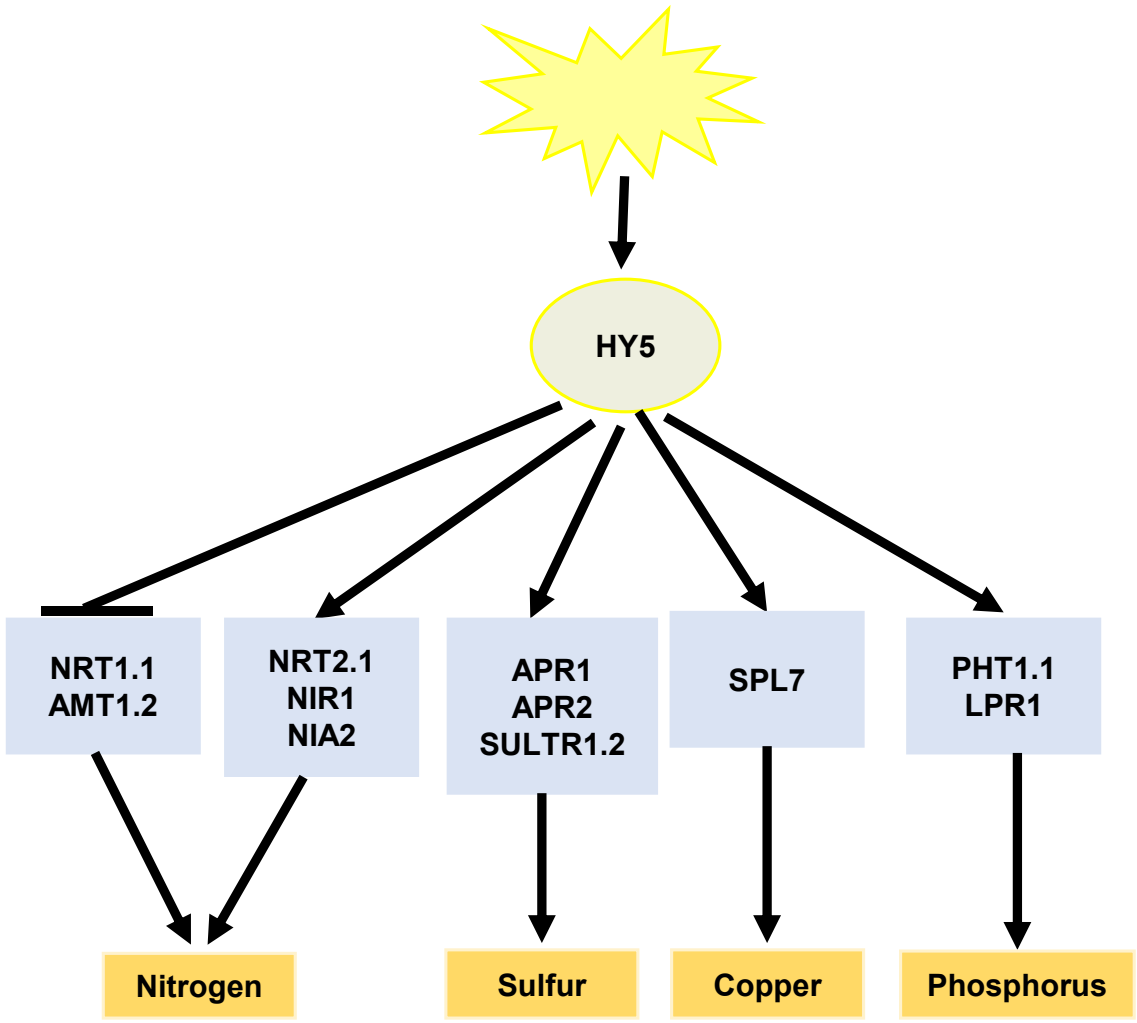

Figure 1.4. HY5 regulates nutrient signaling. Modified from (Gangappa and Botto, 2016; Xiao *et al.*, 2022; Gao *et al.*, 2021)**.** Light activated HY5 regulates nitrogen, sulfur, copper and

phosphorus homeostasis. HY5 acts both as a repressor and activator to regulate the expression of genes involved in nitrogen uptake and assimilation which include *NRT1.1, AMT1.2* (regulated negatively) and *NRT2.1, NIR1, NIA2* (regulated positively). The sulfur assimilation and uptake related genes including *APR1, APR2* and *SULTR1.2* are positively regulated by HY5. It regulates copper homeostasis through SPL7. Phosphorus homeostasis is also positively regulated by HY5 through the activation of *PHT1.1* and *LPR1*. Straight arrows represent activation and blunt arrows represent inhibition.

1.6.4 Copper

HY5 has been reported to mediate a crosstalk between light and copper. It has been found that HY5 interacts with SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 7 (SPL7) which is a Copper-responsive transcription factor. Under changing conditions of light and copper concentration, HY5 and SPL7 together transcriptionally regulate microRNA (MIR408). This leads to differential expression of *MIR408* and its target genes which is essential for maintaining Cu levels under Cu-deficient conditions (Zhang *et al.*, 2014) (Figure 1.4).

1.7 Thesis Objective

The main source of nutrients for plants is soil and the nutrient content in the soil varies because of fluctuations in the temperature, pH, water supply or soil type. Plants must adapt to these variations in the nutrient availability of their surroundings in order to survive. The nutrient fluctuations in the environment trigger plants transcriptional reprogramming. HY5, PIF4 and NF-Y, which are known to be key players of light signalling, were discovered by gene co-expression analysis based on 13 nutrient stress conditions in Arabidopsis as regulators for the overall transcriptome reprogramming. The analysis suggested HY5 to be a regulator of Fe homeostasis. A recent study in tomato (*Solanum lycopersicum* L.*)* shows that HY5 induces Fe-uptake by inducing *SlFER* expression under Fe deficiency (Guo *et al.*, 2021). The role of HY5 has been studied in the regulation of nitrogen, phosphorus, sulfur, and copper homeostasis in Arabidopsis. However, the role of HY5 in regulation of Fe homeostasis has not yet been thoroughly investigated. So, the main objective of the thesis was to investigate the role of HY5 in Fe homeostasis in Arabidopsis. The results, conclusions and future perspectives from the study are described in the following chapters:

Chapter 2: ELONGATED HYPOCOTYL 5 regulates *BRUTUS* and affects iron acquisition and homeostasis in *Arabidopsis thaliana*.

Chapter 3: ELONGATED HYPOCOTYL 5 interacts with POPEYE and regulates iron homeostasis in *Arabidopsis thaliana.*

Chapter 4: Conclusions and future perspectives.

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Chapter 2

ELONGATED HYPOCOTYL 5

regulates *BRUTUS* and affects iron acquisition and homeostasis in *Arabidopsis thaliana.*

This part of the work stated below is a slight modification of the published manuscript entitled, “ELONGATED HYPOCOTYL 5 regulates BRUTUS and affects iron acquisition and homeostasis in Arabidopsis thaliana” authored by **Samriti Mankotia**, Dhriti Singh, Kumari Monika, Muskan Kalra, Himani Meena, Varsha Meena, Ram Kishor Yadav, Ajay Kumar Pandey, and Santosh B. Satbhai which is published in the *Plant Journal*.

Mankotia, S., Singh, D., Monika, K., Kalra, M., Meena, H., Meena, V., Yadav, R.K., Pandey, A.K. and Satbhai, S.B., 2023. ELONGATED HYPOCOTYL 5 regulates BRUTUS and affects iron acquisition and homeostasis in Arabidopsis thaliana. The Plant Journal. 114(6), 1267-1284.

In this chapter, “our” and “we” refers to me and co-authors. My contribution in the paper includes (1) Choosing the objective of the study (2) Analysis and compiling of the literature (3) Designing and performing experiments (4) Interpretation of the findings (5) Creating figures and graphs (6) The manuscript writing and editing.

2.1 ABSTRACT

Iron (Fe) is an essential micronutrient for both plants and animals. Fe-limitation significantly reduces crop yield and adversely impacts on human nutrition. Owing to limited bioavailability of Fe in soil, plants have adapted different strategies that not only regulate Fe-uptake and homeostasis but also bring modifications in root system architecture to enhance survival. Understanding the molecular mechanism underlying the root growth responses will have critical implications for plant breeding. Fe-uptake is regulated by a cascade of basic helix-loop-helix (bHLH) transcription factors (TFs) in plants. In this study, we report that HY5 (Elongated Hypocotyl 5), a member of the basic leucine zipper (bZIP) family of TFs, plays an important role in the Fe-deficiency signalling pathway in *Arabidopsis thaliana*. The *hy5* mutant failed to mount optimum Fe-deficiency responses, and displayed root growth defects under Fe-limitation. Our analysis revealed that the induction of the genes involved in Fe-uptake pathway (*FIT-FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR, FRO2-FERRIC REDUCTION OXIDASE 2* and *IRT1-IRON-REGULATED TRANSPORTER1*) is reduced in the *hy5* mutant as compared with the wild-type plants under Fe deficiency. Moreover, we also found that the expression of coumarin biosynthesis genes is affected in the *hy5* mutant under Fe-deficiency. Our results also showed that *HY5* negatively regulates BRUTUS (*BTS)* and POPEYE (*PYE*). Chromatin immunoprecipitation followed by quantitative polymerase chain reaction revealed direct binding of HY5 to the promoters of *BTS, FRO2 and PYE*. Altogether, our results showed that *HY5* plays an important role in the regulation of Fe-deficiency responses in *Arabidopsis*.

2.2 INTRODUCTION

Iron (Fe) is an indispensable micronutrient for both plants and animals. It plays an irreplaceable role in many crucial processes like respiration, photosynthesis, hormone biosynthesis, pathogen defense and chlorophyll biosynthesis (Hänsch *et al*., 2009; Balk *et al*., 2014). Although Fe is present in abundance in soil, most of it is unavailable for absorption to plants. At the basic and neutral pH in the well-aerated soils, it tends to form insoluble crystals of ferric (Fe^{3+}) oxyhydrates, which cannot be taken up by the plants (Thomine *et al*., 2011)

For efficient Fe-uptake from the soil, plants adopted two types of strategies: reduction-based strategy (strategy I); and chelation-based strategy (strategy II). *Arabidopsis* utilizes reduction-based strategy for Fe-uptake from soil (Kobayashi *et al*., 2012; Brumbarova *et al*., 2015). In this strategy, protons are released into the rhizosphere from the root PLASMA MEMBRANE

PROTON ATPASE 2 (AHA2), which acidifies the rhizosphere and, thus, increases the Fe solubility. In addition to this, Fe^{3+} is also chelated and mobilized by coumarin family phenolics released into the rhizosphere by the ABC transporter PDR9, also named ABCG37 (Tsai *et al.*, 2017). The resulting solubilized Fe^{3+} is reduced to Fe^{2+} by the ferric reductase (FRO2) enzyme (Connolly *et al.*, 2003). The Fe^{2+} is taken inside the root epidermal cells by the IRT1 transporter (Vert *et al.*, 2002; Santi *et al*., 2009; Fourcroy *et al.*, 2016).

In Fe-deficient growth conditions, expression of Fe-uptake genes is induced in plants to improve the Fe absorption from the root rhizosphere. FIT heterodimerizes with subgroup Ib basic helix-loop-helix (bHLH) transcription factors (TFs) including bHLH38, bHLH39, bHLH100 and bHLH101, and induces the expression of *FRO2* and *IRT1* (Yuan *et al.*, 2008; Wang *et al.*, 2013; Liang *et al.*, 2017). The expression of subgroup Ib bHLH TFs is also induced under Fe-deficiency and their expression is activated by subgroup IVc bHLH TFs [bHLH34, bHLH104, bHLH105 (ILR3- IAA-LEUCINE RESISTANT3), and bHLH115] (Li *et al.*, 2016; Liang *et al.*, 2017). ILR3, in addition to its role as a transcriptional activator, also acts as a transcriptional repressor by interacting with POPEYE (PYE), and negatively regulates the expression of genes involved in Fe transport (*NAS4-NICOTIANAMINE SYNTHASE 4*), storage (*FER1, FER3, FER4, VTL2-VACUOLAR IRON TRANSPORTER-LIKE 2*) and assimilation (*NEET*) (Tissot *et al.*, 2019). The subgroup IVc bHLH TFs are regulated at protein level. The BTS, which is a E3 ubiquitin ligase, is involved in regulating the subgroup IVc bHLH TFs at protein level (Long *et al.*, 2010; Selote *et al.*, 2015). It degrades subgroup IVc bHLH TFs under Fe-sufficient conditions, and thus, negatively regulates Fe-uptake to avoid Fe overload (Selote *et al.*, 2015). The BRUTUS LIKE1 (BTSL1) and BTSL2, which are two closely related RING E3 ubiquitin ligases, also negatively regulate Fe homeostasis by directly targeting FIT for degradation by the 26S proteasomal pathway (Rodríguez-Celma *et al.*, 2019). bHLH121 (URI-UPSTREAM REGULATOR OF IRT1) has also been found to positively regulate Fe homeostasis by interacting with bHLH IVc TFs (Kim *et al.*, 2019; Gao, Robe, Bettembourg, *et al.*, 2020; Lei *et al.*, 2020).

The mechanisms behind the perception of Fe-deficiency stress and the transmission of this signal to activate the Fe-deficiency response are not completely understood. Many reports suggest that hormones and small signalling molecules like nitric oxide (NO) also play an important role in regulating the expression of genes involved in Fe-signaling. In Strategy I plants, auxin and ethylene concentration increase in Fe-deficient roots, where they have been implicated in the activation of several Fe-related genes, like FIT, FRO2, IRT1 and others

(Romera *et al.*, 2006; Lucena *et al.*, 2006; Waters *et al.*, 2007; García *et al.*, 2010; García *et al.*, 2011; Lingam *et al.*, 2011; Meiser *et al.*, 2011; Yang *et al.*, 2013; Yang *et al.*, 2014; Lucena *et al.*, 2015; Bacaicoa *et al.*, 2011). Ethylene synthesis is reported to be regulated negatively by LODIS (LONG DISTANCE IRON SIGNAL). LODIS acts as a signal moving from shoots to roots leading to a decrease in ethylene accumulation under Fe-sufficient conditions (García *et al.*, 2018). NO has also been found to positively regulate the expression of genes involved in Fe-uptake genes in tomato (Graziano *et al.*, 2007). In Arabidopsis, NO has been found to act downstream to auxin to induce FRO2 activity under Fe-deficiency (Chen *et al.*, 2010). IRON MAN peptides (IMAs), a class of small peptides induced under Fe-deficiency, have been recently proposed to be involved in Fe-signaling. They play a role in activating Fe deficiency responses by interacting with BTS and promoting the accumulation of bHLH105 and bHLH115 (Grillet *et al.*, 2018b; Li *et al.*, 2021).

Elongated Hypocotyl 5 (HY5) is a member of the basic leucine zipper (bZIP) family of TFs and is a crucial regulator of photomorphogenesis. *hy5* mutant has less chlorophyll content, elongated hypocotyl and increased number of lateral roots as compared with the WT plants (Oyama *et al.*, 1997; Ang *et al.*, 1998; Holm *et al.*, 2002). It is also known to positively regulate copper, sulfur, and phosphorous signalling pathways (Jonassen *et al.*, 2008; Lillo, 2008; Lee *et al.*, 2011; Yanagisawa, 2014; Zhang *et al.*, 2014; Huang *et al.*, 2015). There are some reports that indicate *HY5* to have a broader role in Fe signalling (Brumbarova and Ivanov, 2019; Vélez-Bermúdez and Schmidt, 2022). A study predicted HY5, PIF4, and NF-Y to be master regulators responsible for remodelling the Arabidopsis transcriptome in response to fluctuating nutrient availability. In this study, gene coexpression analysis was done on thirteen different nutrient conditions and HY5 is speculated to be a master regulator of Fe homeostasis. (Brumbarova *et al.*, 2019). Many Fe-responsive genes contain HY5 binding sites in their promoters in *Arabidopsis* (Vélez-Bermúdez *et al.*, 2022). Recently, in tomato, HY5 has been shown to be involved in regulating Fe-uptake. The phytochrome B (PHYB) gets activated by red light and induces HY5, which then moves from shoot to roots and triggers Fe-uptake by inducing *FER* expression (Guo *et al.*, 2021). However, the role of HY5 in Fe-signaling has not been studied in detail till now.

In this study, we report that *HY5* is involved in regulating Fe-deficiency responses in *Arabidopsis*. *HY5* function is crucial for primary root growth under Fe-limiting conditions. We found that HY5 is required for regulating the expression of genes involved in Fe acquisition and homeostasis, which includes *IRT1*, *FIT*, *FRO2, PYE* and *BTS,* as well as genes involved in

coumarin biosynthesis, *CYP82C4*, *S8H*, *F6'H1* and *BGLU42*. Our data also indicate that HY5 acts as a negative regulator of *BTS* and *PYE* to modulate Fe-deficiency responses in *Arabidopsis*. We conclude that HY5 acts as a key player to regulate genes involved in Fe homeostasis pathway in *Arabidopsis*.

2.3 RESULTS:

2.3.1 *hy5* mutant has an impaired Fe-deficiency response.

To investigate the role of *HY5* in Fe homeostasis, we used a T-DNA insertional mutant of *hy5* and compared its phenotype with the WT plants. It is known that the primary root length of WT plants increases under mild Fe-deficiency, remains unchanged under moderate Fe-deficiency and decreases under severe Fe-deficiency (Gruber *et al.*, 2013). In addition to the changes in root length, the chlorophyll content is also known to get reduced under Fe-deficiency conditions. Our phenotypic analysis revealed that either there was a significant increase in the primary root length of the WT seedlings when grown on -Fe as compared with the +Fe or it remains unchanged under -Fe (Figure 2.1a, b and Figure 2.2a). In contrast to the WT, the *hy5* mutant root length was consistently and significantly decreased when grown on -Fe as compared to +Fe (Figure 2.1a, b). The overall growth of the *hy5* mutant was stunted and leaves were more chlorotic as compared to the WT under -Fe. This indicates that the reduction in chlorophyll content is more in case of *hy5* mutant as compared to WT. To confirm this, we calculated the percentage reduction in chlorophyll content of WT and *hy5* mutant grown on -Fe media compared with those grown on +Fe for 10 days. We found that *hy5* mutant has significantly higher reduction (86%) in chlorophyll content as compared with WT plants (48%) (Figure 2.1c). These results suggest that *hy5* mutant is more sensitive to -Fe as compared with the WT.

To confirm that the phenotype observed in *hy5* mutant was indeed due to loss of *HY5* function, we complemented the *hy5* mutant plants by transforming them with a vector containing coding sequence of enhanced green fluorescent translationally fused at C-terminus of the HY5 coding sequence. The resulting cassette was placed under *HY5* promoter (*ProHY5:HY5:eGFP*). We selected two independent T3 complementation transgenic lines P-13 and P-8 (*ProHY5:HY5:eGFP/hy5*). In order to confirm the lines, we checked the HY5-eGFP protein expression in the *ProHY5:HY5:eGFP/hy5* (P-13 and P-8) complementation lines using confocal microscopy. We found that HY5-eGFP fusion protein is uniformly expressed in all the cell layers in root (Figure 2.3a). *ProHY5:HY5:eGFP/hy5* (P-13 and P-8) lines showed only

17.94% and 15.65% reduction in root length under Fe-deficient condition, as opposed to 42.83% reduction of root length observed in *hy5* mutant (Figure 2.1a, b). We also measured the percentage reduction in chlorophyll content of the WT, *hy5* and the *ProHY5:HY5:eGFP/hy5* (P-13 and P-8). The percentage reduction in chlorophyll content of the rescue lines was slightly less but not significantly less as compared with the *hy5* mutant and it was more as compared to the WT (Figure 2.1c). To determine the role of HY5 on Fe accumulation in vivo, we performed Perls as well as Perls/Dab staining. We found that *hy5* mutant roots accumulated less Fe as compared with WT. In *ProHY5:HY5:eGFP/hy5* line, the Fe content appears to be more than *hy5* but less than WT (Figure 2.1d, e). These results indicate that rescue lines partially complemented the *hy5* mutant phenotype.

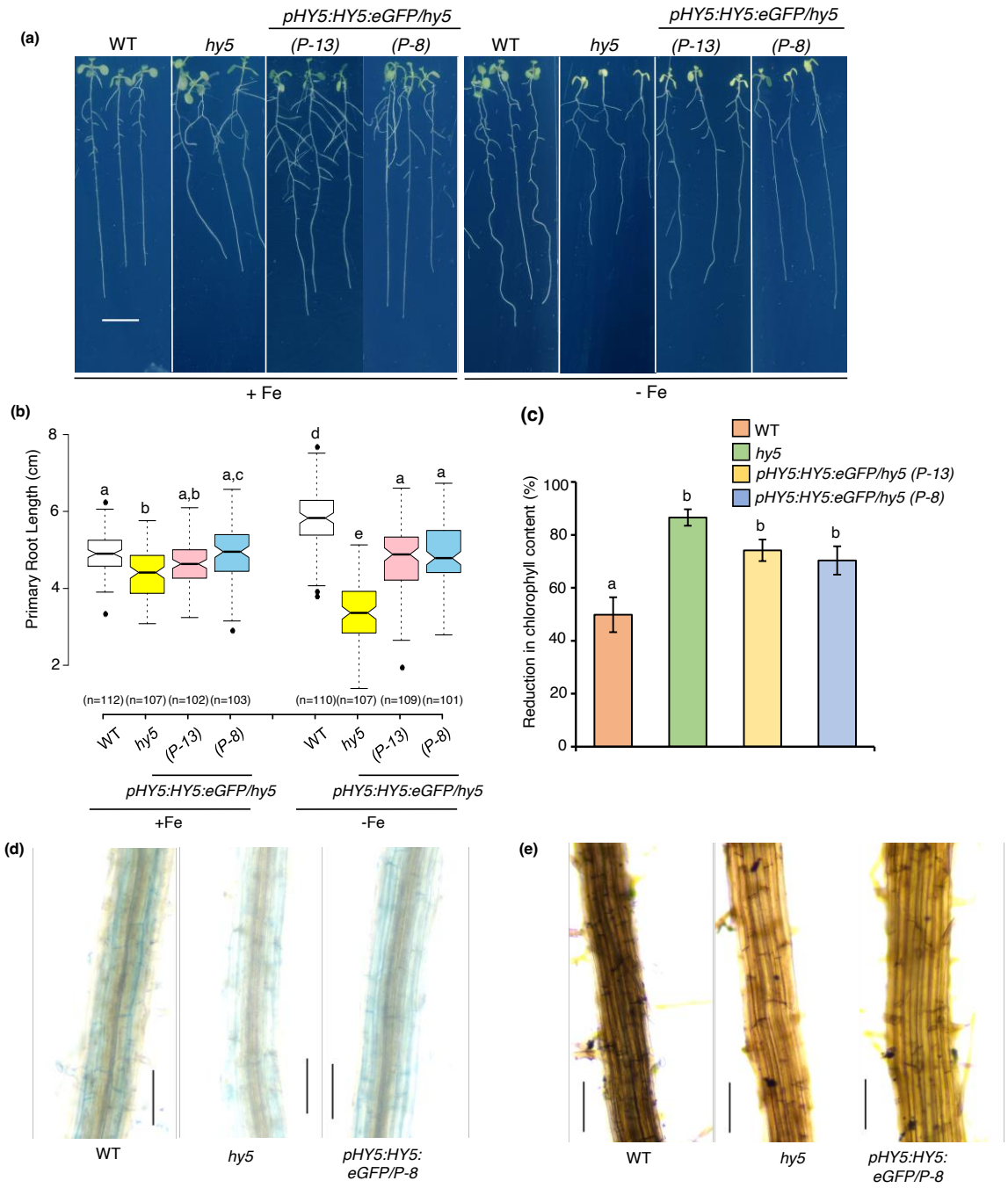

Figure 2.1. *hy5* mutant has less tolerance to Fe deficiency. (a) Phenotypes of the Arabidopsis wild type (WT), *hy5* and *pHY5:HY5:eGFP/hy5* (P-13 and P-8) grown for 10 days on Fe-sufficient and Fe-deficient medium. Scale bars, 1 cm. (b) Boxplot of root length of the wild type (WT), *hy5* and *pHY5:HY5:eGFP/hy5* (P-13 and P-8) grown for 10 days on Fe-sufficient and Fe-deficient medium. (c) Percentage reduction in chlorophyll content of the wild type (WT), *hy5* and *pHY5:HY5:eGFP/hy5* (P-13 and P-8) grown on –Fe as compared to those grown on +Fe. Data shown is an average of three independent experiments. Each experiment consists of 4 biological replicates and each replicate consists of a pool of around six seedlings. Error

bars represent ±SEM. Means within each condition with the same letter are not significantly different according to one-way ANOVA followed by post hoc Tukey test, $P < 0.05$. (d) Perls stained and Perls/Dab stained (e) maturation zone of WT, *hy5* and *pHY5:HY5:eGFP/hy5* (P-8) grown on Fe-sufficient medium for 5 days. Bars = 100µm.

Next, we used *35S:HA:HY5/hy5* line and compared its phenotype with WT and *hy5* plants. We found that it completely rescued the root length phenotype as well as chlorotic phenotype of *hy5* mutant (Figure 2.2a, b, c). The Perls and Perls/Dab staining revealed that Fe content of the *35S:HA:HY5/hy5* was also comparable to WT plants (Figure 2.2d, e).

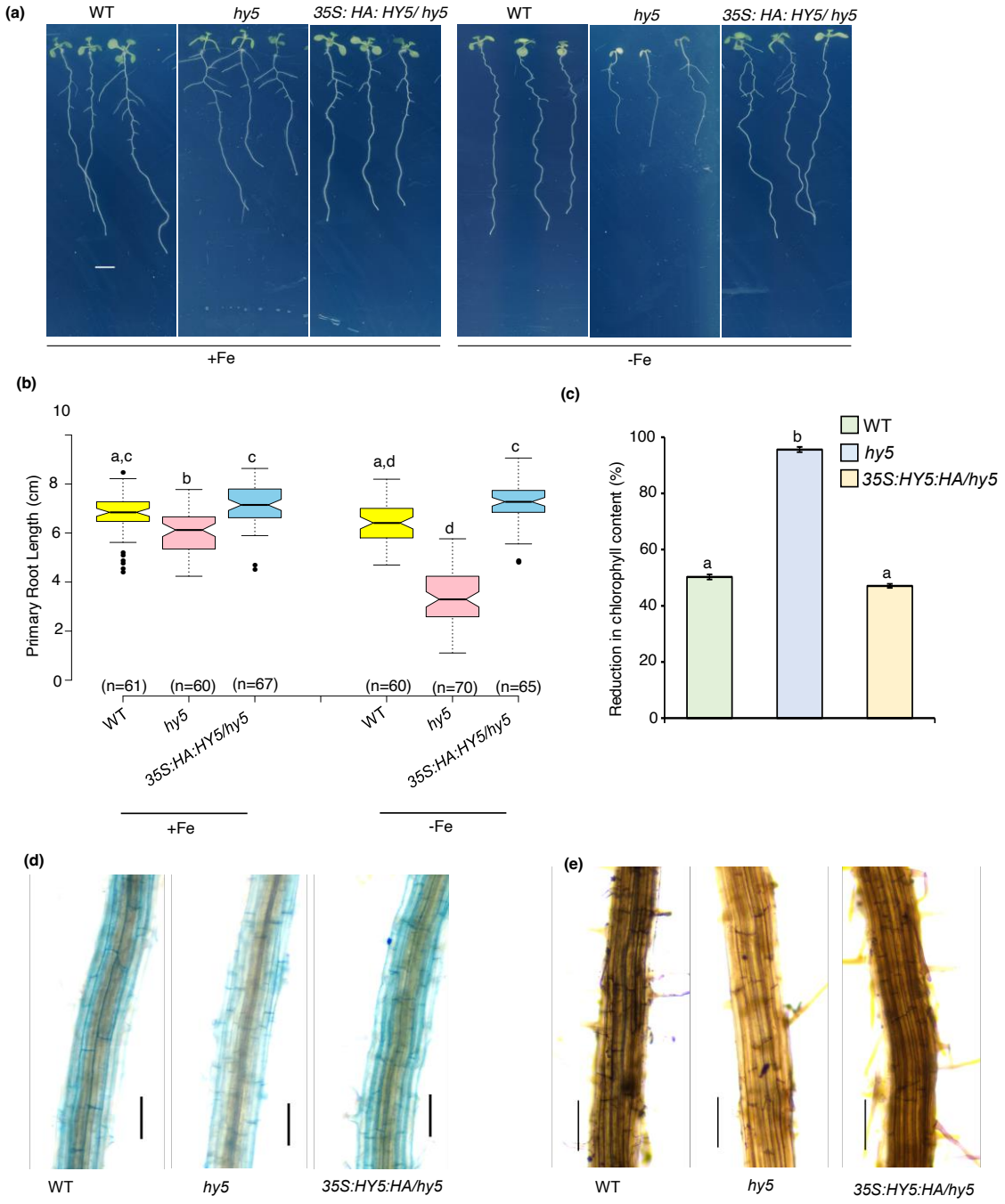

Figure 2.2. *35S:HA:HY5/hy5* rescues the *hy5* mutant phenotype. (a) Phenotypes of the *Arabidopsis* wild type (WT), *hy5* and *35S:HY5:HA/hy5* grown for 10 days on Fe-sufficient and Fe-deficient medium. Scale bars, 1 cm. (b) Boxplot of root length of the wild type (WT), *hy5* and *35S:HY5:HA/hy5* grown for 10 days on Fe-sufficient and Fe-deficient medium. (c) Percentage reduction in chlorophyll content of the wild type (WT), *hy5* and *35S:HY5:HA/hy5* grown on –Fe as compared to those grown on +Fe. Data shown is an average of three

independent experiments. Each experiment consists of 3 to 4 biological replicates and each replicate consists of a pool of around six seedlings. Error bars represent ±SEM. Means within each condition with the same letter are not significantly different according to one-way ANOVA followed by post hoc Tukey test, $P < 0.05$. (d) Perls and Perls/Dab stained maturation zone of WT, *hy5* and *35S:HY5:HA/hy5A/hy5* grown on Fe-sufficient medium for 5 days. Bars = 100µM.

Next, we wanted to check whether *HY5* gets induced at transcript or protein level under -Fe. For this, we performed quantitative real time-reverse transcription-polymerase chain reaction (qRT-PCR) to check *HY5* expression at transcript level. We found it to be slightly upregulated (~1.5-fold) at transcript level under -Fe (Figure 2.3b). We also performed western blot to compare levels of endogenous HY5 in the WT under +Fe and -Fe. We found that endogenous HY5 increases slightly in WT under -Fe as compared to +Fe (Figure 2.3c).

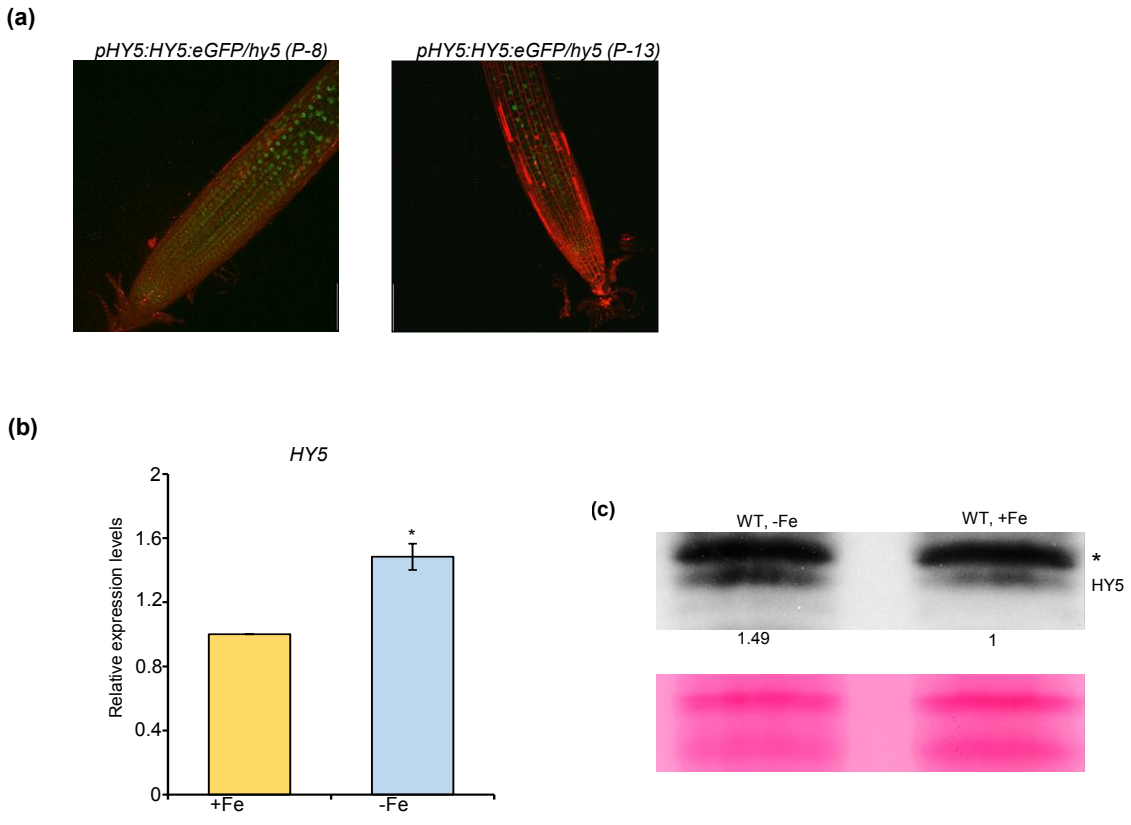

Figure 2.3. (a) Confocal microscopy of roots from the complementation lines *pHY5:HY5:eGFP/hy5 (P-13 and P-8).* Seedlings grown on +Fe media for 7 days were stained with PI for imaging (Scale bar:100µM). **(b)** Expression levels of *HY5.* Relative expression was determined by qRT-PCR in WT seedlings grown on +Fe media for 6 days and transferred to both +Fe and –Fe (+300µM Fz) for three days. Data shown is an average of three biological

replicates (n=2 technical replicates). Each biological replicate consists of pooled RNA extracted from roots of ~ 90 seedlings. Error bars represent ±SEM.*Significant difference by Student's t test (P≤0.05). **(c)** Western blot analysis. WT seedlings grown on +Fe for four days were transferred to both +Fe and –Fe(+300µM Fz) for three days. Native HY5 was visualized with an anti-HY5 antibody. The numbers written below the gel lanes represent the relative protein levels of HY5 under –Fe as compared to +Fe determined from band intensity using ImageJ software and normalized relative to ponceau staining. Two independent experiments were repeated with similar results. * Cross-reacting band.

HYH, a *HY5* homolog, acts redundantly with *HY5* in regulating nitrogen signalling, hypocotyl growth, lateral root development, and light signalling in plants (Oyama *et al*., 1997; Ang *et al.*, 1998; Holm *et al.*, 2002; Gangappa *et al.*, 2016). In order to find out whether *HYH* has any role in Fe-deficiency, we analysed root growth phenotype of *hyh hy5*, WS (Wassilewskija) and *hy5/hyh* double mutant plants under -Fe conditions. The *hyh* mutant showed similar primary root length to WT (WS) plants unlike *hy5* mutant (Figure 2.4). The *hy5* mutant from Ws background displayed a significant inhibition of primary root growth under –Fe similar to the phenotype observed in *hy5* mutant from Col background. This further confirms the involvement of HY5 in root growth under –Fe (Figure 2.1a, b and 2.4). Moreover, the root length of *hy5/hyh* double mutant plants was comparable with that of *hy5* mutant, indicating that *HY5* functions independently of *HYH* under -Fe conditions.

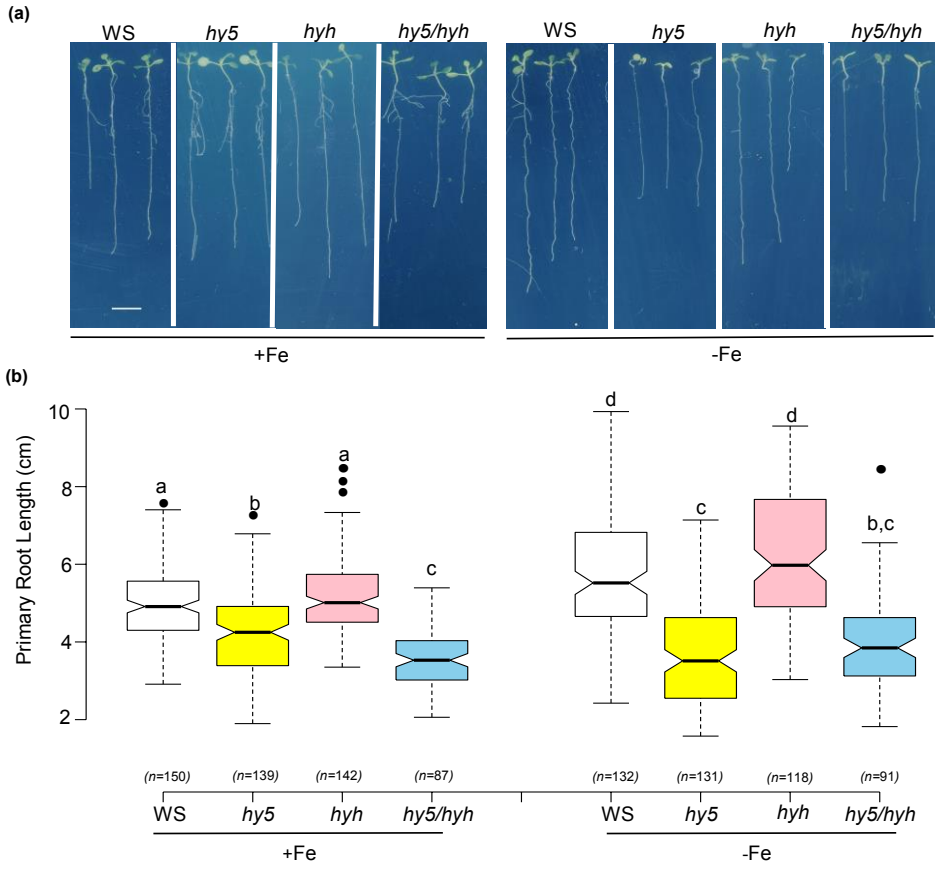

Figure 2.4. HY5 regulates primary root growth under –Fe independently of HYH. (a) Phenotypes of the *Arabidopsis* wild type (WS), *hy5*, *hyh* and *hy5hyh* grown for 10 days on Fe-sufficient and Fe-deficient medium. Scale bars, 1cm. **(b)** Boxplot of **r**oot length of the wild type (WS), *hy5*, *hyh* and *hy5hyh* grown for 10 days on Fe-sufficient and Fe-deficient medium. Means within each condition with the same letter are not significantly different according to one-way ANOVA followed by post hoc Tukey test, $P < 0.05$.

Next, we checked whether *hy5* mutant shows Fe-deficiency specific growth responses in soil. For this, WT, *hy5* and *ProHY5:HY5:eGFP/hy5* (P-13 and P-8) were grown on control soil (pH 5.0 to 6.0) and alkaline soil (pH 7.0 to 8.0) to create Fe-limiting conditions because Fe solubility decreases with an increase in pH. We found that under alkaline soil conditions, *hy5* mutant was more chlorotic as compared with the WT, which was partially rescued in the *ProHY5:HY5:eGFP/hy5 (P-13 and P-8)* lines (Figure 2.5). Moreover, the chlorotic phenotype of *hy5* mutant was also rescued when external Fe (Fe-EDDHA) was provided. Altogether, these results indicate that *hy5* mutant is more sensitive to -Fe conditions as compared to the WT.

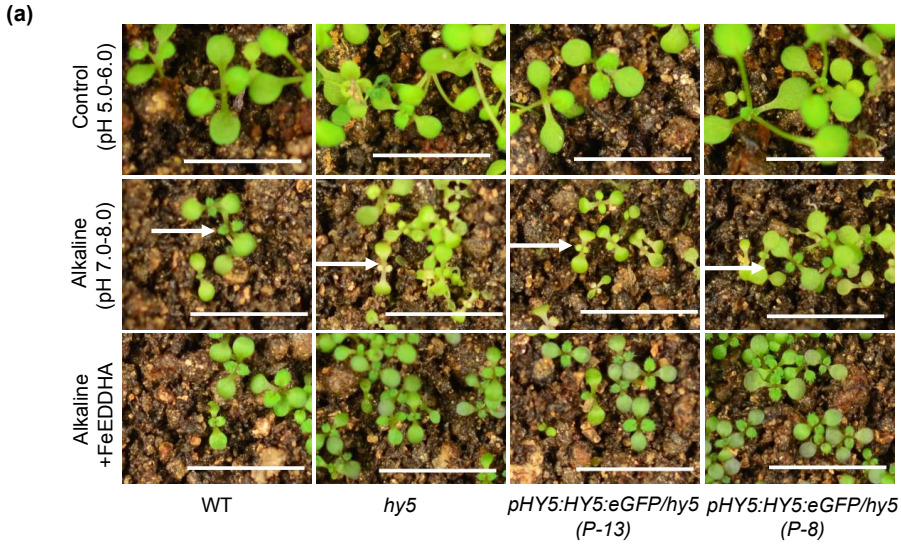

Figure 2.5. Phenotypes of the WT, *hy5, pHY5:HY5:eGFP/hy5 (P-13 and P-8)* grown on normal soil (pH 5.0-6.0), alkaline soil (pH 7.0-8.0) and alkaline soil watered with Fe-EDDHA (Ethylenediamine di-2-hydroxyphenyl acetate ferric) for two weeks. Bars =10mm.

Recently, it has been shown that regulation of Fe uptake by *HY5* is *PHYB*-dependent in tomato (Guo *et al.*, 2021). To check whether *PHYB* function is important for root growth under -Fe, we examined the phenotype of *phyB-9* mutant and compared it with the WT and *hy5* mutant plants. We found that *phyB* mutant phenotype was similar under both +Fe and -Fe conditions, suggesting that *PHYB* function is not critical for root growth under –Fe in *Arabidopsis* (Figure 2.6).

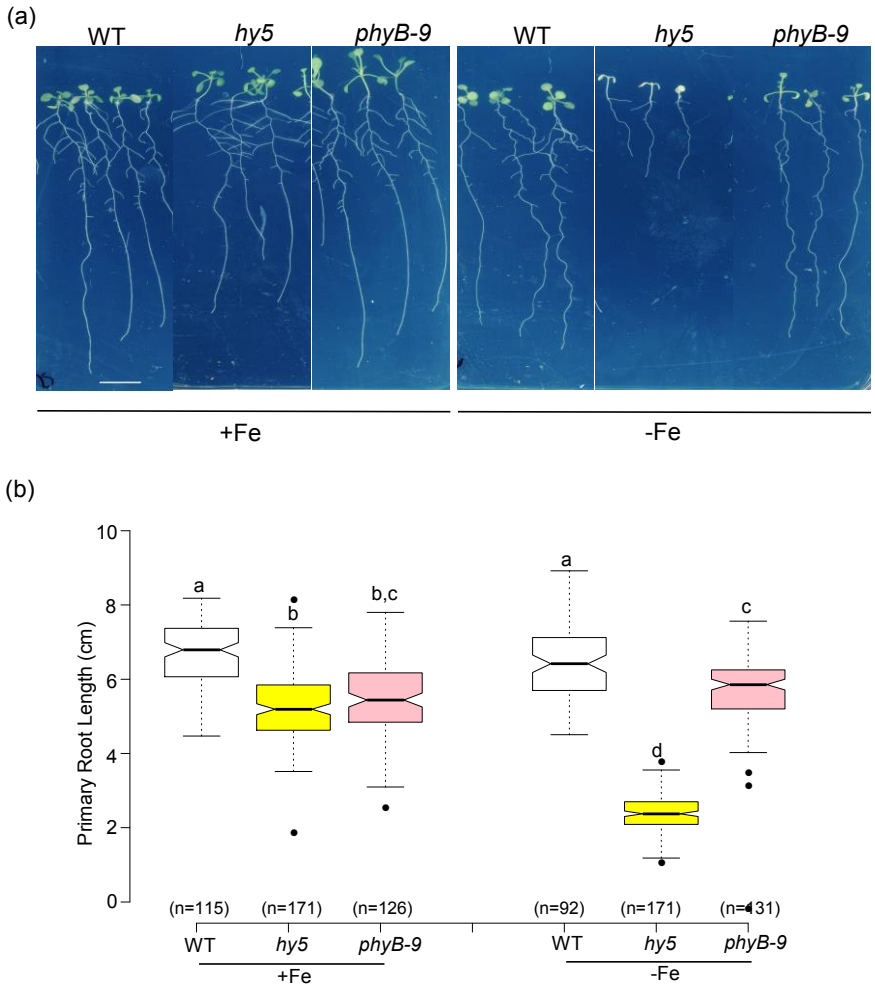

Figure 2.6. *HY5* regulates primary root growth under –Fe conditions independently of *PHYB*. (a) Phenotypes of the *Arabidopsis* wild type (WT), *hy5*, *hyh* and *phyB-9* grown for 10 days on Fe-sufficient and Fe-deficient medium. Scale bars, 1cm. **(b)** Boxplot of **r**oot length of the wild type (WT), *hy5*, *hyh* and *phyB-9* grown for 10 days on Fe-sufficient and Fe-deficient medium. Means within each condition with the same letter are not significantly different according to one-way ANOVA followed by post hoc Tukey test, $P < 0.05$.

2.3.2 HY5 regulates the expression of Fe-deficiency-responsive genes.

Our findings revealed that *hy5* mutant has reduced tolerance to Fe-deficiency. To investigate the role of HY5 in Fe acquisition and homeostasis, we checked the expression of Fe-deficiency-responsive genes in the *hy5* mutant. Expression of *FIT*, *IRT1* and *FRO2* was significantly reduced in the *hy5* mutant compared with the WT plants under Fe-deficiency (Figure 2.7a). This indicates that *HY5* function is important for the optimum induction of key genes involved

in Fe-uptake under Fe-deficient conditions. The expression of genes related to Fe homeostasis (*BTSL1*, *BTSL2, IMA1, IMA2, IMA3*) and subgroup Ib bHLH TFs (*bHLH38, bHLH39, bHLH100 and bHLH101*) was not affected significantly in the mutant compared with the WT (Figure 2.8 and Figure 2.7b).

Next, we hypothesized that HY5, which is an important central regulator of diverse plant responses, may affect genes involved in excess Fe accumulation in plants and thereby could influence some of them, like PYE, a TF that negatively regulates Fe transport, storage and assimilation (Long *et al.*, 2010). To test this, we checked the transcript level of *PYE* and found it was significantly more induced in the *hy5* mutant compared with the WT (Figure 2.7c). This indicates that *HY5* negatively regulates *PYE* expression. The qRT-PCR results indicate that there was reduced induction of *IRT1* in the *hy5* mutant compared with WT under –Fe conditions. To further understand how *hy5* mutation affects the expression pattern of *IRT1* in roots, we used *ProIRT1:GUS* line. We compared *ProIRT1:GUS* activity in WT and *hy5* background grown on both +Fe and –Fe for 8 days. Under +Fe conditions there was no observable reporter activity in both the WT and the mutant plants. However, under -Fe, we observed a strong induction of the reporter gene activity in WT plants grown on –Fe media, which is in agreement with previously published reports (Vert *et al.*, 2002; Blum *et al.*, 2014). Importantly, in *hy5* mutant plants, we observed significantly lower induction of reporter gene activity as compared with WT plants under -Fe (Figure 2.7d). This result clearly indicates that *HY5* is important for optimum induction of *IRT1* under –Fe conditions.

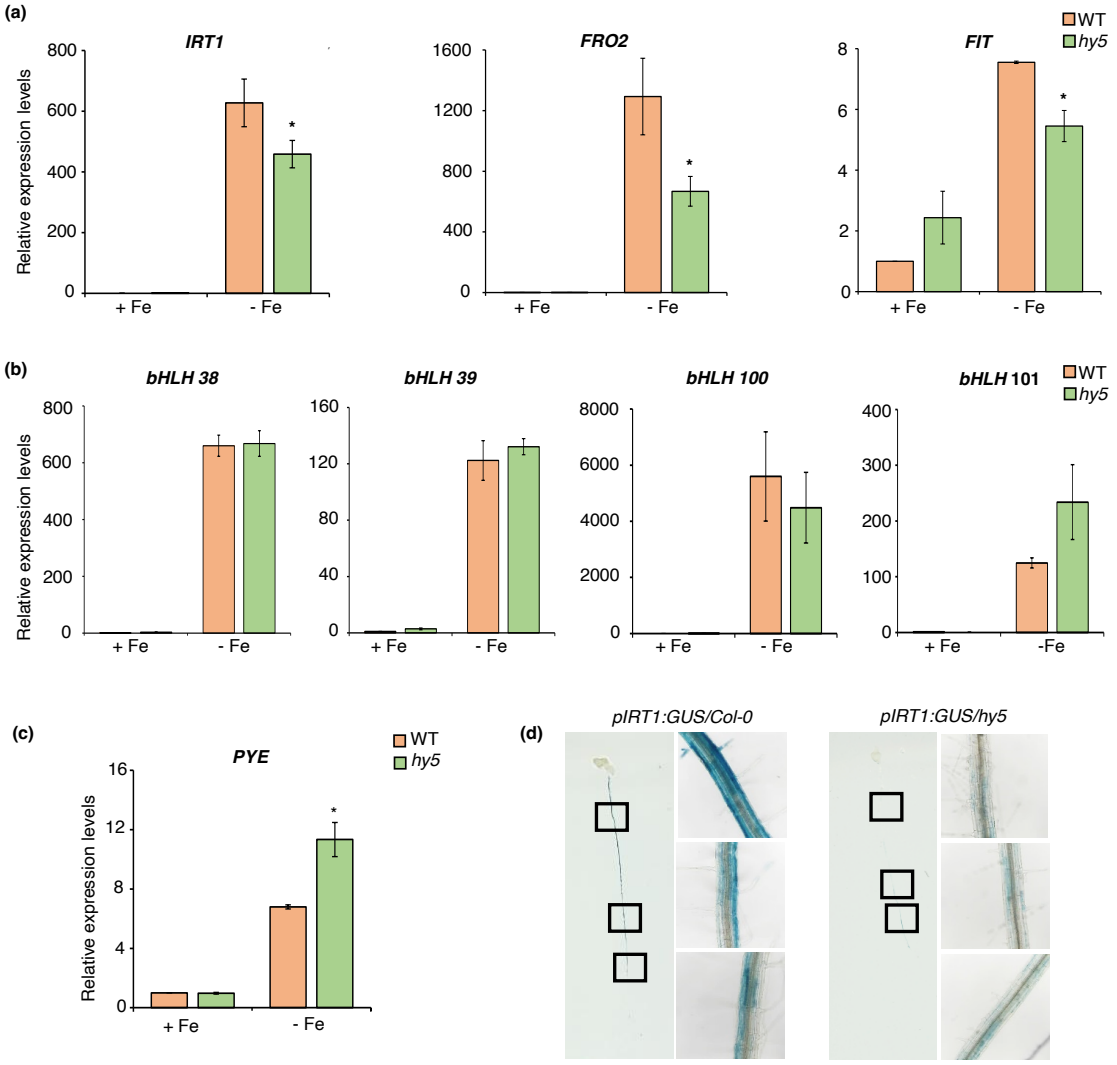

Figure 2.7. Expression of Fe-related genes in the *hy5* mutant. (a) Expression levels of *IRT1, FRO2* and *FIT*. (b) Expression levels of subgroup Ib bHLH TFs (bHLH 38, 39,100 and 101). (c) Expression level of *PYE*. Relative expression was determined by qRT-PCR in WT and *hy5* mutant seedlings grown on +Fe media for 6 days and transferred to both +Fe and –Fe (+300µM Fz) for three days. Data shown is an average of three biological replicates (n=2 technical replicates). Each biological replicate consists of pooled RNA extracted from roots of ~ 90 seedlings. Error bars represent ±SEM.*Significant difference by Student's t test (P≤0.05). (d) Analysis of pIRT1:GUS gene activity in WT Col-0 and *hy5* mutant seedlings expressing it. Plants were grown on –Fe media for 8 days and were stained for GUS activity.

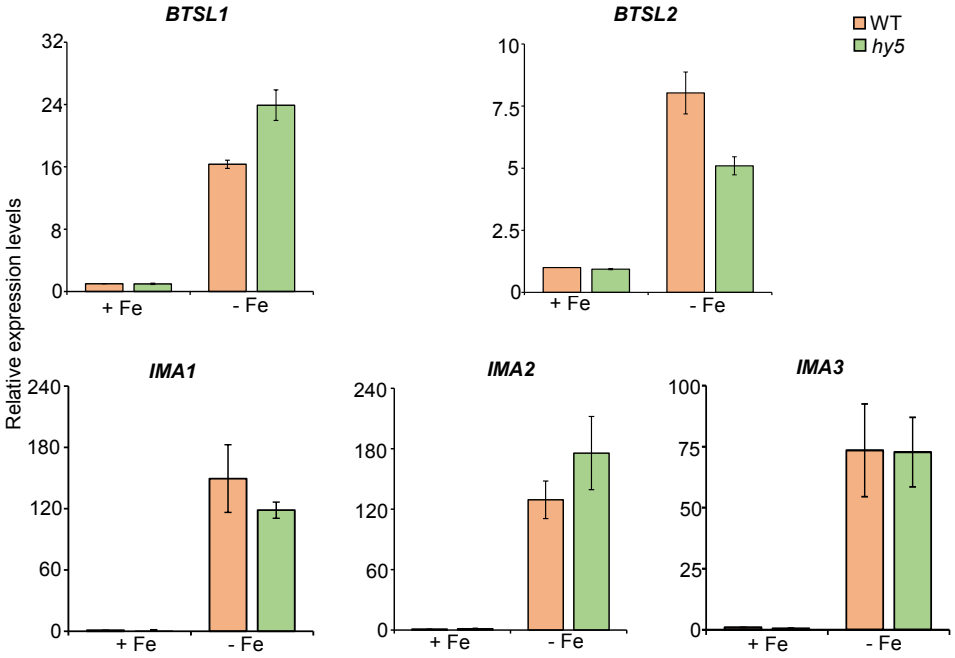

Figure 2.8. Expression levels of genes related to Fe homeostasis in the *hy5* mutant. Expression levels of *BTSL1*, *BTSL2, IMA1, IMA2* and *IMA3*. Relative expression was determined by qRT-PCR in WT and *hy5* mutant seedlings grown on +Fe media for 6 days and transferred to both +Fe and –Fe (+300µM Fz) for three days. Data shown is an average of three biological replicates. Error bars represent ±SEM.*Significant difference by Student's t test (P≤0.05).

Because *FRO2* induction was significantly lower in the *hy5* mutant as compared with the WT, we also checked ferric-chelate reductase (FCR) activity in the mutant. FCR activity was found to be significantly less induced in the mutant as compared with the WT under -Fe (Figure 2.9). Collectively, these results indicate that *HY5* positively regulates the expression of genes involved in Fe-uptake under -Fe.

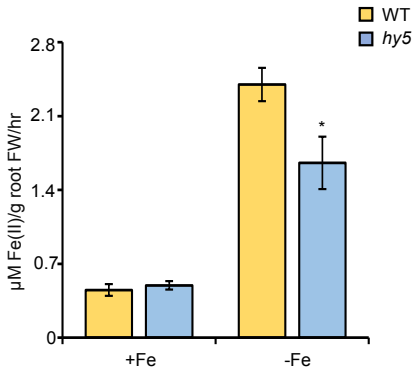

Figure 2.9. Ferric-chelate reductase activity of the wild type (WT) and *hy5* mutant. Plants were grown for 5 days on +Fe and transferred to +Fe-sufficient or –Fe (+300µM Fz) for 3 days. Data shown is an average of three independent experiments. Each experiment consists of five to six biological replicates and each biological replicate consists of a pool of around 50 roots. Error bars represent ±SEM. Significantly different (*) according to one-way ANOVA followed by post hoc Tukey test, $P < 0.05$.

Next, to find out whether HY5 directly binds to the promoter of *IRT1, FRO2, FIT* and *PYE*. We performed *in silico* analysis of the promoter sequence of *IRT1, FRO2, FIT* and *PYE*. We found HY5 binding motifs on the promoter of *IRT1, FRO2, FIT* and *PYE*. The *IRT1* promoter has one G-box which is 756 bp upstream (region I) and one CG-Hybrid which is 1667 bp upstream (region II) to the ATG (Figure 2.10a). The *FIT* promoter contains one CA-Hybrid which is 178 bp upstream (region I), and one Z-box and CA-Hybrid which is within 689 to 743 bp (region II) upstream to ATG (Figure 2.10b). The *FRO2* promoter has one CA Hybrid which is 1373 bp upstream (region I), and also one Z-box and one G-box within 1478-1499 bp upstream (region II) to ATG (Figure 2.11a). The PYE promoter contains one G-box which is 532 bp upstream (region I) and one CG-Hybrid which is 618 bp upstream (region II) to ATG (Figure 2.11b). We performed chromatin immunoprecipitation (ChIP)-qPCR experiments using anti-GFP antibody to determine whether HY5 binds to their promoter or not. For ChIP experiments, *Pro:HY5:HY5:YFP/hy5* as well as WT (*35S:eGFP*) was used. Our result show a significant enrichment of HY5 on the promoter of *FRO2* at both region I and region II, and region I of *PYE* promoter (Figure 2.11b). We did not find enrichment of HY5 on the promoter of *FIT* and *IRT1*. These results suggest that HY5 binds directly to the promoter of *FRO2* and

PYE and regulates their expression. However, further studies are still needed to find out how HY5 impacts genes downstream to PYE.

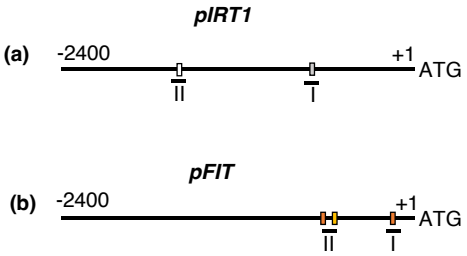

Figure 2.10. (a) Schematic diagram of the promoter of the *IRT1* and *FIT.* White boxes represent CG-Hybrid, grey boxes represent G-boxes, orange boxes represent CA-Hybrid and yellow boxes represent Z-boxes. Lines under the boxes represent region I and region II.

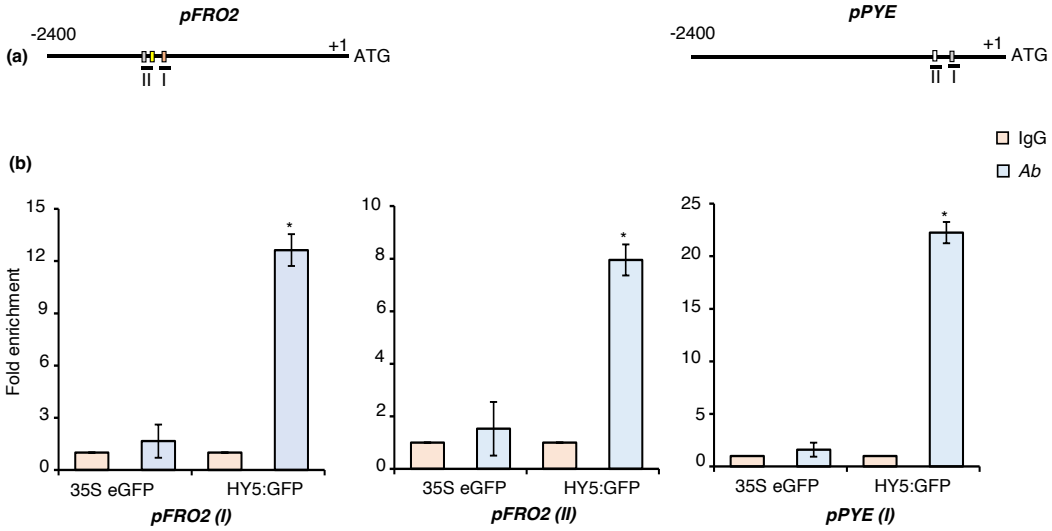

Figure 2.11. HY5 directly binds on *FRO2 and PYE* promoter. (a) Schematic diagram of the promoter of the *FRO2* and *PYE.* White boxes represent CG-Hybrid, grey boxes represent G-boxes, orange boxes represent CA-Hybrid and yellow boxes represent Z-boxes. Lines under the boxes represent sequences detected by ChIP qPCR. (b) ChIP-qPCR showing relative

enrichment of the *FRO2 and PYE* regulatory regions bound by HY5. The ChIP assay was performed using *pHY5::HY5:YFP/hy5* and *35S:eGFP* seedlings grownon +Fe media for 10 days. Error bars represent ±SEM. *Significant difference by Student's t test (P≤0.05).

2.3.3 HY5 functions upstream to BTS

BTS acts as a negative regulator of Fe-uptake by controlling bHLH subgroup IVc protein levels through proteasomal degradation (Selote *et al.*, 2015). Our qRT-PCR analysis showed that *BTS* is induced slightly more in the *hy5* mutant as compared with the WT under +Fe, and the induction becomes significantly higher under -Fe (Figure 2.12a). To find whether *HY5* has ability to regulate *BTS* directly, we did *in silico* analysis of the promoter sequence of *BTS.* We found HY5 binding motifs, three G-boxes, one CG-Hybrid within 406 to 488 bp upstream to ATG (region I) and one CG-Hybrid 1400 bp upstream (region II) to ATG in the promoter of *BTS* (Figure 2.12b). Based on this, we hypothesized that HY5 may bind directly to the *BTS* promoter. To confirm this, we performed ChIP-qPCR experiments using anti-GFP antibody. For ChIP experiments, *Pro:HY5:HY5:YFP* transgene rescuing *hy5* mutant phenotype as well as WT (*35S:eGFP*) was used. We found significant enrichment of region I of *BTS* promoter with HY5-eGFP protein in ChIP assay (Figure 2.12b). This observation indicates that HY5 directly binds to the promoter of *BTS*.

To further investigate the role of HY5 in regulating *BTS* expression *in planta*, we expressed *ProBTS:H2B:YFP* reporter construct in WT and crossed it with *hy5* mutant and checked YFP fluorescence. *ProBTS:H2B:YFP* showed increased fluorescence in *hy5* mutant in both +Fe and –Fe conditions (Figure 2.12c). We selected two regions of the maturation zone for observing the signal as the signal was clearly visible in the maturation zone. We also quantified the signal in these regions of the maturation zone. In region I, the signal was found to be more in the *hy5* mutant under both +Fe and -Fe conditions, but it was significantly higher only under -Fe conditions (Figure 2.13a). In region II, we found the signal to be significantly higher in the *hy5* mutant under both +Fe and -Fe conditions (Figure 2.13b). To further validate our observation, we crossed *ProBTS:GUS* line with *hy5*. We compared *ProBTS:GUS* activity in the WT and *hy5* background grown on both +Fe and –Fe for 8 days. Similar to, *ProBTS:H2B:YFP,* in the *hy5* mutant, we observed a stronger induction of the reporter gene (in the maturation zone) as compared with the WT plants under -Fe conditions (Figure 2.12d). Altogether, these observations indicate that *HY5* negatively regulates the expression of *BTS.*

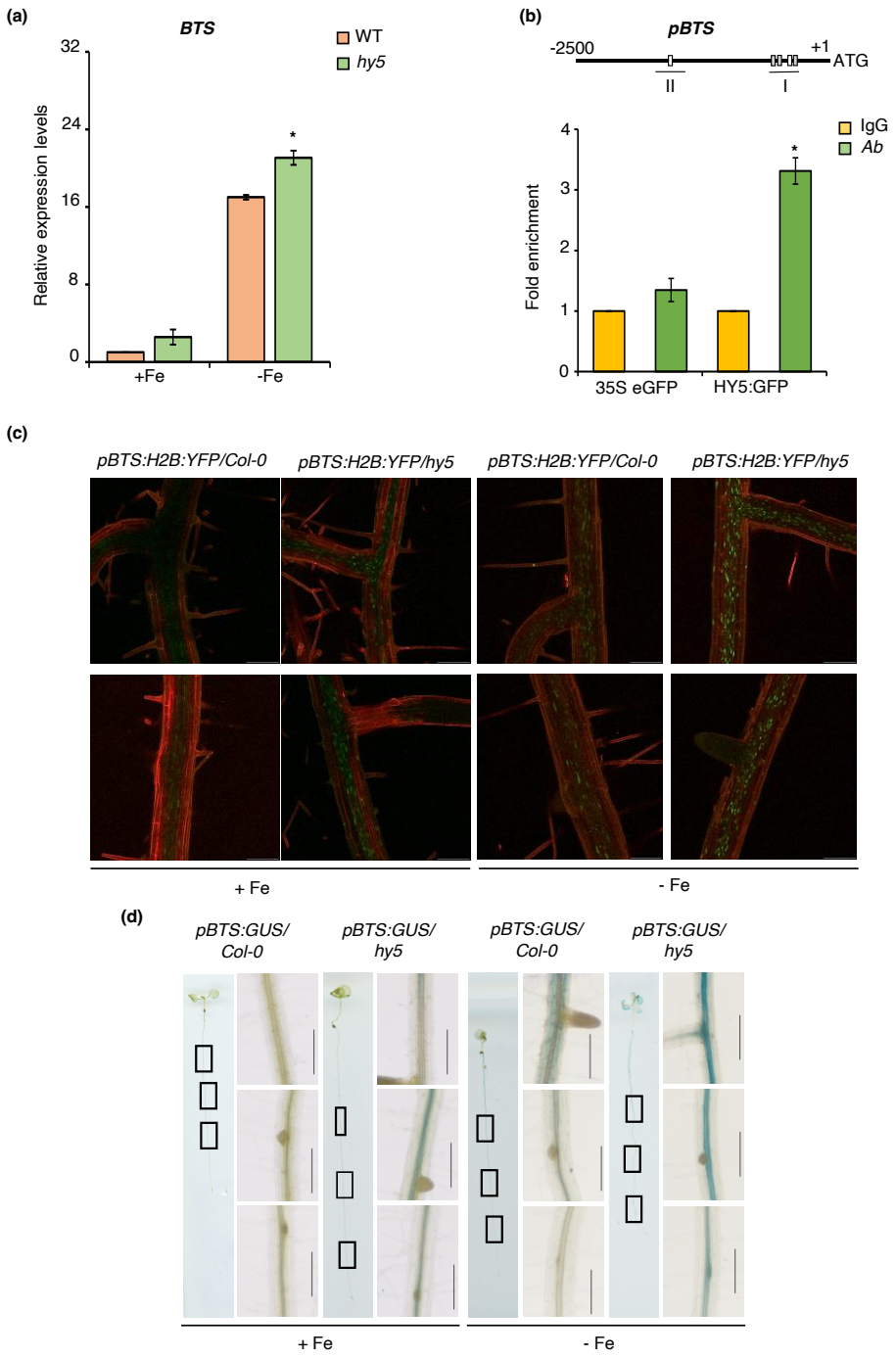

Figure 2.12. *HY5* directly binds on *BTS* promoter and regulates its expression. (a) Expression levels of *BTS*. Relative expression was determined by qRT-PCR in WT and *hy5* mutant seedlings grown on +Fe media for 6 days and transferred to both +Fe and –Fe (+300µM Fz) for three days. Data shown is an average of three biological replicates (n=2 technical replicates). Each biological replicate consists of pooled RNA extracted from ~ 90 seedlings. Error bars represent ±SEM.*Significant difference by Student's t test (P≤0.05). (b) Schematic diagram of the promoter of the *BTS* and ChIP-qPCR showing relative enrichment of the *BTS* regulatory regions bound by HY5. White boxes represent CG-Hybrid, grey boxes represent G-

boxes. Lines under the boxes represent sequences detected by ChIP qPCR. The ChIP assay was performed using *pHY5::HY5:YFP/hy5* and *35S:eGFP* seedlings grown on +Fe media for 10 days. Error bars represent ±SEM. (c) Analysis of *pBTS:H2B:YFP* expression in in WT Col-0 and *hy5* mutant seedlings grown on +Fe and –Fe for 8 days using confocal microscopy (d) Analysis of *pBTS:GUS* gene activity in WT Col-0 and *hy5* mutant seedlings expressing it. Plants grown under +Fe and –Fe media for 8 days were stained for GUS activity.

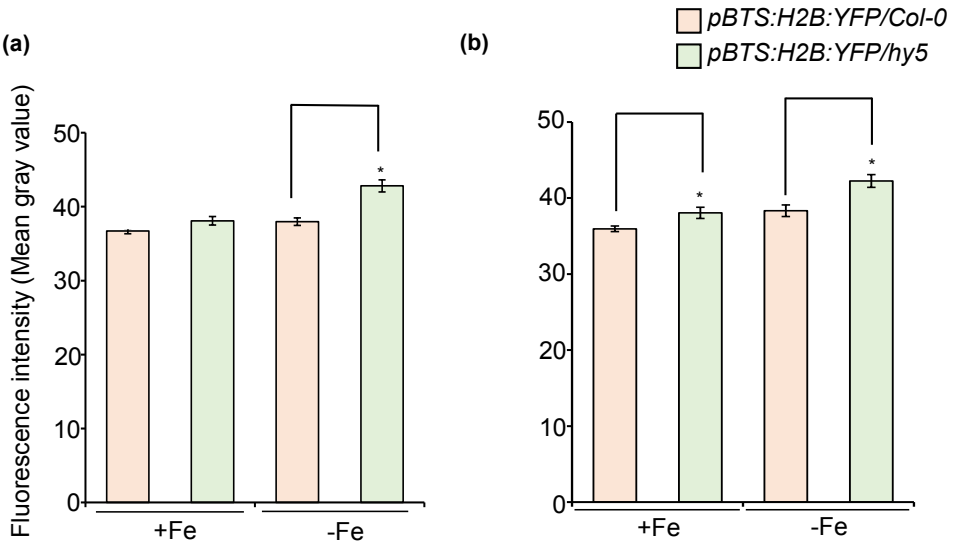

Figure 2.13. Quantification of *pBTS:H2B:YFP* intensity. Intensity was measured from confocal images of both region I (**a**) and region II (**b**) of the maturation zone. Error bars represent ±SEM.*Significant difference by Student's t test (P≤0.05).

To further validate that *BTS* is a direct downstream target of *HY5*, we generated *hy5/bts* double-mutant by crossing *bts* and *hy5* mutant plants. Under control (+Fe) growth conditions, we did not observe a significant difference in the primary root length between the *hy5/bts* double-and the single-mutant. However, under –Fe conditions, the *hy5/bts* double-mutant showed a phenotype similar to *bts* mutant and did not show a decrease in primary root length as *hy5* mutant (Figure 2.14a, b). We also checked the phenotype on alkaline soil, and we found that on alkaline soil the leaves of *hy5/bts* double-mutant were not chlorotic like *hy5* mutant (Figure 2.15a). We also measured the percentage reduction in the chlorophyll content of WT, *hy5*, *bts* and *btshy5* under -Fe conditions as compared to the +Fe. We found that the percentage reduction in the chlorophyll content was highest in case of *hy5* mutant, lowest in case of *bts* but in case of *btshy5* double-mutant the reduction was similar to WT (Figure 2.15b). The Fe

levels were also found to be highest in the *bts* mutant, lowest in the *hy5* mutant, and similar to the WT in the *hy5/bts* double-mutant (Figure 2.14e,f and Figure 2.15c). These results indicate that *bts* mutation suppresses the *hy5* mutant phenotype or *BTS* is epistatic to *HY5.*

To confirm that the suppression of *hy5* mutant phenotype in the double mutant is because of lack of *bts* expression in the *hy5/bts* double-mutant, we generated three independent *BTS* overexpression lines under *hy5* background (P6, P1 and P3) by transforming *Pro35S:BTS* into the *hy5* mutant. Next, we confirmed the *BTS* expression in the overexpression lines by performing qRT-PCR and found that P1 and P3 showed higher expression level (Figure 2.16). We further assessed the phenotype of *Pro35S:BTS/hy5* plants using two independent T3 lines (P1 and P3), and found that the *Pro35S:BTS/hy5* plants clearly showed a phenotype similar to *hy5* under -Fe (Figure 2.14c, d). We also performed Perls and Perls/Dab staining to compare the Fe levels in the WT, *hy5* and *Pro35S:BTS/hy5.* We found that in the *Pro35S:BTS/hy5,* the Fe levels were similar to the *hy5* mutant (Figure 2.14g and h) . Taken together, these results confirm that *HY5* functions upstream to *BTS*.

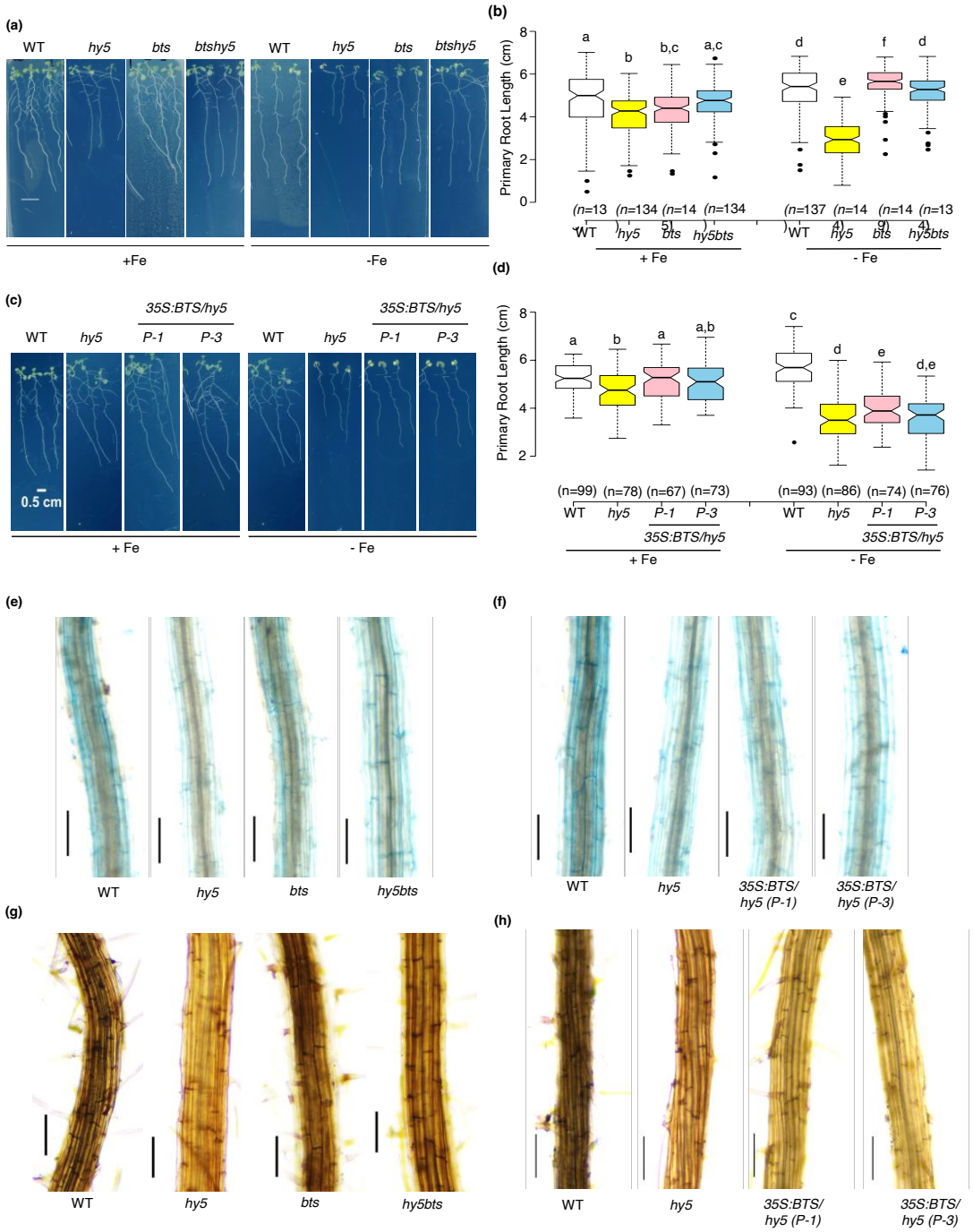

Figure 2.14. *HY5* acts upstream to BTS. (a) Phenotypes of the *Arabidopsis* wild type (WT), *hy5*, *bts* and *hy5bts* grown for 10 days on Fe-sufficient and Fe-deficient medium. Scale bars, 0.5 cm. (b) Boxplot of root length of the wild type (WT), *hy5*, *bts* and *hy5bts* grown for 10 days on Fe-sufficient and Fe-deficient medium. (c) Phenotypes of the *Arabidopsis* wild type (WT), *hy5* and *35S:BTS/hy5 (P-1 and P-3)* grown for 10 days on Fe-sufficient and Fe-deficient medium. (d) Boxplot of root length of the wild type (WT), *hy5* and *35S:BTS/hy5 (P-1 and P-3)* grown for 10 days on Fe-sufficient and Fe-deficient medium. Means within each condition with the same letter are not significantly different according to one-way ANOVA followed by

post hoc Tukey test, P < 0.05. (e, f) Perls stained and (g, h) Perls/Dab stained maturation zone of WT, *hy5*, *bts* and *hy5bts* grown on Fe-sufficient medium for 5 days.

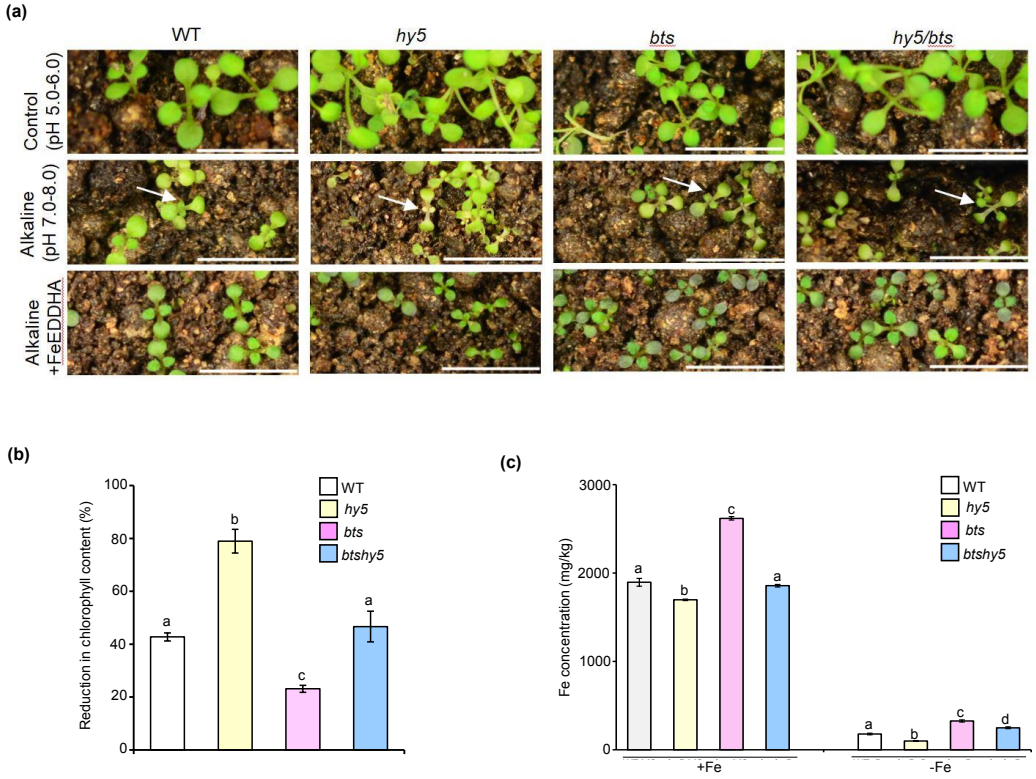

Figure 2.15. *bts* mutation suppresses the *hy5* mutant phenotype. (a) Phenotypes of the WT, *hy5, bts* and *hy5bts* grown on normal soil (pH 5.0-6.0), alkaline soil (pH 7.0-8.0) and alkaline soil watered with Fe-EDDHA (Ethylenediamine di-2-hydroxyphenyl acetate ferric) for two weeks. Bars =10mm. White arrows indicate true leaves undergoing chlorosis**. (b)** Percentage reduction in chlorophyll content of the wild type (WT), *hy5*, *bts* and *hy5bts* grown on –Fe as compared to those grown on +Fe**. (c)** Fe content of seedlings grown on both +Fe and –Fe for 2 weeks. Means within each condition with the same letter are not significantly different according to one-way ANOVA followed by post hoc Tukey test, P < 0.05.

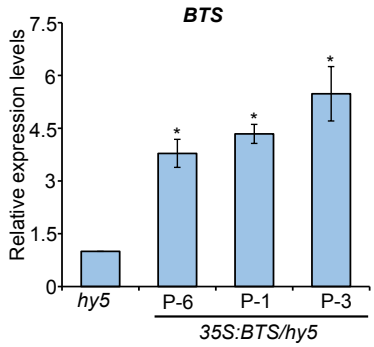

Figure 2.16. Overexpression of *BTS* in the *hy5* mutant. qRT-PCR analysis of *BTS* expression in *hy5* mutant and BTS overexpression lines under *hy5* mutant background (P-6, P-1 and P-3). Relative expression was determined by qRT-PCR in *hy5* and *35S:BTS/hy5* (P-6, P-1 and P-3) seedlings grown on +Fe media for one week. Data shown is an average of two biological replicates (n=2 technical replicates). Each biological replicate consists of pooled RNA extracted from ~ 90 seedlings. Error bars represent ±SEM.*Significant difference by Student's t test (P≤0.05).

2.3.4 Expression of Fe-mobilizing coumarin biosynthesis genes is affected in the *hy5* mutant under Fe-deficiency.

HY5 is known to induce the expression of flavonoid biosynthesis genes and promote flavonoid accumulation (Oyama *et al.*, 1997; Holm *et al.*, 2002; Shin *et al.*, 2007; Song *et al.*, 2008; Stracke *et al.*, 2010). The coumarin production and secretion increases under Fe-deficiency. Because FCR activity and the expression of Fe-uptake genes was compromised in the *hy5* mutant under Fe deficiency, we also analysed the expression of genes involved in coumarin biosynthesis and secretion. Our data indicates that induction of *F6'H1* (the gene which is involved in the first step of coumarin biosynthesis) expression in response to Fe deficiency was significantly reduced in the *hy5* mutant as compared with the WT plants (Figure 2.17a) (Rodríguez-Celma *et al.*, 2013; Schmid *et al.*, 2014). The same pattern was observed for *S8H* and *CYP82C4*, which play a key role in the biosynthesis of fraxetin and sideretin (the main Fe-mobilizing coumarins) respectively (Figure 2.17a) (Rajniak *et al.*, 2018; Siwinska *et al.*, 2018; Tsai *et al.*, 2018). We next analysed the expression of *BGLU42* (β-glucosidase) and *PDR9* (ABC transporter), which are involved in the secretion of coumarins in the rhizosphere (Fourcroy *et al.*, 2014; Zamioudis *et al.*, 2014). *BGLU42* expression was reduced in the *hy5* mutant under Fe-deficiency, but *PDR9* expression was not affected significantly in the *hy5*

mutant compared to the WT (Figure 2.17b). The expression levels of *MYB10* and *MYB72* that regulate the expression of some of the coumarin biosynthesis and secretion genes was not affected in the *hy5* mutant as compared to the WT (Figure 2.17c) (Palmer *et al.*, 2013; Zamioudis *et al.*, 2014). These results indicate that expression of Fe-mobilizing coumarin biosynthesis genes is affected in the *hy5* mutant under Fe deficiency.

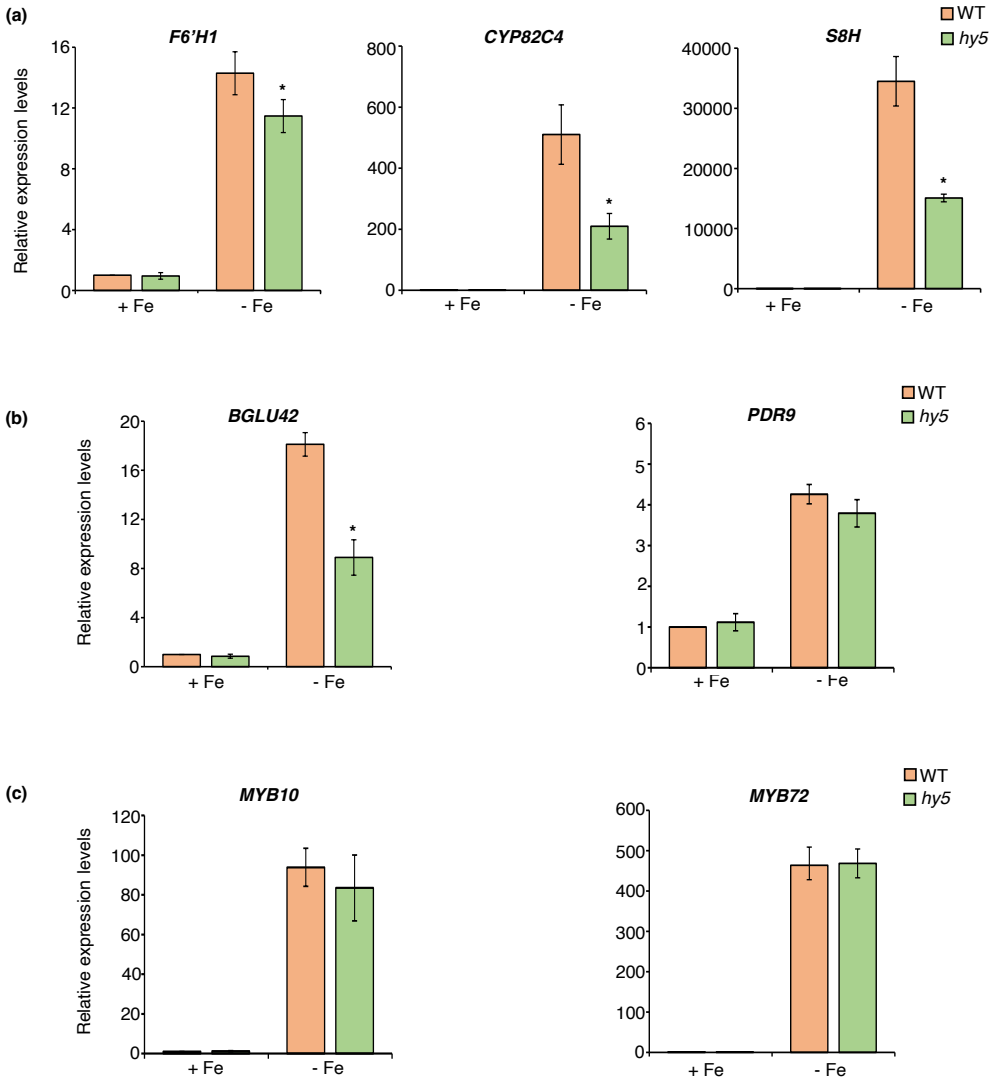

Figure 2.17. **Expression of coumarin-related genes in the *hy5* mutant.** (a) Expression levels of *F6'H1*, *CYP82C4* and *S8H* involved in Coumarin biosynthesis. (b) Expression levels of *BGLU42* and *PDR9* involved in coumarin secretion. (c) Expression levels of *MYB10* and *MYB72* genes involved in transcriptional control of coumarin biosynthesis and secretion. Relative expression was determined by qRT-PCR in WT and *hy5* mutant seedlings grown on +Fe media for 6 days and transferred to both +Fe and –Fe (+300µM Fz) for three days. Data

shown is an average of three biological replicates (n=2 technical replicates). Each biological replicate consists of pooled RNA extracted from roots of ~ 90 seedlings. Error bars represent ±SEM.*Significant difference by Student's t test (P≤0.05).

Coumarins are known to fluoresce when exposed to 365nm UV light (Dorey *et al.*, 1997; Ahn *et al.*, 2010). We analysed the fluorescence in the roots of *hy5* mutant, and compared with the WT under both +Fe and -Fe conditions. In WT seedlings, fluorescence increased under -Fe conditions, but in the case of *hy5* mutant fluorescence was reduced under -Fe conditions, which is in agreement with the qRT-PCR results (Figure 2.18). These observations clearly suggest that root secreted fluorescent coumarins were reduced in the *hy5* mutant as compared with WT under -Fe conditions.

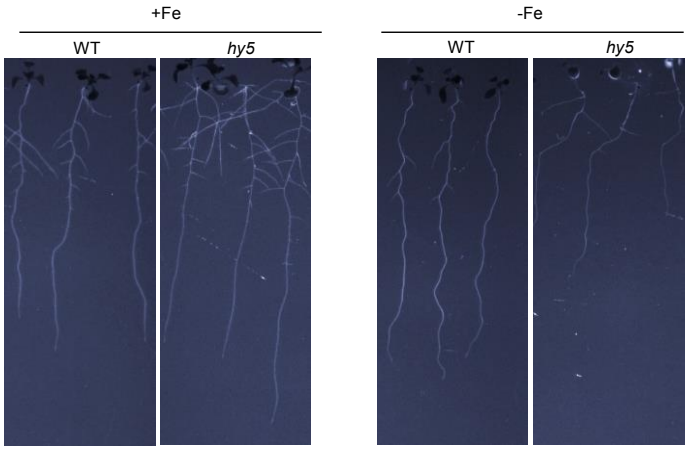

Figure 2.18. Roots of *hy5* mutants show reduced fluorescence under -Fe. Visualization of fluorescent phenolic compounds produced and secreted by the roots of 10 day old WT and *hy5* seedlings grown for 10 days on +Fe and –Fe. The contrast/brightness was adjusted for good visualization.

2.3.5 Discussion

Plants utilize transcriptome reprogramming to maintain Fe homeostasis under varying Fe conditions. Several of the bHLH family TFs play an important role in regulating the expression of genes involved in maintaining Fe homeostasis (Hindt *et al.*, 2012). In *Arabidopsis*, *HY5* is known to regulate expression of genes involved in sulphur, nitrogen and copper uptake. It regulates sulfur uptake by activating the expression of *ADENOSINE 5'-PHOSPHOSULFATE REDUCTASE 1 (APR1)*, *APR2* and *SULFATE TRANSPORTER 1;2 (SULTR1;2)* involved in sulfur uptake (Lee *et al.*, 2011). *HY5* along with *HYH* promotes nitrogen signalling by positively regulating *NITRATE REDUCTASE 2* (*NIA2*) and *NITRITE REDUCTASE 1* (*NIR1*) and negatively regulates nitrate uptake genes *NITRATE TRANSPORTER 1.1* (*NRT1.1*) and

AMMONIUM TRANSPORTER 1;2 (*AMT1;2*). *HY5* regulates copper signalling through SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 7 (*SPL7*), which regulates the expression of copper uptake genes (Zhang *et al.*, 2014).

In our study, we demonstrate that HY5 plays a vital role in regulation of Fe deficiency responses in *Arabidopsis*. Our phenotypic analysis revealed that *hy5* mutant is more sensitive to -Fe conditions. We found that *hy5* mutant has significantly shorter roots and higher reduction in the chlorophyll content as compared to the WT plants under -Fe conditions (Figure 2.1a, b, c). Furthermore, the root length phenotype was partially rescued in the complementation lines *ProHY5:HY5:eGFP/hy5* (P-13 and P-8) (Figure 2.1a, b). The percentage reduction in chlorophyll content under -Fe was not significantly different in the complementation lines *ProHY5:HY5:eGFP/hy5* (P-13 and P-8) as compared to the *hy5* mutant. In the alkaline soil also, we found that *hy5* mutant was more chlorotic and the chlorotic phenotype was partially rescued in the complementation *ProHY5:HY5:eGFP/hy5* (P-13 and P-8) lines. Moreover, we found that the chlorotic phenotype of the *hy5* mutant was recovered when exogeneous Fe was supplied (Figure 2.5). Importantly, the *hy5* mutant root length as well as chlorotic phenotype was completely rescued in the *35S:HA:HY5/hy5* (Figure 2.2a, b, c). Taken together, these results indicate that *hy5* mutant is more sensitive to -Fe conditions, or HY5 positively regulates root growth and chlorophyll synthesis under -Fe conditions.

The Fe content analysis by Perls and Perls/Dab staining revealed that *hy5* mutant has less Fe as compared to the WT plants, and it was found to be recovered fully in *35S:HA:HY5/hy5* line (Figure 2.1d, e and Figure 2.2d, e). This indicates that *HY5* is essential for Fe accumulation in roots, and this reduced accumulation of Fe in *hy5* mutant might explain the growth defects observed under Fe-deficiency.

HYH (*HY5* homolog) is known to play overlapping roles and act redundantly with *HY5* in the regulation of hypocotyl and lateral root growth (Holm *et al.*, 2002; Gangappa *et al.*, 2016). In nitrogen signalling, *HYH* is known to act with *HY5* in positively regulating the expression of nitrate reductase involved in nitrogen assimilation (Jonassen *et al.*, 2008). Our observation that *hyh* mutant has a phenotype similar to WT under Fe-deficiency suggests that *HY5* functions independently of *HYH* in regulation of Fe-deficiency responses in *Arabidopsis* (Figure 2.4). Very recently, it has been shown that HY5 regulates Fe-uptake in a PHYB-dependent manner in tomato (Guo *et al.*, 2021). HY5 activates the expression of *FER,* which leads to upregulation of transcripts involved in Fe-uptake (Guo *et al.*, 2021). Our phenotypic analysis show that

phyB-9 mutant phenotype is not affected by Fe status, which suggests that *PHYB* function is not critical in the regulation of Fe-deficiency responses in *Arabidopsis* (Figure 2.6).

The Fe-deficiency responses are governed by a complex regulatory network. FIT is one of the crucial regulators of the Fe deficiency responses. It is upregulated at transcriptional level under Fe-deficiency. It interacts with bHLH Ib TFs to trigger the expression of Fe-uptake-associated genes (i.e. *IRT1* and *FRO2*). Here, we found that *HY5* positively regulates the expression of *FIT* as well as *IRT1* and *FRO2* under Fe deficiency (Figure 2.7a, d). Similarly, the FCR activity was also found to be less induced in the *hy5* mutant as compared to the WT under -Fe conditions (Figure 2.9). We also found that HY5 directly binds to the promoter of *FRO2* (Figure 2.11b).

Fe homeostasis in plants is tightly regulated to supply required amounts of this element for an optimal growth while avoiding excess accumulation to prevent oxidative stress. Transcriptional regulatory cascade plays important role controlling Fe homeostasis, and the expression of genes involved in Fe-uptake is induced under Fe deficiency and turned off under Fe-excess conditions. BTS, an E3 ubiquitin ligase, plays an important role in maintaining Fe homeostasis by promoting degradation of bHLH105 and bHLH115 under Fe-sufficient conditions. Both bHLH105 and bHLH115 positively regulate the expression of Fe-uptake genes (Selote *et al.*, 2015). *BTSL1* and *BTSL2* are two *BTS* paralogs, and they also negatively regulate Fe-uptake by targeting FIT for degradation (Hindt *et al.*, 2017; Rodríguez-Celma *et al.*, 2019). The regulation of *BTS* expression is important to avoid Fe overload. In our study, we found that HY5 binds to the *BTS* promoter and negatively regulates *BTS* expression under Fe-deficiency (Figure 2.12a, b). We also demonstrate that the *bts* mutation in the *hy5* mutant background suppresses *hy5* mutant phenotype, suggesting that *BTS* acts downstream to *HY5* (Figure 2.14a, b). Based on these results, we hypothesized that the HY5 inhibits BTS under Fe-deficiency because in the *hy5/bts* double mutant, the *hy5* mutant phenotype is rescued. This was further supported by the sensitive phenotype of *35S:BTS/hy5* (*BTS* overexpression in the *hy5* mutant) similar to the *hy5* mutant under -Fe (Figure 2.14c, d). Altogether, these results indicate that HY5 acts upstream to *BTS* in the Fe-signalling pathway and negatively regulates its expression. Similarly, we also found that HY5 negatively regulates *PYE* expression directly under -Fe (Figure 2.7c and Figure 2.11b). Further studies are required to explore the impact of HY5 on genes regulated by PYE under -Fe.

Coumarins have recently emerged as key players for Fe-uptake particularly in high pH (Robe *et al.*, 2021). They aid in the mobilisation of Fe in the soil (Tsai *et al.*, 2017). The production

as well as the secretion of coumarins into the rhizosphere increases under Fe deficiency. The expression of coumarin biosynthesis genes (i.e. *CYP82C4* and *S8H*) is activated by FIT/bHLH Ib complexes (Schmid *et al.*, 2014; Tsai *et al.*, 2017; Tsai *et al.*, 2018). The MYB10 and MYB72 TFs as well as FIT/bHLH Ib complexes regulate the expression of *F6'H1* involved in coumarin biosynthesis as well as *BGLU42* and *PDR9* involved in coumarin secretion (Palmer *et al.*, 2013; Tsai *et al.*, 2017). In our study, we found that *hy5* mutant showed lower induction of *CYP82C4, S8H, F6'H1* and *BGLU42* as compared with WT under Fe-deficiency that resulted in lower coumarin production and secretion in *hy5* mutant (Figure 2.17a, b and Figure 2.18).

HY5 is known to suppress auxin and ethylene signaling by negatively regulating auxin signaling pathway and ethylene biosynthesis, respectively (Cluis *et al.*, 2004; Sibout *et al.*, 2006; Li *et al.*, 2011). Ethylene is also known to enhance HY5 degradation by increasing COP1 accumulation in the nucleus (Yu *et al.*, 2013). Further research is required to completely understand whether the auxin and ethylene signals inducing the expression of Fe-uptake genes under Fe-deficiency are HY5 dependent or independent.

Based on our results and existing literature, we propose a model depicting the crucial involvement of HY5 under Fe-deficiency responses in *Arabidopsis* (Figure 2.19). HY5 positively regulates the expression of *FIT* as well as other downstream genes such as *IRT1* and *FRO2* that are involved in Fe-uptake, and *S8H, CYP82C4, F6'H1, BGLU42* involved in coumarin biosynthesis and secretion. Additionally, *HY5* acts as a negative regulator of *BTS and PYE*. Importantly, it directly binds to the promoter of *BTS*, *PYE* and *FRO2* to regulate their expression.

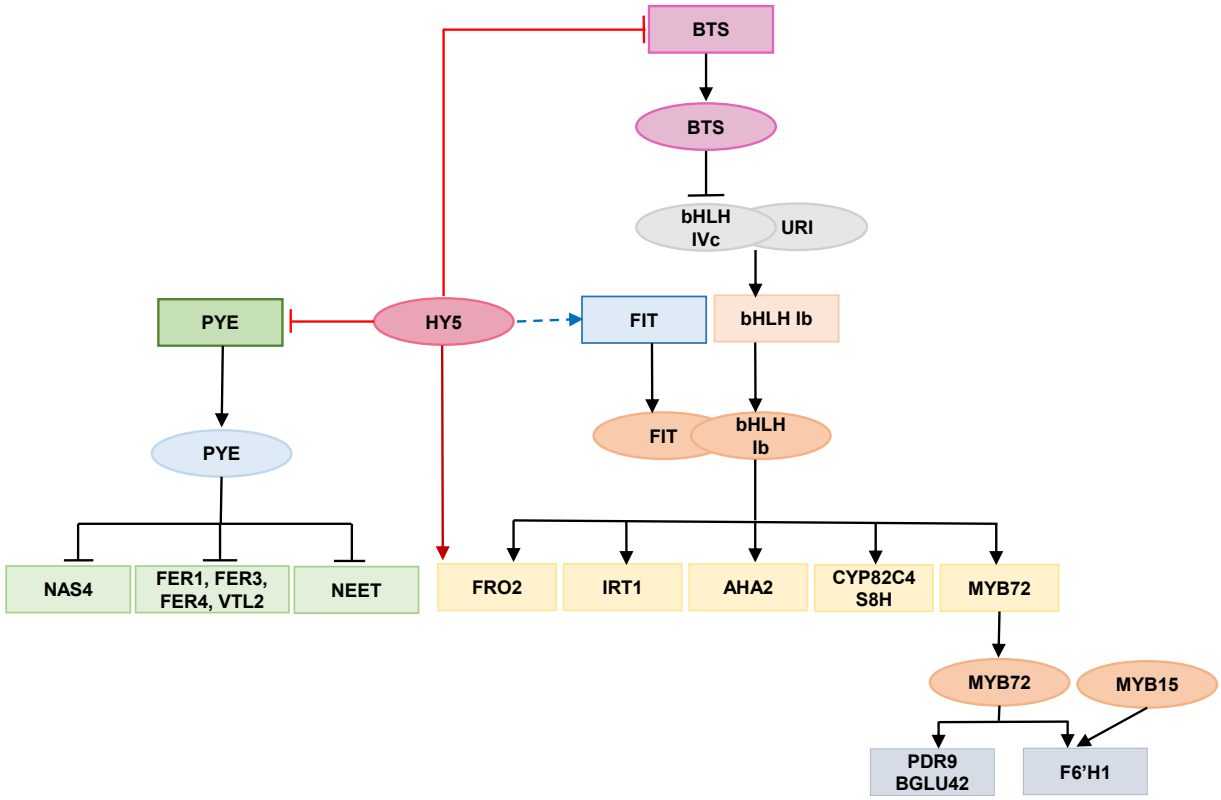

Figure 2.19. Model describing the role of HY5 in maintaining Fe acquisition and homeostasis. HY5 negatively regulates the expression of *BTS* and *PYE*, and positively that of *FRO2* directly. It acts upstream of *BTS* and negatively regulates its expression. BTS is known to degrade bHLh IVc TFs which activate the expression of bHLH Ib TFs under Fe-deficient conditions. These Ib bHLH TFs along with FIT, either directly or indirectly, positively regulates the expression of many genes involved in Fe acquisition, such as *IRT1*, *FRO2* (involved in Fe uptake) and *CYP82C4*, *S8H*, *BGLU42* and *F6'H1* (involved in coumarin biosynthesis). *HY5* negatively regulates PYE expression which is known to repress *NAS4*; *FER1, FER3, FER4, VTL2*; and *NEET* (involved in Fe transport, storage and assimilation respectively). Oval boxes represent protein and rectangular boxes represent mRNA. Dotted lines represent the possible mechanism of regulation that are also in agreement with the data.

Several studies have revealed that HY5 can be both an activator and a repressor (Ang *et al.*, 1998; Lee *et al.*, 2007; Ruckle *et al.*, 2007; Kindgren *et al.*, 2012; Delker *et al.*, 2014; Xu *et al.*, 2016; Gangappa *et al.*, 2016; Norén *et al.*, 2016; Zhang *et al.*, 2017; Gangappa *et al.*, 2017; Nawkar *et al.*, 2017; Burko *et al.*, 2020; Yadukrishnan *et al.*, 2020). Our results so far

clearly indicates that HY5 acts both an activator and a repressor to regulate the genes involved in Fe regulatory network.

In summary, our findings using gene expression analysis, genetic analysis and ChIP experiments provide novel evidence that HY5 regulation of *BTS* is critical for the Fe-deficiency responses in *Arabidopsis*. HY5 functions as a direct transcriptional repressor of *BTS.* Additionally, HY5 also negatively regulate the expression of *PYE* directly under Fe-deficiency. On the other hand, HY5 induces the expression of *FRO2* directly. Several other key genes (*S8H, CYP82C4, F6'H1,* and *BGLU42)* involved in the Fe regulatory network are also positively regulated by HY5 either by directly binding to their promoter or with the help of other factors. Further experiments will be required to understand whether HY5 directly regulates the other key genes involved in Fe regulatory pathway in addition to *BTS, PYE* and *FRO2*. Moreover, interaction studies with bHLH TFs involved in Fe-signalling pathway will shed the light on whether HY5 interacts with them and regulates the genes involved in Fe homeostasis. The knowledge gained on HY5 function and its role in Fe homeostasis by regulating *BTS* in *Arabidopsis* can be further extended to crop plants.

2.3.5 Materials and Methods:

Plant Materials and Growth Conditions

In this study, Columbia (Col-0) and Wassilewskija (WS-0) ecotypes of *Arabidopsis thaliana* were used as WT. The mutant/transgenic lines used in this study were *hy5* [SALK_056405; (Saini *et al.*, n.d.)*, bts-1* [SALK_016526; (Long *et al.*, 2010)], *pye* [SALK_021217; (Long *et al.*, 2010)] and *phyB-9* ((Reed *et al.*, 1993) *35S:HA:HY5/hy5* (Job *et al.*, 2018) in the Col-0 background. In addition, following mutant lines were kindly provided by Roman Ulm: *hy5, hyh* and *hy5/hyh* in the WS background and *pHY5:HY5:YFP/hy5* in the Ler background. *hy5* single-mutant was crossed with *bts-1* to generate *hy5/bts* double-mutant. The *hy5* allele was also introduced into *pIRT1:GUS* line (Blum *et al.*, 2014) and *pBTS:GUS* line (Selote *et al.*, 2015) by crossing. The single- as well as double-mutant were confirmed by genotyping. The primers used for genotyping are listed in Table 2.1. Seeds were surface sterilized using 5% sodium hypochlorite followed by 70% ethanol and stratified in dark at 4 ºC for 3 days. Plants were grown on ½ Murashige and Skoog media (Caisson labs, USA) with Fe (+Fe) or ½ Murashige and Skoog media (Caisson labs, USA) without Fe (-Fe). The pH of media was adjusted to 5.7 with KOH. To create Fe deficiency conditions, 300µM of [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate] Ferrozine (Sigma-Aldrich) was added in the -Fe media. For seedling growth, plants were grown under long-day conditions, 16 h light and 8 h dark at 22ºC

with 50% humidity and a light intensity of 100-110 µmol/cm^2/s. Soil was made as a mixture of soilrite, perlite and compost (3:1:1). CaO (Sigma-Aldrich) (7.8 gm CaO/kg) was mixed in the soil to generate alkaline pH soil.

Phenotypic Analyses

For phenotypic characterization, seeds were sown on ½ MS media with Fe (+Fe) and also on ½ MS media without Fe (-Fe). The plates were kept vertically in the growth chamber, and 10 -day-old seedlings growing on the plates were scanned using Epson Perfection V600 with 1200dpi resolution. The root length was quantified using ImageJ 1.52a software (National Institutes of Health).

Chlorophyll Content Measurement

The chlorophyll content was measured using seedlings grown on +Fe and -Fe for 10 days. It was measured by extracting chlorophyll using 1ml of 80% acetone from leaf tissues of five to six seedlings and incubating in dark for 24 hours using the formula (mg/g) = $\underline{(20.3 \times A_{645} + 8.04 \times A_{663}) \times V}$/ W X 10^3 (Aono *et al.*, 1993).

FCR Assay

The FCR assay was performed as previously described with some modifications (Ying Yi *et al.*, 1996) . Seeds were grown for 5 days on +Fe, and then transferred to both +Fe and -Fe (+300 µM Ferrozine) for 3 days. Roots from 30-40 seedlings were collected in 700 µl assay solution consisting of 0.1mM Fe (III)-EDTA and 0.3mM ferrozine in distilled water. An identical assay solution without roots was used as blank. The FCR activity is determined by taking absorbance at 562 nm with the help of a spectrophotometer using the formula (µM Fe (II)/g root FW/hr = (A/28.6) x V/ Root FW.

Fe histochemical staining

For Fe staining, 5-day-old seedlings grown on +Fe were vacuum infiltrated with a solution containing 1% (v/v) HCL and 1% (w/v) K-ferrocyanide for 5 minutes. Seedlings were then washed with water five times, observed and photographed using NIKON ECLIPSE Ni U microscope.

For diaminobenzidine (DAB) intensification, after Perls staining the seedlings were kept for incubation in methanol that contains 10mM Na-azide and 0.3% (v/v) H_2O_2 for 1 hour. The seedlings were washed with 100mM Na phosphate buffer (pH 7.4) and kept for incubation in

the same buffer which contains 0.025% (w/v) DAB and 0.005% (v/v) H_2O_2. The seedlings were washed with water to stop the reaction. The stained seedlings were then photographed using NIKON ECLIPSE Ni U microscope.

Iron measurements

Elemental analysis of the root tissues was performed using inductive coupled plasma-mass spectrometry (ICP-MS, Agilent, USA). The tissue samples were first dried at 60°C overnight, and 100 mg of finely grounded tissue was subsequently acid digested (HNO_3) in the microwave-assisted digestion system. The digested samples were diluted using dilution factor 40x with 5% HNO_3. After dilution, the elementals concentrations of Fe was estimated. At least two biological replicates were used for each treatment and genotypes.

RNA Extraction and qRT-PCR

Total RNA was extracted from roots of seedlings grown on ½MS for 6 days and transferred to +Fe or -Fe for 3 days using Plant RNeasy kit (Qiagen) following manufacturer's protocol and digested with DNase I to remove genomic DNA. cDNA was synthesised using RevertAidTM First Strand cDNA Synthesis Kit (Thermo) from RNA (2µg). qPCR was performed using a LightCycler 480 II (Roche) and TB GreenTM Premix Ex. The β-tubulin was used as a reference gene and relative expression levels were calculated using comparative threshold cycle method *(ΔΔCT*). The primers used are listed in Table 2.2.

Histochemical GUS Staining

The GUS staining was done using GUS-expressing seedlings grown on +Fe or -Fe for 8 days. For visualization of GUS expression, seedlings were incubated in staining solution (100 mM Na_2HPO_4, 100 mM NaH_2PO_4, 2 mM $K_4[Fe(CN)_6]$, 2 mM $K_3[Fe(CN)_6]$, 0.2 Triton X-100, and 2 mM 5-bromo-4-chloro-3-indolyl glucuronide, pH 7.0) and vacuum was applied for 25 minutes followed by incubation at 37°C for 2 to 3 h. After incubation, the seedlings were stored in 70% ethanol before imaging. Images were taken using NIKON ECLIPSE Ni U microscope.

Confocal Microscopy

For confocal microscopy, seedlings grown on ½ MS for 7 days were stained with 10 µM propidium iodide for 45 seconds. For GFP excitation, a 488nm laser was used, and propidium iodide was excited with 561nm laser, and emission spectra were collected at 500-530 nm and

600-650nm, respectively. Imaging was performed in Z-stack mode with 1-µm step size using a SP8 upright confocal microscope (Leica).

Immunoblots

Protein was isolated from seedlings grown on +Fe for 4 days, and transferred to both +Fe and -Fe (+300µM Fz) for 3 days. The seedlings were harvested and crushed using liquid nitrogen and 50 mM TRIS-HCl (pH 6.8), 50 mM DTT, 4% (w/v) SDS, 10% (v/v) glycerol, 1% (w/v) polyvinylpolypyrrolidone (PVPP), 5 mM PMSF. The samples were vortexed followed by centrifugation at 14,000 rpm for 15 min. The protein concentration was determined using Bradford assay. For HY5 detection (Chai *et al.*, 2015), 50 µg of protein was loaded on 12% SDS-polyacryl-amide gel electrophoresis gel, and gel was run at 0.3mA at RT for 1 hr 40 min. The separated proteins were then transferred to poly-vinylidene difluoride (PVDF) membrane (10V, 1 hr 30 min). The PVDF membrane was blocked with 5% (w/v) milk powder and 1% (w/v) polyvinylpyrrolidone (PVP-40) in TRIS-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 1 hr followed by incubation with primary antibody anti-HY5 (+Agrisera, AS12-1867) diluted 1:1000 in TBST at 4ºC overnight. Membrane was washed in TBST for 1 hr. After washing, membrane was incubated with an HRP-conjugated secondary antibody (Sigma-Aldrich; AP307P) diluted 1:10,000 in TBSTM. Membrane was washed again for 1hr followed by detection with ECL select HRP substrate (Bio-Rad; #170-5060).

Fluorescence

Seeds were sterilized and sown on +Fe and -Fe for 10 days. Fluorescence of 10-day-old plants was observed using a Gel Doc XR+ System (Biorad) with an excitation wavelength of 365nm (Vanholme *et al.*, 2019).

Plasmid constructs and transgenic lines

For overexpression lines, the coding sequence of *BTS* was amplified from cDNA. The forward primer was designed with a CACC overhang to facilitate directional cloning of the CDS fragment into pENTR/D-TOPO. The resulting clone was sequence verified and used to set up an LR reaction with the destination vector (pMDC32) to generate *35S:BTS*. The construct was introduced into *Agrobacterium tumefaciens GV3101* and transformed into WT Col-0 background by the floral dip method. Single gene insertions were detected by T2 3:1 segregation ratio on media containing hygromycin followed by homozygous line selection at T3.

For *pHY5:HY5:eGFP* construct, the *HY5* gene was amplified from cDNA and cloned in pENTR/D-TOPO. The resulting clone was digested with PspOMI and StuI, and the *HY5* coding sequence was cloned at the N terminus of eGFP in pENTR/D/TOPO eGFP vector. The native promoter (750 bp upstream of the translational start codon) was amplified from gDNA and cloned into SacI and KpnI restriction sites in pAtExp7 containing attR1 and attR2 recombination sites. The resulting clone was used to set up a LR reaction with pENTR/D/TOPO HY5-eGFP to create *pHY5:HY5-eGFP,* which was then transformed into *hy5* mutant. The primers used are listed in Table 2.3.

ChIP-qPCR

The ChIP was performed according to the method described previously (Gendrel *et al.*, 2005). *pHY5:HY5:YFP/ hy5* and *35S:eGFP* in the WT Ler background grown and germinated on ½ MS media for 7 days and then transferred to ½ +Fe and ½ -Fe for 3 days were fixed using 1% formaldehyde. Nuclei isolation was done followed by shearing using a Qsonica 800R ultrasound sonicator with 30 cycles of 70% pulse amplitude for 15sec followed by 45sec pulse off time. The protein-A magnetic beads (1614013, SureBeads) and anti-GFP antibody (a290, Abcam) were used to pull down HY5-DNA complexes. Rabbit IgG was used as a negative control. The immunocomplexes bound to beads were washed and eluted from beads followed by reverse crosslinking. The qPCR was set up using precipitated chromatin. The fold enrichment was determined by normalizing against negative control (IgG). The primers used are listed in Table 2.4.

Statistical Analysis

Data was expressed as means ± SEM. P-value was calculated by student's t test or one-way ANOVA followed by post-hoc Tukey HSD Test. P-value ≤ 0.05 was considered as statistically significant.

Acknowledgments

The authors thank Roman Ulm for providing WS*, hyh, hy5*, *hyh/hy5* and *pHY5:HY5:YFP/hy5* (Ler) seeds. The authors are grateful to Rumen Ivanov and Petra Bauer for providing the *pIRT1:GUS* seeds; Terry A. Long for *pye*, *bts* and *pBTS:GUS;* and Janneke Balk for *bts* seeds. The authors are also thankful to Ferenc Nagy, Maria Agustina Mazzella, Saurav Datta and Sreeram Gangappa, for *phyB-9* seeds. The authors thank Prince Saini and Asis Kumar for their technical help in qRT-PCR and ChIP-qPCR experiments. The authors are grateful to all the

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Author contributions:

SM and SBS conceived the study and designed the experiments. SM performed all the experiments with the help from DS, KM, MK, HM and VM. SM, SBS, RKY and AKP analysed the data. SM prepared the figures and wrote the first draft. SBS supervised the work, edited the manuscript, and provided the overall direction. All authors discussed the results and commented on the manuscript.

Tables

Table 2.1. Primers used in genotyping.

bts-1, LP	CCAAATGCGTTCGTAGGTAAG
bts-1, RP	TCAGATTTACACAAATTTGCAGC
hy5, LP	TTCACTCTCGATATCCGTTCG
hy5, RP	ATGCGAGTGAATGACCATTTC
LBb1.3	ATTTTGCCGATTTCGGAAC

Table 2.2. Primers used for qRT-PCR.

qbHLH38 F	ACGGTGCCGGAGATAACCTA
qbHLH38 R	GTCGGTCACGTTCACTAGCA
qbHLH39 F	CCGTTCATGTCTTCCTGCCT
qbHLH39 R	GCCTTTGGTGGCTGCTTAAC
qbHLH100 F	CTCCCACCAATCAAACGAAGAAG
qbHLH100 R	TGTTTTGGTCGGTGTAAACGAG
qbHLH101 F	AAGAAGATCGAGGAGCGGTG

qbHLH101 R	TGTTTTGGTCGGTGTAAACGAG
qRT PYE FP	CAGGACTTCCCATTTTCCAA
qRT PYE RP	CTTGTGTCTGGGGATCAGGT
qRT FRO2 FP	GCCACATCTGCGTATCAAGTT
qRT FRO2 RP	TCCCAAACAAGCTACGACCA
qRT FIT FP	CAGTCACAAGCGAAGAAACTCA
qRT FIT RP	CTTGTAAAGAGATGGAGCAACACC
qRT BTS FP	GCTCTGGCACAAGTCAATCA
qRT BTS RP	CGTTCATCAAATGCCGATAA
qRT IRT1 FP	GAATGTGGAAGCGAGTCAGCGA
qRT IRT1 RP	GATCCCGGAGGCGAAACACTTA
qRT TUBULIN FP	CGACAATGAAGCTCTCTACGA
qRT TUBULIN RP	AAGTCACACCGCTCATTGTT
qRT MYB10 FP	GGGGAAATCTTGGTGGAGCA
qRT MYB10 RP	AGGAGGAACCTGGCTATCGT
qRT MYB72 FP	TCGAGAGGTAACCAAATCGCA
qRT MYB72 RP	CAGCTGTCTCCTCAAGTCGG
qRT NAS4 FP	GGCTTCGACGTTGTGTTCTT
qRT NAS4 RP	AGCAAAGCACCAGGAGACAT
qRT At-NEET FP	TCGTTGTCACCGAGCTTTCC
qRT At-NEET RP	ACGTCCCCGACCTCCAA
qRT’F6'H1 FP	TGATATCTGCAGGAATGAAACG
qRT’F6'H1 RP	GGGTAGTAGTTAAGGTTGACTC
qRT S8H FP	CCGAGACACTTGGCTTCTT
qRT S8H RP	CAGCAGCTCCACCGAAACA
qRT CYP82C4 FP	AGGCTCAGTATCGTCGGAG
qRT CYP82C4 RP	TTTCTATGTCTGAATCCTCGACG

qRT PDR9 FP	GTCTTGGACACTCAACGGGT
qRT PDR9 RP	ATCTTGCAACCGTCGTGGAT
qRT BGLU42 FP	ATGGCCTGGGAACTGAAGTC
qRT BGLU42 RP	ATTTGTCCAACCTCCGATTG
qRT IMA1 FP	ATGTCTTTTGTCGCAAACTT
qRT IMA1 RP	CACCACCATTCTCACTATATG
qRT IMA2 FP	TGCTTCCGTGGTGTATGTTG
qRT IMA2 RP	CAAGAAAACTCGAGACACAATC
qRT IMA3 FP	GGCAGGCTATACGAATCAAC
qRT IMA3 RP	GTTCTATGTCAAGAAGCACA
qRT BTSL1 FP	GGCAATGAAGATGGATTTGG
qRT BTSL1 RP	TCATATGGAACCGTTGCTGA
qRT BTSL2 FP	CGGGGCAGAATCCATCTTAT
qRT BTSL2 RP	GTTGCAACAAGGAGCAAGAAG

Table 2.3. Primers used in Cloning.

pHY5 FP/Sac1	CACCGAGCTCTCTAATGTTAACGTTGAGATGG
pHY5 RP/Kpn1	AAGGTACCTTTTCTTACTCTTTGAAGATCG
HY5 CDS FP	CACCATGCAGGAACAAGCGACTAGCTC
HY5 CDS RP/ StuI*	TAAAAGGCCTAAGGCTTGCATCAGCATTAGAACC
BTS CDS, FP/SpeI	CACCACTAGTATGGCGACGCCGTTACCAGAT
BTS CDS,RP/StuI-STOP	ATAATAGGCCTTCAGGATGAGGTTGAGCAGTCC

Table 2.4. Primers used for ChIP qPCR.

pBTS ChiP FP	CTCCTTCTAACTCCGAGAAC
pBTS ChiP RP	GAAAATGAATAAAAGTGCTTGG
pBTSChiP II FP	GAAAAAAAAGGAATGTGTTG
pBTSChiP II RP	TTGATGAAGTAGAAAATGGTG
pPYE CHIP I FP	GACGTGTCCATGAGAGATGA
pPYE ChiP I RP	TTTTTGGAGGAAGAAGGTCC
pPYE ChiP II FP	TTAAACCACTCTTCCACTCG
pPYE ChiP II RP	TTAAGTGGACACGTGAGTTC
pFRO2 ChIP FP	AACCAGATTCCTTACAACCA
p FRO2 ChIP RP	ATTAGCAACAAGAGTCATAC
pFRO2 ChiP II FP	TGACATATTTTGTTGACACG
pFRO2 Chip II RP	TAAGGAATCTGGTTCGTGTT
pIRT1 ChiP II FP	CAATATGGAAAATCTCCCCA
pIRT1 CHIP II RP	ACCTCCCACGTTTTCATATG
pFIT ChiP RP	GAGTGAGTATGAAATAAACTG
pFIT ChiP FP	CACGACAAATTAATCATTCG
pFIT ChiP II FP	AACAAGAATATATGTGGATC
pFIT ChiP II RP	CTCATAATATATCCTGTTAC

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Chapter 3

ELONGATED HYPOCOTYL 5 interacts with *POPEYE* and regulates iron homeostasis in *Arabidopsis thaliana.*

Chapter 3: ELONGATED HYPOCOTYL 5 interacts with *POPEYE* and regulates iron homeostasis in *Arabidopsis thaliana.*

3.1 Abstract:

Iron (Fe) is an essential trace element for nearly every all organisms. Alterations in Fe uptake, transport, storage, and assimilation in plants leads to defects in their growth and development which ultimately affects plant productivity. In Arabidopsis, Fe homeostasis is regulated by the basic helix-loop-helix (bHLH) family of transcription factors (TFs). Among them, the bHLH TF POPEYE (PYE) plays an essential role in regulating iron transport, storage, and assimilation. Recently, a basic leucine zipper (bZIP) TF, ELONGATED HYPOCOTYL (HY5) has been shown to play an essential role in regulation of Fe uptake and homeostasis. In this study, we report that HY5 interacts with PYE and regulates Fe homeostasis. Genetic analysis suggests that PYE and HY5 have additive roles in regulation of seedling growth under Fe limiting conditions. Transcriptional analysis reveals that HY5 acts as a transcriptional repressor of genes involved in Fe transport including *YSL3, FRD3 NPF5.9, YSL2,* and *OPT3*. Chromatin immunoprecipitation followed by quantitative polymerase chain reaction reveals that HY5 directly binds on the promoter of these genes involved in Fe transport. Further investigation revealed that HY5 and PYE directly interact at the same region on *PYE* and *NAS4* promoter. Taken together, we showed that the interaction between PYE and HY5 is important for maintaining Fe homeostasis under Fe limiting conditions.

3.2 Introduction

Iron (Fe) homeostasis is crucial for optimum growth and development of plants. Fe deficiency in plants affects photosynthesis, nutrient transport, immunity, and mitochondrial respiration which ultimately leads to defects in plant growth and development (Balk and Schaedler, 2014; Hänsch and Mendel, 2009a) . On the other hand, Fe excess is toxic for plants, and it leads to inhibition of root growth, leaf bronzing, necrosis, and biomass reduction (Zhang *et al.*, 2018) . Therefore, in order to maintain Fe homeostasis, plants have various mechanisms which regulate Fe uptake, transport, storage, and assimilation.

In *Arabidopsis thaliana* (Arabidopsis) a reduction-based mechanism also known as Strategy I ensures optimum Fe uptake from the soil (Kobayashi and Nishizawa, 2012b; Brumbarova *et al.*, 2015). The Strategy I is mainly dependent on the activity of ATpase2 (AHA2), FERRIC REDUCTION OXIDASE2 (FRO2) and IRON-REGULATED TRANSPORTER1 (IRT1). The release of H^+ ions in the rhizosphere by AHA2 decreases the pH of the rhizosphere, enhancing

the solubility of insoluble Fe^{3+} present in the soil (Santi and Schmidt, 2009b). The solubility of Fe^{3+} is also enhanced by the coumarins released into the rhizosphere by the PLEIOTROPIC DRUG RESISTANCE (PDR9) transporter (Fourcroy *et al.*, 2014). FRO2 enzyme reduces this soluble Fe^{3+} into Fe^{2+} which is taken inside the cell with the help of IRT1 transporter (Ying Yi and Mary Lou Guerinot, 1996; Nigel j. Robinson *et al.*, 1999; Eide *et al.*, 1996; Vert *et al.*, 2002). Once inside the root, Fe is translocated to the shoot followed by redistribution to sink tissues and excess Fe is stored in the vacuoles to avoid Fe toxicity. Fe is considered to be transported in chelation form with citrate and nicotianamine (NA). Fe is transported from root to shoot through xylem in the form of Fe^{3+}-citrate complex. FERRIC REDUCTASE DEFECTIVE 3 (FRD3) is involved in the transport of citrate in the xylem (Durrett *et al.*, 2007). OLIGOPEPTIDE TRANSPORTER 3 (OPT3) mediates the transport of Fe from xylem to phloem and facilitates Fe distribution from source to sink tissues (Stacey *et al.*, 2002; Stacey *et al.*, 2008). YELLOW STRIPE-LIKE (YSL) proteins are responsible for the transport of Fe-NA complexes. YSL2 mediates the transport of Fe through vascular system (DiDonato *et al.*, 2004). YSL1 and YSL3 are involved in transport of Fe to seeds and senescent leaves (Waters *et al.*, 2006).

The Fe homeostasis is regulated at the transcriptional level by a cascade of basic leucine zipper (bHLH) transcription factors (TFs). The expression of major Fe uptake genes *IRT1, FRO2* and *AHA2* is induced under -Fe which is under the control of FIT and bHLH Ib (bHLH38, bHLH39, bHLH100, and bHLH101) TFs (Yuan *et al.*, 2008; Wang *et al.*, 2013; Liang *et al.*, 2017). The expression of bHLH Ib TFs is also induced under -Fe by the bHLh IVc TFs (bHLH34, bHLH105/ILR3, bHLH104, and bHLH115) and bHLH121/URI (Li *et al.*, 2016; Liang *et al.*, 2017; Kim *et al.*, 2019; Gao, Robe and Dubos, 2020). The bHLh IVc TFs are known to be regulated at the protein level by the activity of BRUTUS (BTS). BTS is a E3 ubiquitin ligase, and it degrades bHLh IVc TFs under Fe sufficient conditions to avoid Fe overload (Selote *et al.*, 2015). Interestingly, under Fe deficient conditions, IRON MAN PEPTIDES (IMAs) interact with BTS which stabilizes bHLh IVc TFs under Fe deficient conditions (Grillet *et al.*, 2018b; Li *et al.*, 2021). POPEYE (PYE), a transcriptional factor belonging to bHLh IVb group is also upregulated under Fe deficiency (Long *et al.*, 2010). PYE is known to act as transcriptional repressor to regulate Fe homeostasis in plants. PYE contains an EAR motif (DLNxxP) in its C-terminal end which is one of the most common forms of transcriptional repression motif found in plants. The PYE-mediated transcriptional repression of genes under Fe deficiency is essential for maintaining Fe homeostasis. PYE is known to interact with ILR3

and directly negatively regulate the expression of *NAS4* (involved in iron transport)*, FER1, FER2, FER4* and *VTL2* (involved in iron storage) and *NEET* (iron assimilation) (Tissot *et al.*, 2019). Recently, we have shown that ELONGATED HYPOCOTYL 5 (HY5), a bZIP TF act as both transcriptional activator and repressor to regulate Fe deficiency responses. HY5 has been found to positively regulate the expression of Fe uptake genes which include *FRO2, IRT1* and *FIT* and negatively regulate the expression of *BTS* and *PYE* (Mankotia *et al.*, 2023). It is known that depending on its binding partner HY5 can function both as a transcriptional activator and repressor (Ang *et al.*, 1998; Delker *et al.*, 2014; Gangappa and Botto, 2016; Gangappa and Kumar, 2017; Burko *et al.*, 2020). However, the binding partners of HY5 in the regulation of Fe homeostasis are not studied at all. Moreover, the role of HY5 in regulation of Fe transport and storage has not been studied till now.

In this study, we performed yeast two-hybrid (Y2H) assay followed by bimolecular fluorescence complementation (BiFC) and found that HY5 interacts with PYE. We found that *pyehy5* double mutant was more sensitive to Fe deficiency as compared to both the single mutants. Transcriptional analysis together with chromatin immunoprecipitation (ChIP) assays revealed that HY5 also directly negatively regulates the expression of genes involved in Fe transport including *YSL3, FRD3 NPF5.9, YSL2,* and *OPT3.* Interestingly, HY5 and PYE were found to directly bind at the same region on *PYE* and *NAS4* promoter. Our findings indicate that HY5, together with PYE and independently possibly by interacting other TFs regulates the expression of several genes to maintain Fe homeostasis.

3.3 Results:

3.3.1 HY5 Physically interacts with PYE.

HY5 has been shown to act both as a transcriptional activator and repressor depending on its binding partner (Ang *et al.*, 1998; Burko *et al.*, 2020; Delker *et al.*, 2014; Gangappa and Kumar, 2017; Gangappa and Botto, 2016; Nawkar *et al.*, 2017; Ruckle *et al.*, 2007; Xu *et al.*, 2016; Yadukrishnan *et al.*, 2020; Zhang *et al.*, 2017). We therefore performed yeast two-hybrid of HY5 with the major bHLH TFs involved in regulating the iron deficiency response to identify potential interacting partners of HY5. Y2H assays were performed using HY5 fused to the binding domain of GAL4 as bait and bHLH TFs (bHLH34, bHLH105/ILR3, bHLH104, bHLH115, bHLH38, bHLH39, bHLH100, bHLH101, PYE, FIT, BTS) fused to the activation domain of GAL4 as prey. HY5 fused to the activation domain of GAL4 was used as a positive control as HY5 is known to interact with itself to form homodimers. We found that HY5

interacts with some of the bHLH TFs in one or two Y2H assays but only PYE was found to be interacting with HY5 consistently in all the three independent assays (Figure 3.1a). To further confirmed our results, the interaction between HY5 and PYE was further analyzed using bimolecular fluorescence complementation (BiFC) assays. For the BiFC assays, PYE was fused to the N-terminal part of YFP (nYFP-PYE) and HY5 was fused with C-terminal part of YFP (HY5-cYFP). HY5 was also fused with N-terminal part of YFP (nYFP-HY5) which is used as a positive control. We observed strong signal when HY5-cYFP was assayed with nYFP-PYE and nYFP-HY5, whereas no signal was observed when HY5-cYFP was assayed with nYFP and -nYFP-PYE was assayed with cYFP (Figure 3.1b). This assay confirms the interaction observed by Y2H experiment.

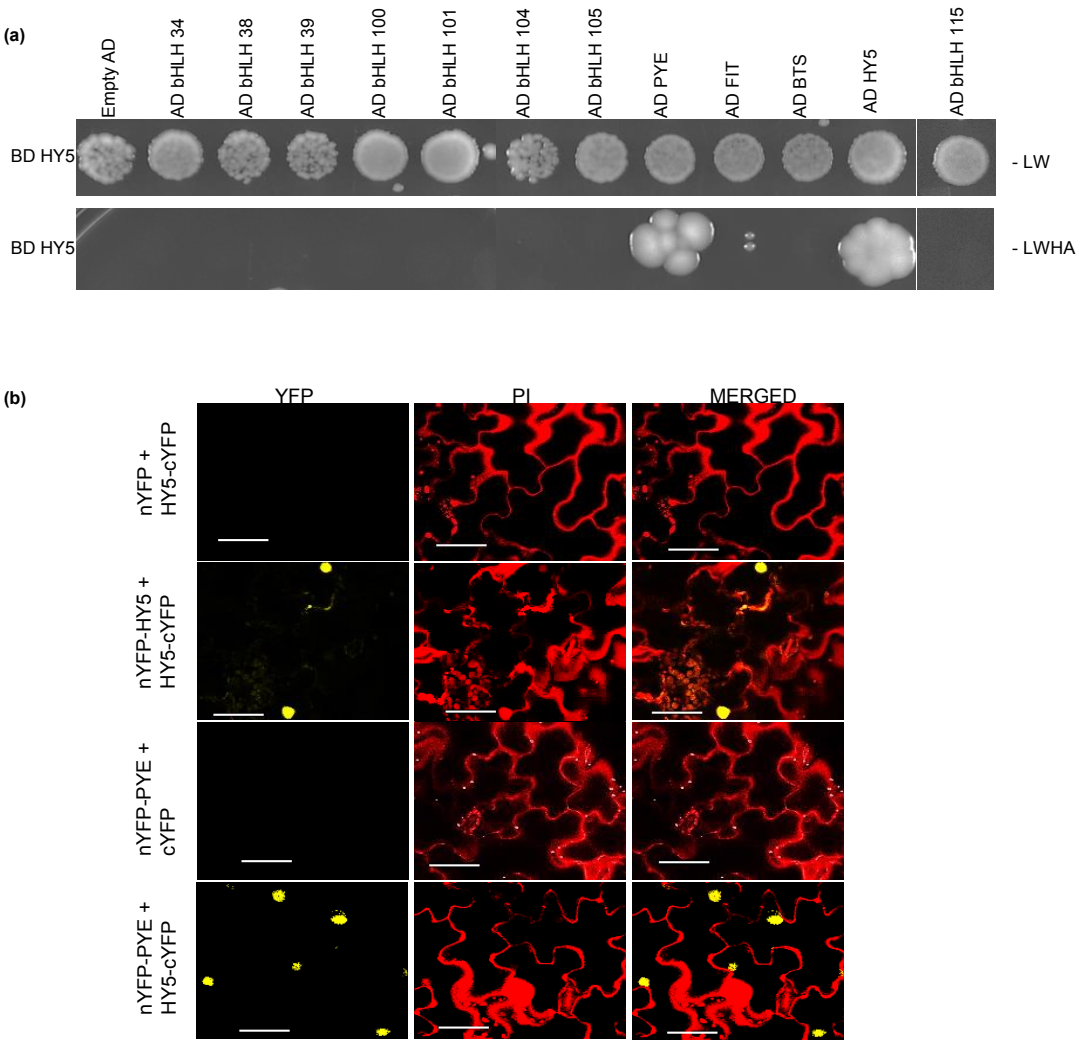
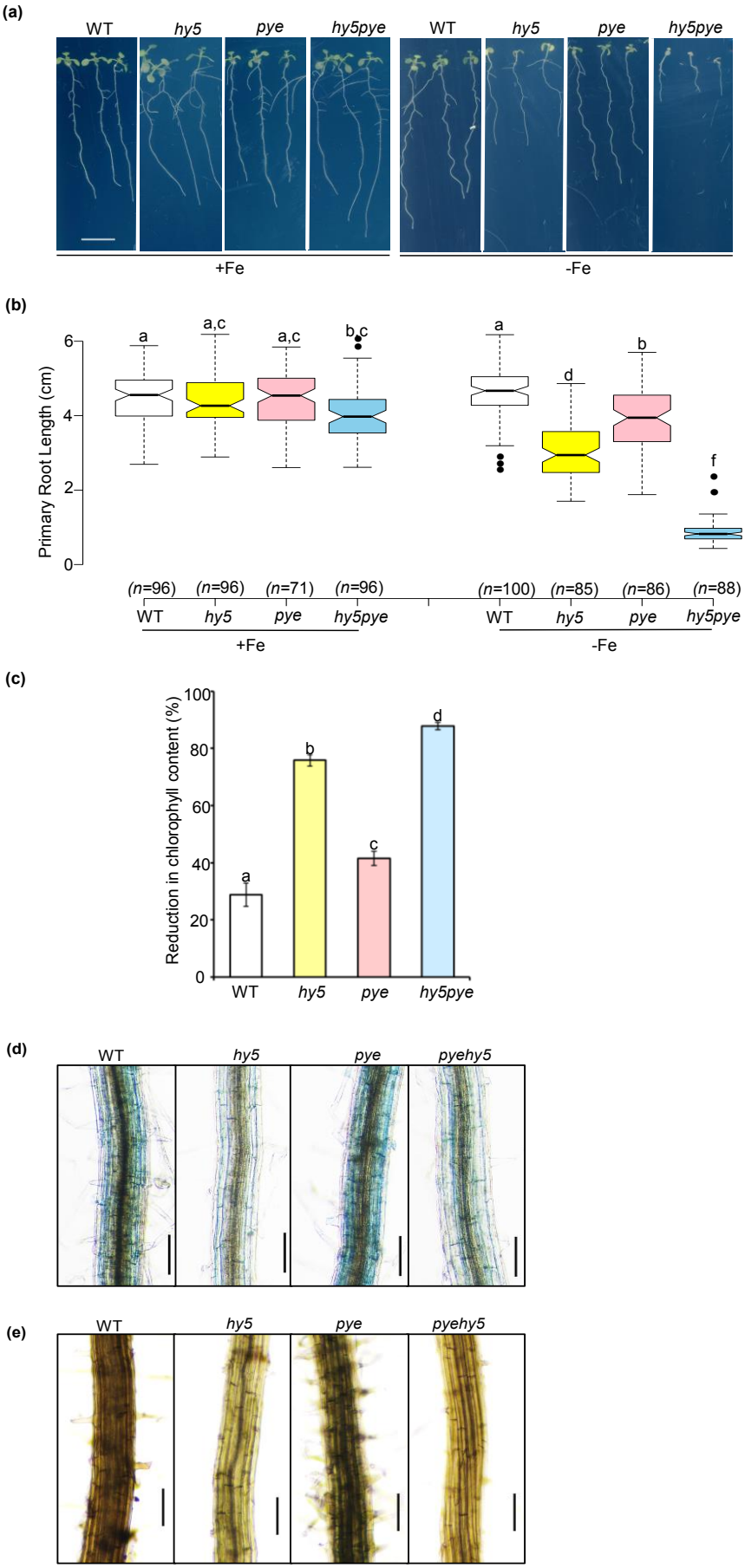


Figure 3.1. HY5 and PYE interact with each other. (a). Y2H assays. bHLH34, bHLH38, bHLH39, bHLH100, bHLH101, bHLH104, bHLH105, PYE, FIT, BTS and bHLH115 fused

with the GAL4 activation domain (AD) and HY5 with the GAL4 binding domain (BD) were cotransformed in the yeast strain. The different yeast strains were plated on non-selective media (-LW) and also on selective media (-LWHA). AD alone was used as negative control. The growing colonies on the selective media represent positive Y2H interacting colonies and they were identified after 7 days of growth. H, histidine; L, leucine; W, tryptophan, A, adenine. **(b).** BiFC assays. PYE was fused with N-terminal part of YFP (nYFP) and HY5 was fused with both N-terminal and C-terminal part of YFP (cYFP) prior to infiltration into *Nicotiana benthamiana* leaves and analysis was done using confocal microscopy. nYFP and cYFP alone were used as a negative control. Bar =100μM.

3.3.2 Genetic interaction exist between HY5 and PYE.

Considering the finding that HY5 interacts with PYE. We next wanted to understand the genetic interaction between them. For this, we generated *hy5pye* double mutant by crossing the corresponding single mutants and compared its phenotype with the wild type (WT) as well as both the *hy5* and *pye* single mutants. When grown on -Fe, both the *hy5* and *pye* single mutants were more sensitive to -Fe as compared to the WT which is in accordance with the previously published reports (Long *et al.*, 2010; Mankotia *et al.*, 2023) (Figure 3.2a). Furthermore, we observed that the *hy5pye* double mutant was more sensitive under -Fe as compared to both the single mutants and has significantly shorter roots when grown on -Fe as compared to +Fe conditions (Figure 3.2b). The percentage reduction in the chlorophyll content of *hy5pye* double mutant grown on -Fe as compared to those grown on +Fe was also found to be higher as compared to both the single mutants (Figure 3.2c). Next, we checked Fe content in the roots of *hy5pye* double mutant and compared it with the corresponding single mutants and WT. We found that the Fe content is less in the *hy5* mutant, more in the *pye* mutant as compared to the WT which is in accordance with previously published reports and in the double mutant Fe content was less as compared to the WT and *pye* single mutant (Long *et al.*, 2010) (Figure 3.2d,e). Altogether, these results suggest that *HY5* and *PYE* play additive roles in the regulation of seedling growth under -Fe conditions.


(a)
WT
hy5
pye
hy5pye
WT
hy5
pye
hy5pye
+Fe
-Fe
(b)
Primary Root Length (cm)
0
2
4
6
a
a,c
a,c
b,c
a
d
b
f
(n=96)
(n=96)
(n=71)
(n=96)
(n=100)
(n=85)
(n=86)
(n=88)
WT
hy5
pye
hy5pye
WT
hy5
pye
hy5pye
+Fe
-Fe
(c)
Reduction in chlorophyll content (%)
0
20
40
60
80
100
a
b
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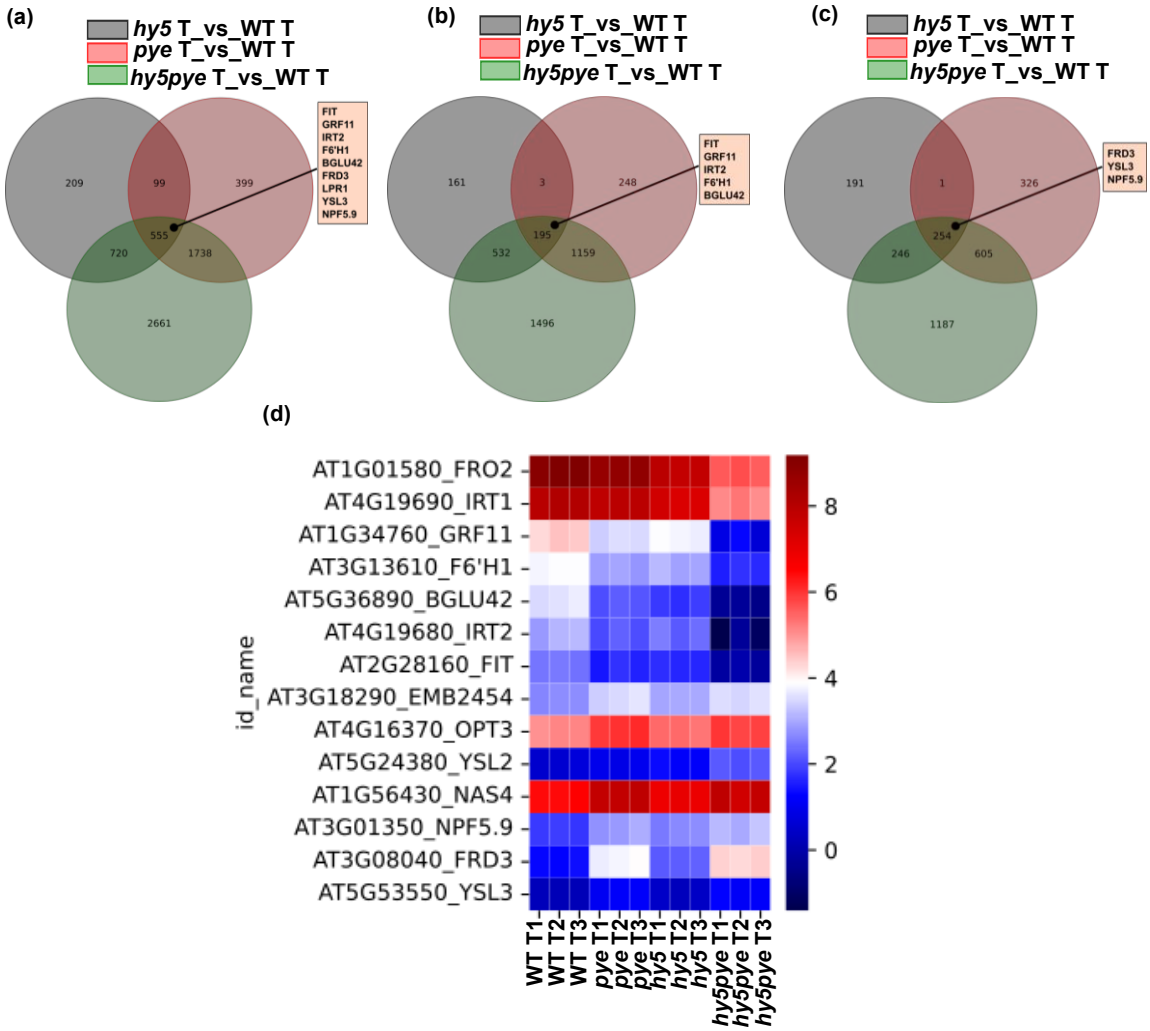
d
WT
hy5
pye
hy5pye
(d)
WT
hy5
pye
pyehy5
(e)
WT
hy5
pye
pyehy5

Figure 3.2. *HY5* and *PYE* play additive roles in regulating Fe deficiency response. (a). Phenotypes of the *Arabidopsis* wild type (WT), *hy5*, *pye* and *hy5pye* grown for 10 days on Fe-sufficient and Fe-deficient medium. Scale bars, 0.5cm. **(b).** Boxplot of root length of the wild type (WT), *hy5*, *pye* and *hy5pye* grown for 10 days on Fe-sufficient and Fe-deficient medium. Means within each condition with the same letter are not significantly different according to one-way ANOVA followed by post hoc Tukey test, $P < 0.05$. **(c).** Percentage reduction in Chlorophyll content of the wild type (WT), *hy5*, *pye* and *hy5pye* grown grown on –Fe as compared to those grown on +Fe. Data shown is an average of three independent experiments. Each experiment consists of 4 biological replicates and each replicate consists of a pool of around six seedlings. Error bars represent ±SEM. Means within each condition with the same letter are not significantly different according to one-way ANOVA followed by post hoc Tukey test, $P < 0.05$. **(d).** Perl's stained and **(e).** Perls/Dab-stained maturation zone of WT, *hy5, pye* and *pyehy5* grown on Fe-sufficient medium for 5 days. Bars = 100µm.

3.3.3 Genome-wide expression analysis showed mis-regulation of Fe deficiency-responsive genes in the *hy5, pye* and *pyehy5* mutant.

Since, HY5 interacts with PYE and *hy5pye* double mutant was more sensitive to -Fe as compared to both the single mutants. We next wanted to find out the genes regulated by HY5 and PYE together. For this, we analyzed the transcriptomic changes in the roots of *pye, hy5 hy5pye* and WT that were grown on +Fe conditions for 6 days and exposed to +Fe or -Fe (+300µM Ferrozine) conditions for 3 days before harvesting. We compared the differentially expressed genes in the *pye, hy5,* and *hy5pye* with the WT (-Fe vs +Fe). We used 1.5-fold difference as a cutoff. We found that 1,583 were differentially expressed genes (DEGs) between the *hy5* T (-Fe) vs WT T (-Fe), 2,791 genes were differentially expressed in the *pye* T (-Fe) vs WT T (-Fe), and 5,674 genes were differentially expressed in the *hy5pye* double mutant (Figure 3.3a). Among these, 555 genes were the common DEGs in the *pye, hy5* and *hy5pye* mutant (Figure 3.3a). Out of common DEGs, 254 genes were commonly upregulated in all, and 195 genes were commonly downregulated in all (Figure 3.3b, c). The genes involved in Fe uptake (*FIT, GRF11, IRT2, F6'H1* and *BGLU42)* were found to be commonly downregulated in the *hy5*, *pye* single as well as *hy5pye* double mutant (Figure 3.3b). The genes involved in Fe transport (*NPF5.9, FRD3* and *YSL3)* were found to be commonly upregulated in all (Figure 3.3c). *NPF5.9, FRD3* and *YSL3* were involved in xylem and phloem transport of Fe. We found that the genes involved in Fe uptake which include *FRO2, IRT1, GRF11, F6'H1, BGLU42, IRT2* and *FIT* were not induced like the WT (T) levels in the *pye* mutant (T) and less induced

in the *hy5* single mutant (T) (Figure 3.3d). In the *hy5pye* double mutant (T), we observed that these genes were even more less induced as compared to both the single mutants (Figure 3.3d). The genes involved in Fe transport which include *OPT3, YSL2, NAS4, NPF5.9, FRD3* and *YSL3* were more induced in the *pye* single mutant and the *hy5pye* double mutant (Figure 3.3d). In the *hy5* mutant, *NPF5.9, FRD3,* and *YSL3* were clearly more induced as compared to the WT. The expression of *OPT3, YSL2,* and *NAS4* was also slightly more induced in the *hy5* mutant as compared to the WT but not highly induced like the *pye* and *hy5pye* double mutant (Figure 3.3d). So, next we performed qRT-PCR to confirm the RNA-seq results and we found that *OPT3, YSL2* and *NAS4* were more upregulated in the *hy5* mutant as compared to the WT but not highly induced like the *pye* and the *hy5pye* double mutant (Figure 3.3e). In addition to these genes, *BTS* which is a E3 ubiquitin ligase, and a negative regulator of Fe uptake genes was also found to be more upregulated in the *hy5* and *pye* mutant which is in accordance with previously published reports, and we found that in the *hy5pye* double mutant it was more induced as compared to the single mutants. Altogether, these results suggests that *hy5pye* is more sensitive to Fe deficiency because the genes involved in Fe uptake are very less induced in the *pyehy5* double mutant as compared to the WT and the genes involved in Fe transport are more induced. To further explore the RNA-seq data, we performed Gene Ontology (GO) analysis using Panther for the commonly differentially upregulated as well as downregulated genes in the *hy5* (T), *pye* (T) and *hy5pye* (T) as compared to the WT (T). This was conducted using genes that were upregulated or downregulated more than 1.5-fold. Major GO clusters for all the upregulated genes are shown in Figure 3.4a and nitrate metabolic process, glutathione metabolic process and nitrate assimilation were among the enriched upregulated GO terms. On the other hand, the response to iron ion starvation, response to nutrients levels, response to abiotic stimulus, and response to light stimulus were among the enriched downregulated GO terms (Figure 3.4b). To further investigate the pathways affected specifically in the *hy5* mutant (T) as compared to the WT (T), we performed GO analysis for the genes differentially expressed only in the *hy5* (T). The GO clusters for the upregulated genes and downregulated genes are shown in Figure 3.4c and 3.4d. GO analysis of genes specifically downregulated in the *hy5* (T) as compared to the WT (T) revealed that an oxidoreductase activity cluster was involved, including *FRO2*, which is directly positively regulated by HY5 (Figure 3.4c). On the other hand, GO analysis of upregulated genes revealed that ABC-type xenobiotic transporter activity, transcription regulator activity and RNA binding clusters were involved (Figure 3.4d).


(a)
hy5 T_vs_WT T
pye T_vs_WT T
hy5pye T_vs_WT T
209
99
399
555
720
1738
2661
FIT
GRF11
IRT2
F6'H1
BGLU42
FRD3
LPR1
YSL3
NPF5.9
(b)
hy5 T_vs_WT T
pye T_vs_WT T
hy5pye T_vs_WT T
161
3
248
195
532
1159
1496
FIT
GRF11
IRT2
F6'H1
BGLU42
(c)
hy5 T_vs_WT T
pye T_vs_WT T
hy5pye T_vs_WT T
191
1
326
254
246
605
1187
FRD3
YSL3
NPF5.9
(d)
AT1G01580_FRO2
AT4G19690_IRT1
AT1G34760_GRF11
AT3G13610_F6'H1
AT5G36890_BGLU42
AT4G19680_IRT2
AT2G28160_FIT
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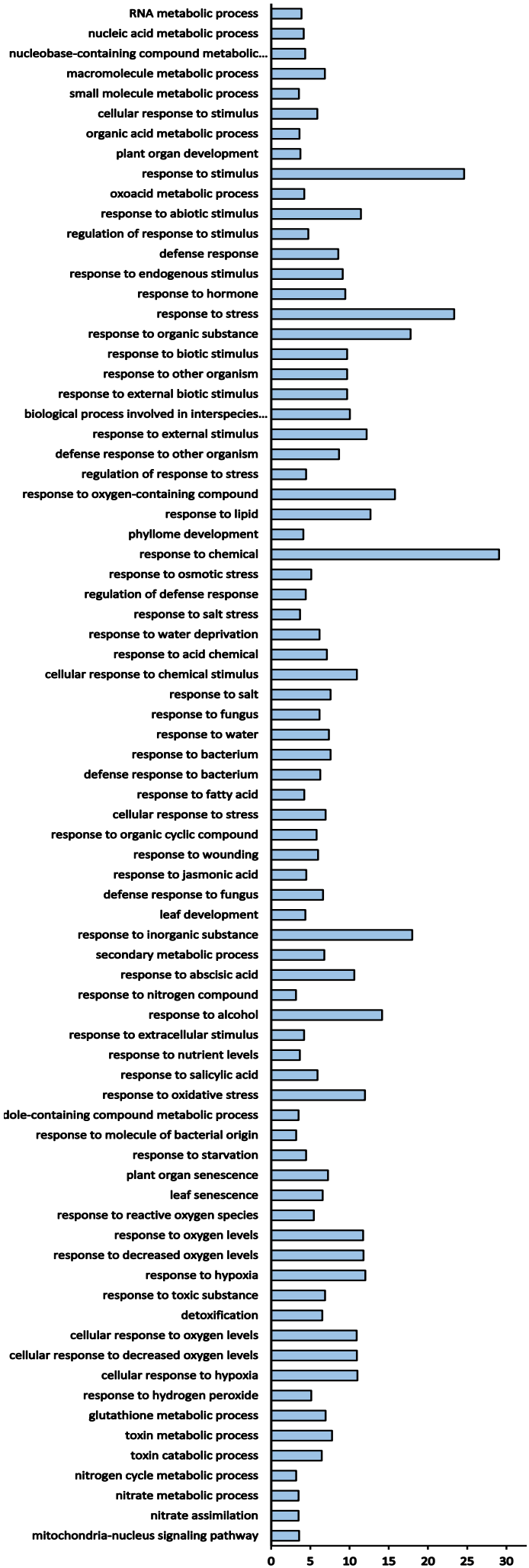
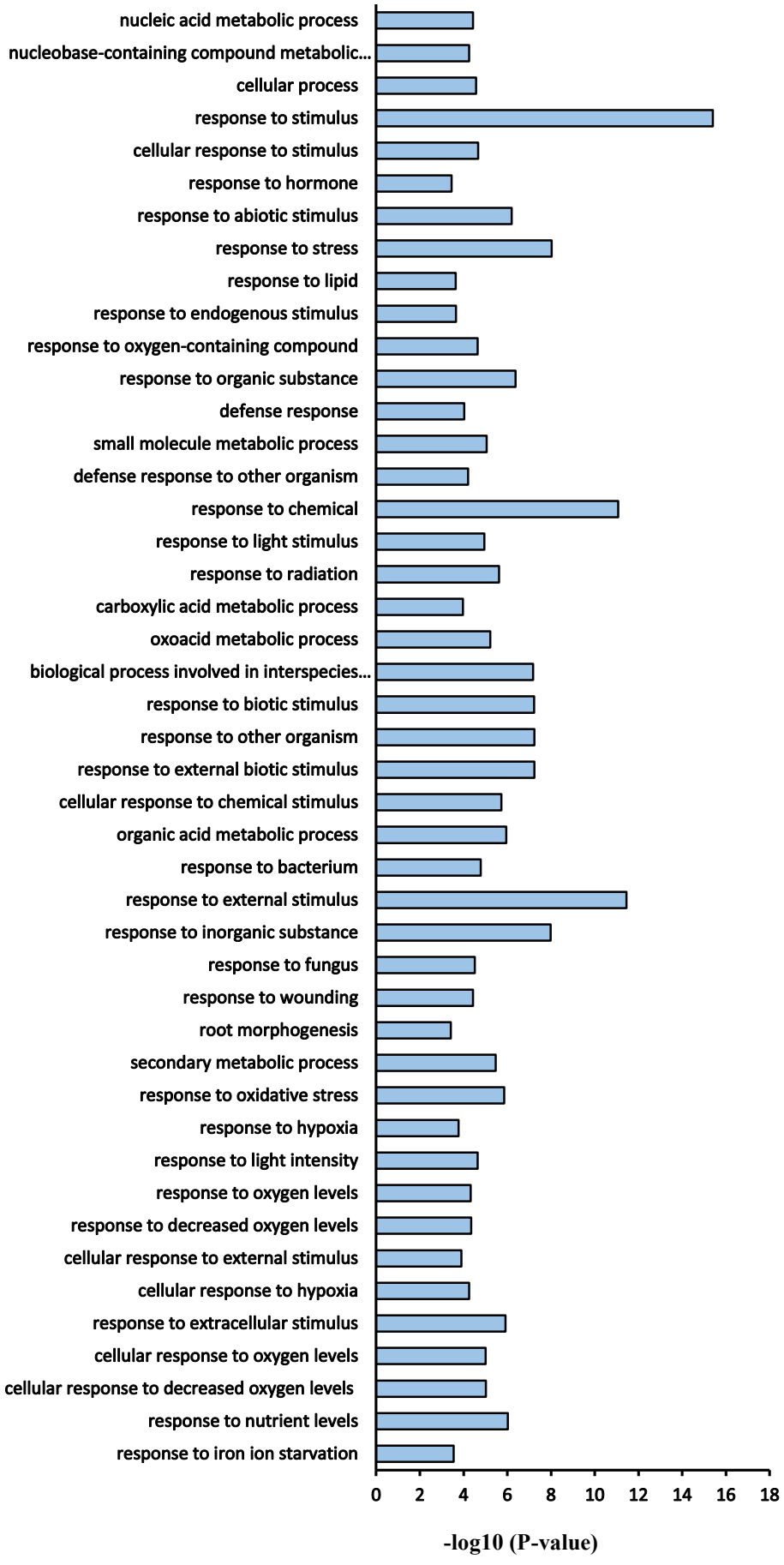
AT4G16370_OPT3
AT5G24380_YSL2
AT1G56430_NAS4
AT3G01350_NPF5.9
AT3G08040_FRD3
AT5G53550_YSL3
id_name
8
6
4
2
0
WT T1
WT T2
WT T3
pye T1
pye T2
pye T3
hy5 T1
hy5 T2
hy5 T3
hy5pye T1
hy5pye T2
hy5pye T3
(e)
WT
hy5
pye
hy5pye
OPT3
YSL2
NAS4
Relative expression levels
400
300
200
100
0
35
28
21
14
7
0
1400
1200
800
400
0
a
b
c
d
+ Fe
- Fe

Figure 3.3. Fe deficiency-responsive genes are misregulated in the *pye, hy5* and *pyehy5* double mutant. (a). Venn diagram showing overlap of differentially expressed genes (DEGs; fold change; |FC| > 1.5) in the *hy5*, *pye* and *pyehy5* (T) versus WT (T). (**b).** Venn diagram showing overlap of commonly downregulated genes in the*hy5*, *pye* and *pyehy5* (T) versus WT (T). **(c)**. Venn diagram showing overlap of commonly upregulated genes in the*hy5*, *pye* and *pyehy5* (T) versus WT (T). **(d).** Heat map of selected Fe deficiency responsive genes. The color bar on the right side demonstrates the log2 (FoldChange) of a given gene in response to iron deficiency for a given genotype. **(e)**. Expression levels of *OPT3, YSL2*, and *NAS4.* Relative expression was determined by qRT-PCR in WT, *hy5, pye* and *pyehy5* double mutant grown on +Fe for days, and then transferred to both +Fe and –Fe (+300μM Fz) for 3 days. Data shown is an average of three biological replicates (n=2 technical replicates). Each biological replicate comprises of pooled RNA extracts from roots of 90 seedlings. Error bars represent ±SEM. *Significant difference by Student's *t*-test ($P \leq 0.05$).

(a)


RNA metabolic process
nucleic acid metabolic process
nucleobase-containing compound metabolic...
macromolecule metabolic process
small molecule metabolic process
cellular response to stimulus
organic acid metabolic process
plant organ development
response to stimulus
oxoacid metabolic process
response to abiotic stimulus
regulation of response to stimulus
defense response
response to endogenous stimulus
response to hormone
response to stress
response to organic substance
response to biotic stimulus
response to other organism
response to external biotic stimulus
biological process involved in interspecies...
response to external stimulus
defense response to other organism
regulation of response to stress
response to oxygen-containing compound
response to lipid
phyllome development
response to chemical
response to osmotic stress
regulation of defense response
response to salt stress
response to water deprivation
response to acid chemical
cellular response to chemical stimulus
response to salt
response to fungus
response to water
response to bacterium
defense response to bacterium
response to fatty acid
cellular response to stress
response to organic cyclic compound
response to wounding
response to jasmonic acid
defense response to fungus
leaf development
response to inorganic substance
secondary metabolic process
response to abscisic acid
response to nitrogen compound
response to alcohol
response to extracellular stimulus
response to nutrient levels
response to salicylic acid
response to oxidative stress
dole-containing compound metabolic process
response to molecule of bacterial origin
response to starvation
plant organ senescence
leaf senescence
response to reactive oxygen species
response to oxygen levels
response to decreased oxygen levels
response to hypoxia
response to toxic substance
detoxification
cellular response to oxygen levels
cellular response to decreased oxygen levels
cellular response to hypoxia
response to hydrogen peroxide
glutathione metabolic process
toxin metabolic process
toxin catabolic process
nitrogen cycle metabolic process
nitrate metabolic process
nitrate assimilation
mitochondria-nucleus signaling pathway
0
5
10
15
20
25
30

(b)


nucleic acid metabolic process
nucleobase-containing compound metabolic...
cellular process
response to stimulus
cellular response to stimulus
response to hormone
response to abiotic stimulus
response to stress
response to lipid
response to endogenous stimulus
response to oxygen-containing compound
response to organic substance
defense response
small molecule metabolic process
defense response to other organism
response to chemical
response to light stimulus
response to radiation
carboxylic acid metabolic process
oxoacid metabolic process
biological process involved in interspecies...
response to biotic stimulus
response to other organism
response to external biotic stimulus
cellular response to chemical stimulus
organic acid metabolic process
response to bacterium
response to external stimulus
response to inorganic substance
response to fungus
response to wounding
root morphogenesis
secondary metabolic process
response to oxidative stress
response to hypoxia
response to light intensity
response to oxygen levels
response to decreased oxygen levels
cellular response to external stimulus
cellular response to hypoxia
response to extracellular stimulus
cellular response to oxygen levels
cellular response to decreased oxygen levels
response to nutrient levels
response to iron ion starvation
0
2
4
6
8
10
12
14
16
18
-log10 (P-value)

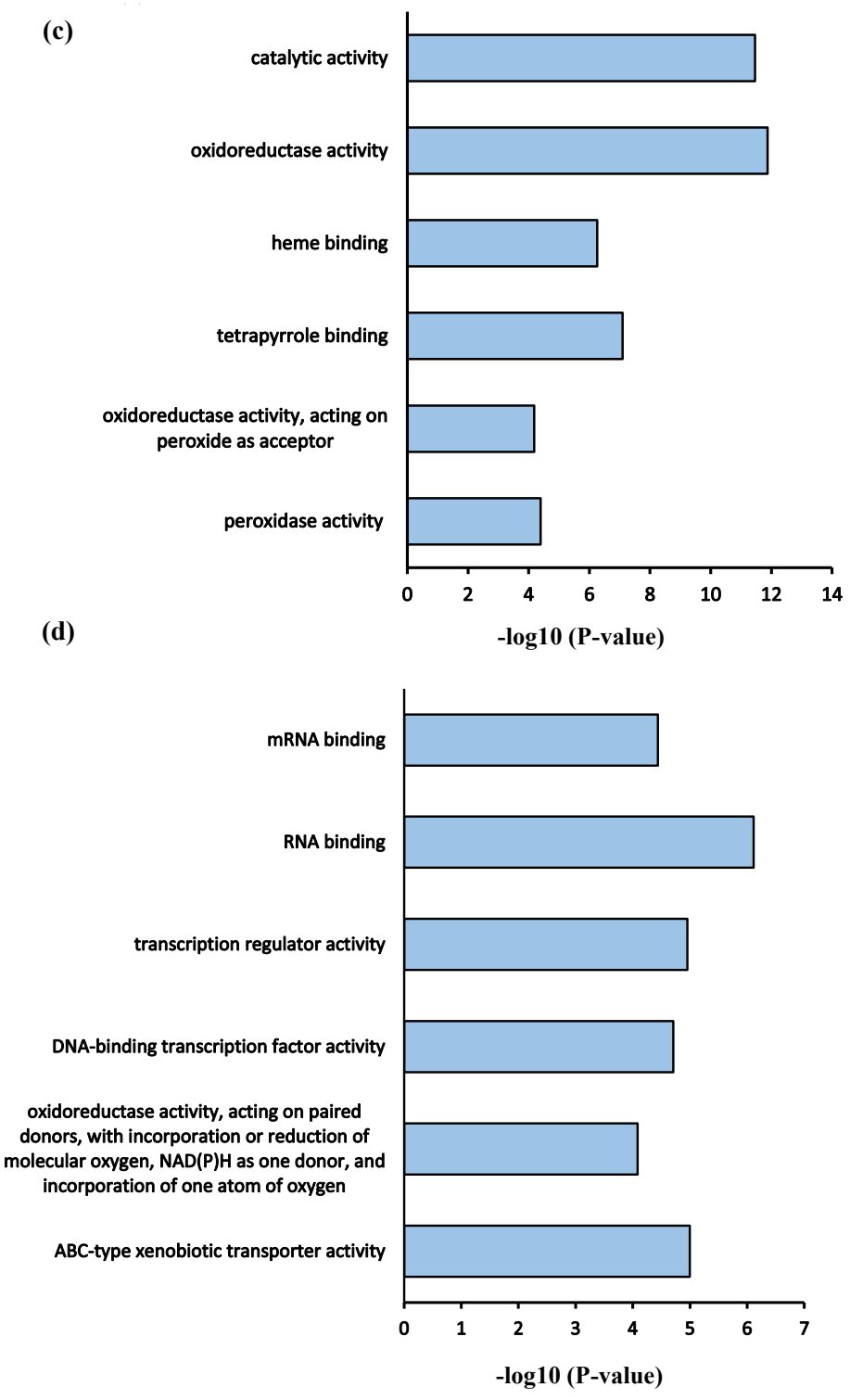


Figure 3.4. GO analysis for the differentially expressed genes. (a) Gene ontology analysis of the genes commonly upregulated genes in the *hy5* (T), *pye* (T) and *hy5pye* (T) as compared to the WT (T). (b) Gene ontology analysis of the genes commonly downregulated genes in the *hy5* (T), *pye* (T) and *hy5pye* (T) as compared to the WT (T). (c) Gene ontology analysis of the

genes specifically downregulated genes in the *hy5* (T) and (d) upregulated genes in the *hy5* (T) as compared to the WT (T).

3.3.4 HY5 directly regulates the expression of genes involved in Fe transport.

Next, we wanted to check whether HY5 directly binds to the promoter of *NPF5.9, FRD3, YSL3, YSL2* and *OPT3* to regulate their expression. For this, we performed in silico analysis of the promoter sequence of *NPF5.9, FRD3, YSL3, YSL2* and *OPT3*. We found HY5 binding motifs on the promoter of these genes (Figure 3.5a). The *YSL3* gene has one E-box which is 189 bp upstream (region I), one CA-Hybrid which is 870 bp upstream (region II) to ATG. The *FRD3* promoter has one T/G-box which is 116 bp upstream (region I), one ACE-element which is 1713 bp (region II) upstream to ATG. The *NPF5.9* promoter has one G-box which is 1529 bp upstream (region I) and one E-box which is 1961 bp (region II) upstream to ATG. The *OPT3* promoter has one G-box which is 145 bp upstream (region I), one G-box which is 344 bp upstream (region II), one T/G-box which is 444 bp upstream (region III), one G-box which is 575 bp ustream, one T/G-box which is 592 bp upstream, one G-box which is 622 bp upstream (region IV) and one CG-Hybrid which is 1056 bp (region V) upstream to ATG. The *YSL2* gene has one A-box which is 904 bp upstream to ATG (Figure 3.5a). Chromatin immunoprecipitation (ChIP)-qPCR was performed using anti-GFP antibody to check whether HY5 directly binds to the promoter of these genes to regulate the expression or not. The *Pro:HY5:HY5:YFP/hy5* and *35S:eGFP* were used for the ChIP experiments. The ChIP-qPCR results revealed that HY5 directly binds to the promoter of *FRD3, YSL3, NPF5.9* at region II (ACE-element, CA-Hybrid box, and E-box respectively), *YSL2* at region I (A-box) and *OPT3* at region IV (G-box, T/G box) (Figure 3.5b).

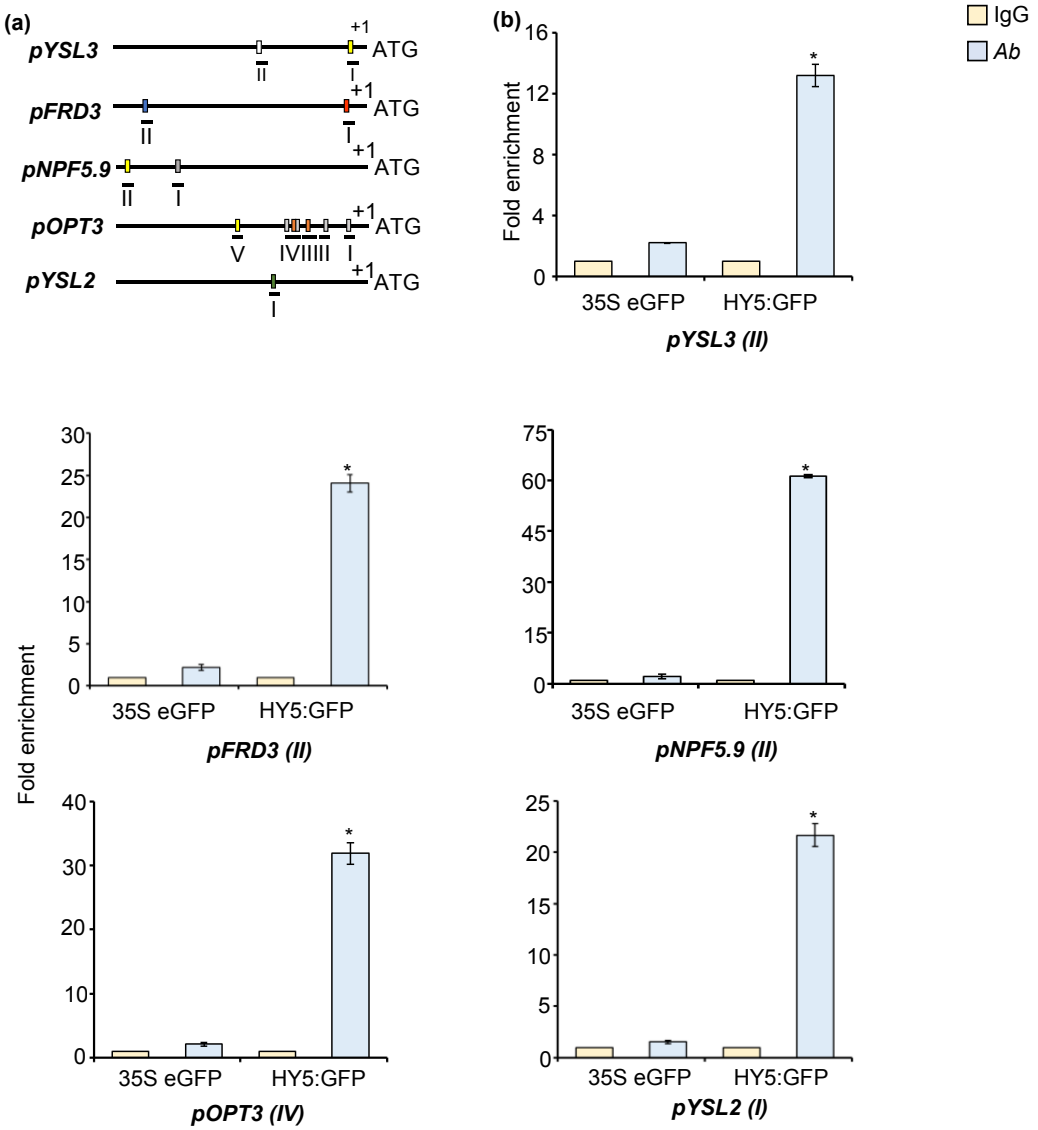


Figure 3.5. HY5 directly binds on *YSL3, FRD3, NPF5.9, OPT3 and YSL2* promoter. (a). Schematic diagram of the promoter of the *YSL3, FRD3, NPF5.9, OPT3* and *YSL2.* White box represent CA-Hybrid boxes, grey boxes represent G-boxes, orange boxes represent T/G-boxes, blue boxes represent ACE-element, green boxes represent A-boxes and yellow boxes represent E-boxes. Lines under the boxes represent sequences detected by ChIP qPCR. **(b).** ChIP-qPCR showing relative enrichment of the *YSL3, FRD3, NPF5.9, OPT3* and *YSL2* regulatory regions bound by HY5. The ChIP assay was performed using *pHY5::HY5:YFP/hy5* and *35S:eGFP* seedlings grown on +Fe media for 10 days. Error bars represent ±SEM. *Significant difference by Student's t test (P≤0.05).

3.3.5 HY5 and PYE interact with the same promoter loci on *PYE* and *NAS4* promoter.

We found that HY5 directly regulates *OPT3, YSL2, YSL3, FRD3* and *NPF5.9* and the expression of these genes was also found to be more induced in the *pye* mutant. PYE is known to bind G-box and E-box (De Masi *et al.*, 2011). So, we hypothesized that HY5 and PYE might together interact *in planta* with the same promoter loci on *OPT3* and *NPF5.9* to regulate the expression of these genes. For this, ChIP experiments were performed on the transgenic line *Pro:PYE:PYE:GFP/pye* using the anti-GFP antibody. The results revealed that PYE does not interact with the same promoter loci as HY5 does on *OPT3* and *NPF5.9* (Figure 3.6a). Therefore, PYE might regulate the expression of these genes indirectly.

In our previous study, we have found that HY5 binds to the region I on *PYE* promoter and negatively regulates its expression. It is known that ILR3 and PYE also interacts with the promoter of *PYE* (Tissot *et al.*, 2019). So, next we wanted to confirm whether PYE also binds to same region I to which HY5 binds. For this, we performed ChIP experiment and we found that PYE also interacts at the same locus as HY5 does on the *PYE* promoter (Figure 3.6a).

As we have found that *NAS4* which is a known direct target of PYE is more induced in the *hy5* mutant as compared to the WT (Tissot *et al.*, 2019) (Figure 3.3e). Next, we wanted to check whether HY5 also directly binds to the *NAS4* promoter region on which PYE binds. To confirm this, we performed ChIP experiment using *Pro:HY5:HY5:YFP/hy5* and *35S:eGFP*. We found that HY5 also interacts at the same region on *NAS4* promoter to which PYE binds (Figure 3.6b). In addition to *NAS4* and *PYE*, PYE is known to act as a direct transcriptional repressor of iron storage (*FER1, FER3 and FER4*) and iron assimilation (*NEET*) genes (Tissot *et al.*, 2019). So, we next compared the expression of these genes in the *hy5* mutant (T) with the *pye* and WT (T). The genes involved in iron storage and assimilation including *FER1, FER3, FER4*, and *NEET* are more expressed in the *pye* (T) as compared to WT (T) which is in accordance with the previously published reports (Figure 3.6c). We found that in the *hy5* mutant (T) the expression is similar to the WT (T) and these genes are not induced in the *hy5* (T). According to a previous study, the expression of *MHR2* and *MHR6,* which are known to be involved in differentiation of root hairs is repressed more in the *pye* mutant. So, we also checked the expression of these genes in the *hy5* mutant and found that these genes are not repressed in the *hy5* mutant (Figure 3.6c).

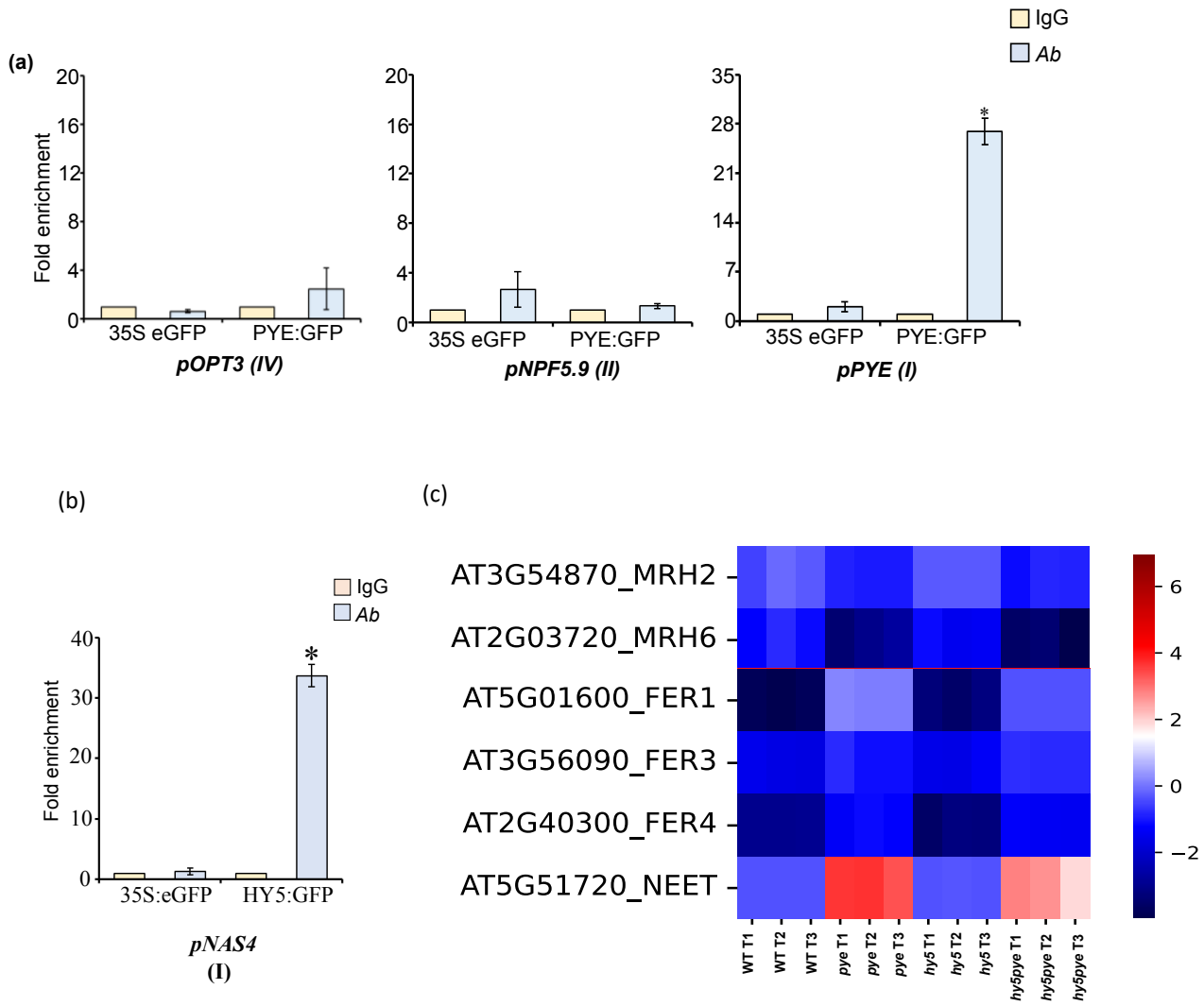


Figure 3.6. PYE and HY5 directly interact at same region on *PYE* and *NAS4* promoter (a). ChIP-qPCR analysis of the binding of the PYE to the promoter regions of the *OPT3*, *NPF5.9* and *PYE* that are targeted by HY5. The ChIP assay was performed using *pPYE::PYE:GFP/pye* and *35S:eGFP* seedlings grown on +Fe media for 10 days. Error bars represent ±SEM. *Significant difference by Student's t test (P≤0.05). **(b).** ChIP-qPCR analysis of the binding of the HY5 to the promoter region of the *NAS4* which is targeted by PYE. The ChIP assay was performed using *pHY5::HY5:YFP/hy5* and *35S:eGFP* seedlings grown on +Fe media for 10 days. **(c).** Heat map of selected Fe related genes. The color bar on the right side demonstrates the log2(Foldchange) of a given gene in response to iron deficiency for a given genotype.

3.4 Discussion

An extensive regulatory network comprising of numerous bHLH TFs is essential for the regulation of Fe homeostasis in plants. Fe uptake, transport, storage, and assimilation must be tightly regulated in order to meet Fe requirements in various tissues and organs. PYE, a bHLH TF belonging to subgroup IVb is known to act as transcriptional repressor to regulate the expression of genes involved in internal mobilization of Fe, transport of Fe from root to shoot, and Fe storage (Long *et al.*, 2010; Tissot *et al.*, 2019). Recently, HY5 belonging to bZIP TF family has been found to regulate some of the genes involved in Fe uptake (Mankotia *et al.*, 2023). However, it is still unclear whether HY5 also regulates other Fe mobilization or storage related genes or not.

In this study, we have done Y2H assay followed by BiFC to identify the interacting partners of HY5. The results revealed that HY5 interacts with PYE (Figure 3.1a, b). The study of *hy5pye* double mutant revealed that the double mutant was more sensitive to Fe deficiency as compared to both the single mutants under -Fe (Figure 3.2). Transcriptional analysis of WT, *hy5, pye,* and *hy5pye* double mutant revealed that the major Fe uptake genes were less induced in the *hy5* as well as *pye* single mutant and they were even more less induced in the *hy5pye* double mutant under -Fe conditions (Figure 3.3). Inversely, the Fe transport related genes were found to be more induced in the *pye, hy5,* and *hy5pye* double mutant under -Fe conditions (Figure 3.3). Taken together, these results suggest that HY5 and PYE have additive roles and HY5 also functions as a repressor of Fe transport genes.

Fe transport from root to shoot and its redistribution from shoots to various sink organs is very crucial for the supply of Fe to various plant parts and ultimately optimum plant growth. Fe is mainly transported and redistributed to different plant parts via xylem and phloem. In our study, we found that some of the key Fe transport genes including *FRD3, OPT3, NPF5.9, YSL3* and *YSL2* were more induced in the *hy5* mutant as compared to the WT under -Fe conditions and HY5 directly binds to the promoter of these genes (Figure 3.3, 3.5). These results indicate that HY5 acts as a direct transcriptional repressor of Fe transport genes including *OPT3, FRD3, NPF5.9, YSL3* and *YSL2* under -Fe.

PYE is known to interact with ILR3 and directly negatively regulate the expression of genes involved in Fe transport (*NAS4*), storage (*FER1, FER3, FER4* and *VTL2*) and assimilation *(NEET).* In addition to this, PYE is also known to repress its own expression (Tissot *et al.*, 2019). In our study, we checked whether PYE also binds on the promoter of other Fe transport genes on the same region as HY5 does and we found that PYE does not bind to the promoter

of Fe transport genes . In our previous study, we have shown that HY5 binds on the promoter of PYE and negatively regulate its expression (Mankotia *et al.*, 2023). Here, we confirmed that PYE also binds to the same region as HY5 does on its promoter (Figure 3.6a). The *NAS4* gene expression was also found to be more induced in the *hy5* mutant as compared to the WT (Figure 3.3e). We found that HY5 also interacts on the same region of *NAS4* as PYE does and negatively regulates its expression (Figure 3.6b). These results suggests that some of the genes including *NAS4* and *PYE* are regulated by PYE and HY5 together, whereas the other Fe related genes are regulated by HY5 and PYE independently, possibly by interacting with some other bHLH TFs.

Based on our results and existing literature, we propose a model describing the role of HY5 in intercellular Fe transport under Fe deficiency in Arabidopsis. Importantly, we found that HY5 directly negatively regulates the expression of genes involved in Fe transport such as *OPT3, YSL2, YSL3, FRD3* and *NPF5.9*. Additionally, HY5 interacts with PYE and they both interact with the same region of *PYE* and *NAS4* promoter.

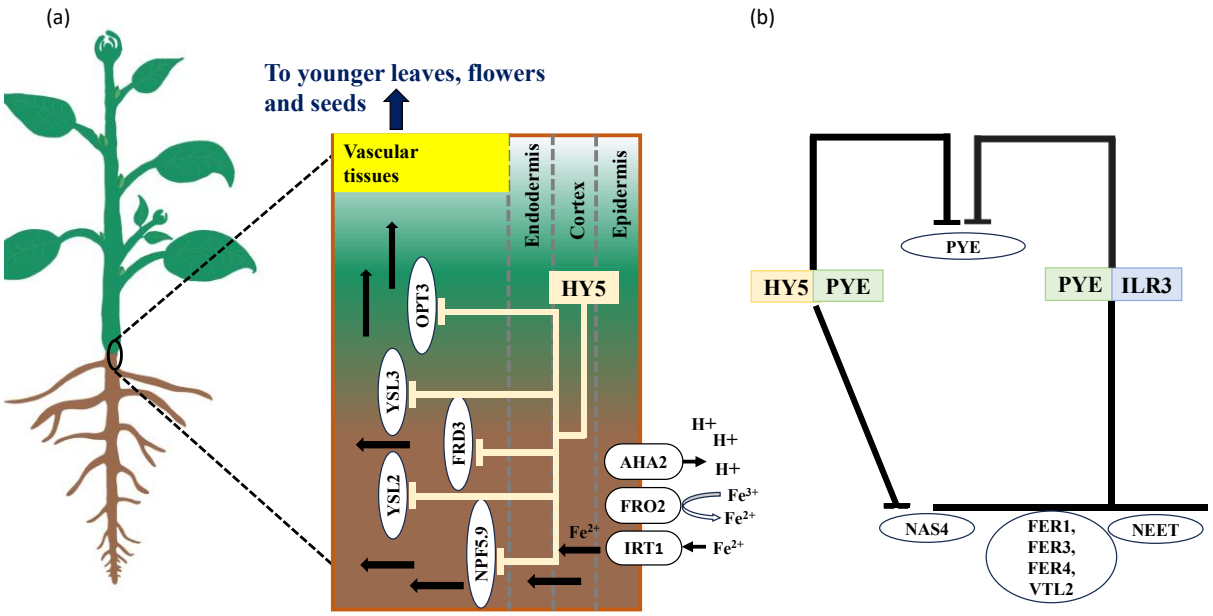


Figure 3.7. Model describing the role of HY5 in regulation of Fe homeostasis. (a) Under Fe deficiency, HY5 acts a direct transcriptional repressor of genes involved in intercellular transport of Fe from the vascular tissues such as *OPT3*, *FRD3*, *NPF5.9*, *YSL2* and *YSL3*. (b) HY5 interacts with PYE. PYE is known to interact with ILR3 and repress the expression of *NAS4* (involved in Fe transport), *FER1, FER3, FER4* and *VTL2* (involved in Fe storage) and

NEET (involved in Fe assimilation) as well as it represses its own expression. PYE and HY5 interact at the same region on the promoter of *PYE* and *NAS4* to negatively regulate their expression. Oval boxes represent mRNA and rectangular boxes represent protein. Dotted lines represent the possible mechanisms of regulation that are also in agreement with the data.

In summary, the data produced in this study and in the previous studies suggest HY5 interacts with PYE, and they play additive as well as independent roles in regulation of Fe deficiency responses. HY5 and PYE were found to together regulate the expression of *NAS4* as well as *PYE*. HY5 acts as a both transcriptional activator and repressor of plant responses to Fe deficiency which ensures the optimum expression of genes involved in maintenance of Fe homeostasis which is crucial for plant growth and development.

3.5 Materials and Methods:

Plant materials and growth conditions

Columbia (Col-0) ecotype of Arabidopsis thaliana was used as WT. The mutant lines used in this study were *hy5* (SALK_056405) and *pye* (SALK_021217) in the Col-0 background. The *pHY5:HY5:YFP/hy5* line was provided by Roman Ulm. The *pPYE::PYE:GFP/pye* was provided by Terri Long (Long *et al.*, 2010). The *hy5pye* double mutant was generated by crossing the *hy5* and *pye* single mutants. The mutants (single as well as double) were confirmed by performing genotyping PCR. The primers which are used for performing the genotyping PCR are listed in Table 3.1. For seed sterilization, 5% sodium hypochlorite and 70% ethanol was used. After sterilization, seeds were kept in 4°C for 3 days. Seeds were sown on ½ Murashige and Skoog media (Caisson Labs, Smithfield, UT, USA) with Fe (+Fe) or without Fe (-Fe). The media pH was maintained to 5.7 with KOH. For making media Fe deficient, 300 μM of [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate] Ferrozine (Sigma-Aldrich, Bangalore, India) was added in -Fe media. Seedlings were grown under long-day conditions, 16 h light and 8 h dark at 22 °C with a light intensity of 100–110 μmol cm^{-2} sec^{-1} and 50% humidity. The soilrite, perlite and compost (3:1:1) mixture was used as soil.

Phenotypic studies

For phenotypic study, the seeds were grown on ½ MS media containing Fe (+Fe) and on ½ MS media without Fe (-Fe). In the growth chamber, the plates were placed vertically and after 10-days, plates were scanned using the Epson Perfection V600 with 1200 dpi resolution. The root

length quantification was performed using ImageJ 1.52a software (National Institutes of Health).

Chlorophyll content determination

For the measurement of chlorophyll content seedling were grown on +Fe and -Fe for 10 days. The chlorophyll was extracted from the leaf tissue of five-six seedlings using 1ml 80% acetone and incubated in dark for 24 h. The chlorophyll content was measured using the formula: $(mg/g) = (20.3 \times A_{645} \pm 8.04 \times A_{663}) \times V/W \times 10^3$ (Aono *et al.*, 1993).

Fe staining

For Fe staining, seedlings grown on +Fe for 5 days were used, and vacuum infiltrated with a solution of 1% (v/v) HCl and 1% (w/v) K-ferrocyanide for 5 min. The seedlings were washed using water for 4 to 5 times and imaged using NIKON ECLIPSE Ni U microscope.

The diaminobenzidine (DAB) intensification was done after Perls staining and for this seedlings were kept in methanol containing 10mM Na-Azide and 0.3% (v/v) H_2O_2 for incubation for 1 hour. After 1 hour incubation, the seedlings were washed using 100mM Na phosphate buffer (pH 7.4) and again incubated in same buffer which contains 0.025% (w/v) DAB and 0.005% (v/v) H_2O_2. The reaction was stopped by washing the seedlings with water. The images of stained seedlings were captured using NIKON ECLIPSE Ni U microscope.

Targeted Yeast Two-Hybrid (Y2H)

For performing Y2H, the AD (pDEST/GADKT7) and BD (pDEST/pGBKT7) constructs of the selected genes were generated by gateway cloning. The constructs were co-transformed into PJ697a, followed by plating on selection plate -2 (-Leu, -Trp). The colonies were obtained after 2 to 3 days, which were dissolved in 100µl of autoclaved millipore water. $O.D._{.600}$ was adjusted to 2.0 in a 96-well plate and spotting was done on -4 (-Leu, -Trp, -His, -Ade) selection plates. The plates were scanned after 6 to 7 days to check growth and identify interacting colonies.

Cloning for BiFC

The CDS of gene of interest was first cloned in pENTR/D/TOPO vector. The pENTR/D/TOPO construct of the gene of interest was used to set up LR reaction with the gateway compatible destination vector pSITE BiFC cEYFP or pSITE BiFC nEYFP. The translational fusion was confirmed by performing restriction digestion and sequencing.

Bimolecular Fluorescence Complementation Assay

The *Nicotiana benthamiana* was grown for 16 hrs light and 8 hrs dark conditions for the bifluorescent complementation assay. The 2-week-old old plant leaves were used for Agrobacterium-infiltration. After 3 days of infiltration, the leaves were screened with the help of a confocal microscope. The vectors containing the N- and C-terminal fragments of YFP respectively were utilized for the generation of N-terminal and C-terminal fusion of PYE and HY5. The constructs of nYFP and cYFP-HY5, nYFP-HY5 and cYFP-HY5, nYFP-PYE and cYFP or of nYFP-PYE and cYFP-HY5 were transformed into Agrobacterium strain GV3101.

Agrobacterium-mediated infiltration

The various constructs in the Agrobacterium were separately inoculated in 5ml LB which contains rifampicin, gentamycin, and spectinomycin. The Agrobacterium having p19 RNAi suppressor gene construct was also inoculated similarly in 5ml LB containing rifampicin, gentamycin, and kanamycin. All the cultures were kept in shaker at 30 ℃ for growth. After 24 hours, the cultures were centrifuged at 4000rpm for 15 minutes. After centrifugation, the supernatant was discarded, and the pellets were suspended in 2ml of infiltration buffer which contains 10mM Mgcl2, 10mM MES/KOH (pH 5.6), 150µM Acetosyringone). The 2ml of infiltration buffer was used to wash the pellet twice. The pellet was finally resuspended in infiltration buffer and OD600 was adjusted in the range of 0.8 to 1.0. The resuspended bacterial cells were finally mixed in a 1:1:1 ratio in a 2ml MCT and kept for incubation for 1 hour at 30℃. The resulting mixture was injected in the abaxial surface of a leaf of *Nicotiana benthamiana.* The plants were kept at 22℃ and leaves were screened after 2 to 3 days using a confocal microscope (Leica SP8 upright).

Confocal microscopy

For confocal microscopy, the leaves were stained with Propidium Iodide (100 µg/ml) in dH20. The leaves were then scanned using Leica SP8 upright laser scanning confocal microscope. Argon laser (488-nm) was used to excite the YFP and Propidium Iodide was excited using 561-nm laser. The emission spectra was collected at 500-530 nm and 600-650 nm, respectively.

RNA isolation and qRT-PCR

The total RNA was isolated from the roots of seedlings which were grown for 6 days on ½ MS and transferred to +Fe or -Fe for 3 days using the Qiagen Plant RNeasy kit following the manufacturer's protocol. The isolated RNA was treated with DNase I for the removal of

genomic DNA. The cDNA was synthesized from 2µg RNA using the RevertAidTM First Strand cDNA synthesis kit (Thermo). The qPCR was set up using the LightCycler 480 II (Roche) and TB GreenTM Premix Ex. The relative expression levels were measured using the comparative threshold cycle method (ΔΔCT) and for the reference gene, β-tubulin was used. The primers used are listed in Table 3.1. The 3µg of isolated RNA was sent for RNA Sequencing.

RNA-Sequencing data analysis

RNA sequencing data were obtained using an Illumina sequencing. Bulk RNA sequencing data was analyzed as per the standard practices. Briefly, QC was performed on all fastq files using FASTQC following which 5' sequences and adapter sequences were removed using trimmomatic (Bolger *et al.*, 2014). The output fastq files were mapped to Arabidopsis thaliana genome (TAIR10) using HISAT2 (Zhang *et al.*, 2021). Subsequently, the counts file was generated using featureCounts (Liao *et al.*, 2014). All downstream analysis was performed in a Python environment. Counts files for all the samples were imported as a dataframe and subjected to basic filtering in which genes having counts less than 10 were removed. Thereafter, FPKM was calculated for all the genes and a second filtering step was applied to remove genes having FPKM < 0.5. Seaborn and matplotlib was used for plotting. Counts file was used to perform DEGs analysis using pyDESEQ2. A p-value cutoff of 0.05 and Fold Change cutoff of 1.5 was applied to identify significant DEGs.

ChIP assay

The ChIP experiment was conducted according to a previously described method (Gendrel *et al.*, 2005). The *pHY5:HY5:YFP/hy5* and *35S:eGFP* was in the Ler background. The *pPYE:PYE:GFP/pye* and *35S:eGFP* was in the Columbia background. The seedlings were germinated and grown on ½ MS media for 10 days. The fixation was done using 1% formaldehyde. The nuclei isolation was done followed by sonication using Qsonica 800R ultrasound sonicator with around 30 cycles of 70% pulse amplitude for 15 sec followed by 45 sec of pulse off time. The protein-G magnetic beads (10004D; Dynabeads) and anti-GFP antibody (a290; Abcam) were used to pull down protein-DNA complexes. IgG was used as a negative control. The beads were washed and the immunocomplexes bound to the beads were eluted and reverse crosslinked. The precipitated chromation was used to set up qPCR. For calculating Fold enrichment, normalization was done against the negative control, i.e., IgG. The primers used are mentioned in Table 3.1.

Statistical analysis

Data was expressed as means of ± SEM. P-values were determined using Student's t-test or one-way ANOVA followed by *post hoc* Tukey HSD test. The P-value of ≤ 0.05 was considered as statistically significant.

Table 3.1: Primers used in the study:

pye-1, LP	TTCAAGACCTCATTCACTGGC
pye-1, RP	GGGGATTGATTATGTTTGGTG
hy5, LP	TTCACTCTCGATATCCGTTCG
hy5, RP	ATGCGAGTGAATGACCATTTC
qRT_*NAS4*_F	GGCTTCGACGTTGTGTTCTT
qRT_*NAS4*_R	AGCAAAGCACCAGGAGACAT
qOPT3-F	AAGCTTACTATAAACAGAGCCTTAGCTT
qOPT3-R	ACAGGATCAACAAGGTACCTCCTC
qRT *YSL2* FP	GGATACTTATTCTTCTCCCTTGTC
qRT *YSL2* RP	CCATCGTTTTTTCCTGCC
pOPT3 Chip FP	CATACTCCTCTTAATAACATTGG
pOPT3 Chip RP	CAGAAAGTGAATGCTGTTAC
pOPT3 Chip II RP	CATTGGGAGGTTCCAAATGG
pOPT3 Chip II FP	AGTGTCAAAAAAACGGGACC
pOPT3 Chip III FP	TTATGTTCTGCGCACACCAC
pOPT3 Chip III RP	TCCAGATCAAAGCTTGTCTC
pOPT3 Chip IV FP	GGACAACCAATAGAAAGTGC

pOPT3 Chip IV RP	CTAGGTGTGGTTAGCTCGTG
pOPT3 Chip V FP	CAAGAGAGATTCATGCATGT
pOPT3 Chip V RP	AACACAGTCTATGTTAGCTG
pYSL2 ChIP FP	TTTTATGTTACCTCCTAACTTAC
pYSL2 ChIP RP	CACTGTTACACAACAACAATATTT
pFRD3 ChIP FP	GGAAACCTTTGTTTTCTTC
pFRD3 ChIP RP	GAAGATATCAATAAGTGTTTCG
pFRD3 ChIP II FP	GCGGTTAACTCTACGATAAC
pFRD3 ChIP II RP	GTTGTATATAGTGCGTGTCG
pYSL3 ChIP FP	TTCAGATAATTATGCTTGGG
pYSL3 ChIP RP	TCAAAACAAAAACCCAAAAG
pYSL3 ChIP II FP	ATGAAGGTTATATATGTGGAG
pYSL3 ChIP II RP	AAGAGAAAAGAGTTTTGGGG
pNPF5.9 ChIP FP	GTTTTTATATGCGACGCCTG
pNPF5.9 ChIP RP	AGGTATCATGTACGAAGAGG
pNPF5.9 ChIP II FP	AATCCACCCTATAATGGCAC
pNPF5.9 ChIP II RP	CTTTCTTTTTTTGTCGAAAGG
pPYE ChIP FP	GACGTGTCCATGAGAGATGA
pPYE ChiP RP	TTTTTGGAGGAAGAAGGTCC
pNAS4 ChiP FP	CGAAATATGAAGACAACACATGC
pNAS4 ChiP RP	TGAGAGTACACGTGCCATCG

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Chapter 4

Conclusions and future perspectives

Chapter 4: Conclusions and future perspectives

Fe availability in the soil determines the growth and development of plants. Fe limitation and excess both are detrimental for plants. Therefore, it is essential to study the regulatory mechanisms that maintain Fe homeostasis in plants. Many TFs belonging to the bHLH family are known to play a crucial role in maintaining Fe homeostasis (Hindt and Guerinot, 2012). In this study, we have shown that a bZIP TF, HY5 also plays a crucial role in maintenance of Fe homeostasis.

Transcriptome reprogramming is one of the sophisticated strategies which plants have evolved to maintain Fe homeostasis under Fe-deficiency. Fe-deficiency triggers the expression of many Fe-uptake related genes which include bHLH TFs (*FIT, bHLH38, bHLH39, bHLH100, bHLH101*), *FRO2, IRT1*, and coumarin biosynthesis as well as secretion related genes (*F6'H1, S8H, CYP82C4, BGLU42, PDR9, MYB10* and *MYB72)* (Liang *et al.*, 2017; Wang *et al.*, 2013; Yuan *et al.*, 2008; Li *et al.*, 2016.). HY5 has been found to positively regulate Fe-deficiency response in Arabidopsis as *hy5* mutant plants were found to be more sensitive to Fe deficiency as evidenced by decrease in root length, FCR activity and chlorophyll content (Figure 2.1 and 2.2). The Fe accumulation was also found to be positively regulated by HY5 (Figure 2.1d and e). HY5 function was found to be crucial for the optimum expression of Fe deficiency responsive genes. It acts both as an activator and repressor to regulate the expression of Fe deficiency responsive genes. HY5 was found to positively regulate the expression of *IRT1, FIT* indirectly and *FRO2* directly which are involved in Fe uptake (Figure 2.7 and 2.11). HY5 acts a direct negative regulator of *BTS* and *PYE* (Figure 2.12 and 2.11). BTS is known to negatively regulate Fe deficiency responses and PYE is known to act as a transcriptional repressor of Fe mobilization genes to maintain Fe homeostasis (Long *et al.*, 2010; Selote *et al.*, 2015). We showed that HY5 acts upstream to BTS in the Fe deficiency responsive signalling pathway (Figure 2.14). The expression of coumarin biosynthesis genes which include *S8H, CYP82C4, F6'H1* and *BGLU42* was also found to be positively regulated by HY5 (Figure 2.17).

In addition to the transcriptional induction of genes related to Fe uptake, the transcriptional repression of genes related to Fe transport, storage, and assimilation which is mediated by the PYE-ILR3 complex is also essential for the maintenance of Fe homeostasis under Fe deficiency (Tissot *et al.*, 2019). In our study, we found that HY5 interacts with PYE, and they play additive roles in regulation of Fe deficiency response as the *hy5pye* double mutant displayed stronger decrease in root growth and chlorophyll content under Fe deficiency than the *hy5* and *pye* single

mutant (Figure 3.1 and 3.2). PYE is known to negatively regulate the expression of many Fe transport related genes either directly or indirectly and in our study, we found that HY5 also acts as a negative regulator of several key genes involved in Fe transport (Figure 3.3). HY5 was found to act as a direct transcriptional repressor of genes involved in intercellular Fe transport including *OPT3, YSL3, YSL2, FRD3* and *NPF5.9* (Figure 3.5). We also found that *NAS4* which is a direct downstream of PYE was more induced in the *hy5* mutant under -Fe (Figure 3.3). Interestingly, HY5 and PYE were found to bind on the same region of *PYE* and *NAS4* promoter (Figure 3.6). Taken together, these results suggest that HY5 interacts with PYE, and they together directly regulate the expression of *PYE* and *NAS4.* On the other hand, the other direct targets of HY5 which include both Fe uptake and Fe transport related genes might be regulated by HY5 by interacting with other Fe related bHLH TFs.

Overall, our study revealed that the loss of HY5 function leads to reduced tolerance to Fe deficiency, reduction in Fe accumulation and a weaker transcriptional iron deficiency response. Further investigation is required to understand whether HY5 directly regulates the expression of coumarin biosynthesis ultimately affecting coumarin content or not. In this study, we have checked the interaction of HY5 with some of the selected bHLH TFs. Further experiments are required to find out whether HY5 interacts with some other known TFs involved in regulation of Fe deficiency response. In tomato, HY5 is known to act downstream to *phyB* and activate the expression of FER (Guo *et al.*, 2021). In Arabidopsis, *phyB* mutant was not found to be sensitive to Fe deficiency unlike *hy5* mutant (Figure 2.7). A recent study has reported that blue light promotes the Fe uptake responses downstream of FIT in Arabidopsis (Trofimov *et al.,* 2022). Further studies are required to fully understand the role of light signalling and determine the photoreceptors involved in the regulation of Fe deficiency response. In the *hy5* mutant, as we have found that the expression of genes related to intercellular transport of Fe was mis-regulated, it will be further interesting to study the impact of loss of *hy5* function on the intercellular distribution of Fe. In addition to the Fe-related genes, the expression of genes involved in some other pathways was also found to be affected in the *hy5* mutant, suggesting a possibility of HY5 in the regulation of these pathways (Figure 3.4c and d). As, the role of HY5 is known to be conserved in nutrient uptake and utilization in different plant species, the knowledge obtained on HY5 function and its role in Fe homeostasis in *Arabidopsis* would be beneficial for further studying its role in Fe homeostasis in crop plants.

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