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"B3 transcription factors regulate iron distribution in *A. thaliana* embryos"

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Por:

SUSANA MARGARITA GRANT GRANT

Director de tesis: Dr. Hannetz Roschzttardtz

Co-Director: Dr. José O'Brien

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Dedicatoria

A mi Familia.
A mi David.
A mis amigues.
Y todos los que apoyaron.
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Abbreviations List

ABA	Abscisic acid
AGRIS	Arabidopsis Gene Regulatory Information Server
ANAP	Arabidopsis Network Analysis Pipeline
cDNA	complementary DNA
ChIP-seq	Chromatin ImmunoPrecipitation sequencing
DAB	3,3′-Diaminobenzidine
DAP-Seq	DNA affinity purification sequencing
DAP	Days After Pollination
DMA	Deoxymugineic acid
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
FBS	Fetal Bovine Serum
GA	Gibberellin
GEO	Gene Expression Omnibus
GRN	Gene Regulatory Network
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
MA	Mugineic acid
NA	Nicotianamina
PBM	Protein Binding Motif
PBS	Phosphate Buffered Saline

PMSF	Phenylmethylsulfonyl fluoride
qRT-PCR	quantitative Reverse Transcription Polymerase Chain Reaction
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SSP	Seed Storage Protein

Resumen

El hierro es un macronutriente esencial para todos los seres vivos. En humanos, la deficiencia de hierro en la dieta es la principal causa de anemia en el mundo. La Organización Mundial de la Salud a propuesto la biofortificación de cultivos como una alternativa para aumenta el contenido de hierro en los alimentos. Uno de los cultivos más consumidos en la dieta humana son las semillas. Sin embargo, poco se conoce sobre la acumulación del hierro en semillas. En *Arabidopsis thaliana* durante la maduración del embrión el hierro se acumula en las vacuolas de la capa celular que rodea la provasculatura.

En *A. thaliana* y otras plantas modelo se han identificado tres factores de transcripción B3, FUS3 (FUSCA3), LEC2 (LEAFY COTYLEDON2) y ABI3 (ABSCISIC ACID INSENSITIVE 3), que cumplen una función crítica en la acumulación de compuestos de almacenaje como proteínas y lípidos en la semilla durante la maduración. La función de los factores de transcripción B3 en la maduración y la acumulación de compuestos de almacenaje ha sido bien estudiaba, sin embargo, la función de estos factores de transcripción B3 en la acumulación de micronutrientes como el hierro no ha sido estudiada.

En este trabajo se estudió el rol de los factores de transcripción B3 en la homeostasis de hierro en semillas de *A. thaliana,* utilizando mutantes para los factores de transcripción B3 y técnicas histológicas y de biología molecular. En este trabajo se determinó que los factores de transcripción B3 participan en la regulación de la distribución de hierro en semillas de *A. thaliana*, pero no así el contenido total de hierro en semilla. Los resultados de este trabajo mostraron que los genes asociados a la homeostasis de hierro cambiaron su expresión en las semillas mutantes para los factores de transcripción B3. Este cambio en la expresión coincide con el fenotipo heterocrónico de las semillas mutantes en los genes de los factores de transcripción B3. Finalmente, a través de una Red de Regulación Génica se encontró un nuevo rol para la vía de respuesta a etileno asociado a la distribución de hierro en semillas. Estos resultados entregan algunas respuestas sobre la regulación de la homeostasis de hierro en semillas, a pesar de que aún quedan muchas preguntas sin respuesta, como, por ejemplo: ¿Cómo el etileno regularía la distribución de hierro en semillas? y ¿Qué factores de transcripción regulan directamente a los genes asociados a la homeostasis de hierro?

Abstract

Iron is an essential micronutrient for humans and other organisms. Its deficiency is one of the leading causes of anemia worldwide. The world health organization has proposed that an alternative to increasing iron content in food is through crop biofortification, which requires full knowledge of iron metabolism in plants. One of the most consume crop are seeds, however little is known about how iron accumulation in seed occurs and is regulated.

In A. thaliana and other plant models, three B3 transcription factors have been identified: FUS3 (FUSCA3), LEC2 (LEAFY COTYLEDON2), and ABI3 (ABSCISIC ACID INSENSITIVE 3). These B3 transcription factors have a critical function in the storage compound accumulation, such as proteins and lipids. Their roles in seed maturation have been well characterized. However, their relevance in micronutrient accumulation and distribution, like iron, remains unknown.

This work studied how seed iron homeostasis is affected in B3 transcription factor mutants using histological and molecular approaches. This work determines that B3 transcription factors regulate iron distribution in *A. thaliana* seeds; however, they do not regulate iron accumulation in seeds. The results show that genes related to iron homeostasis change their expression in the B3 transcription factor mutants. This change in expression in the gene related to iron homeostasis matches the B3 transcription factor mutants heterochronic phenotype. Finally, through a Gene Regulatory Network, a new role of the ethylene pathway in seed iron distribution was discovered. These results give some answers about iron homeostasis in seeds. However, there are still questions without an answer: How does ethylene regulate iron distribution in seeds? Which transcription factors do regulate genes related to iron homeostasis directly?

Chapter I

1. Introduction

There are three kinds of malnutrition (Ritchie and Roser, 2017): (1) Undernourishment when food intake is smaller than the need to keep a healthy weight. (2) Overnourishment, when the intake of food exceeds the healthy amount. (3) Micronutrient deficiency in which the intake of minerals and vitamins is lower than the healthy amount needs correct body development. Micronutrient deficiency or hidden hunger are caused by the deficiency of different kinds of micronutrients that can only be acquired by diet, such as Vitamin A, zinc, folic acid, among others. Moreover, these deficiencies can have a detrimental impact on human development (Imdad *et al*., 2017).

Another essential micronutrient is iron. Iron is a micronutrient critical for human health and development. In fact, iron deficiency is the leading cause of anemia worldwide. Anemia affects 43% of children from 0-5 years old and 38% of pregnant women globally (Stevens *et al*., 2013). Children with anemia have low cognitive development (Stevens *et al*., 2013), working people have reduced productivity (Haas and Brownlie, 2001), and for pregnant women with severe anemia, the outcomes of maternal death and low-weight infant increase (Kozuki *et al*., 2012; Zhang *et al*., 2009). Although anemia has been reduced since the 1990s, worldwide still affects around 800 million people, anemia is especially prevalent in South Asia, central and west Africa (Stevens *et al*., 2013). Acknowledging the problem, the World Health Organization in 2012 specified six global nutrition targets by 2025. One of them is to reduce by 50% the anemia in

women of reproductive age. This decrease will benefit women and should also decrease maternal death and low-weight infants (WHO, 2014).

In order to improve iron deficiency, various strategies have been implemented these years. Iron has been used in soils for fertilization at the agronomic level, but this strategy is expensive and potentially detrimental for the environment (Zhu *et al*., 2007). Fortification of flour with iron is currently used in several countries to fight iron deficiency, and it has shown that fortification of flour covers almost 70% of the population and has a low cost (US$0.12/person/year) (Horton *et al*., 2008). Since 1950 iron fortification in flour is mandatory in Chile (David, 2004).

Anemia in fertile Chilean women (15-49 years) was only 10% in 2003. Later in 2013, anemia rise to 12%, which is half of the world average. However, This increase shows that fortification alone is not the solution (Stevens *et al*., 2013).

The biofortification of crops, usually by genetic engineering, is another strategy use (Horton *et al*., 2008). The selected crops are consumed regularly by poor communities where fortification does not reach. Examples of these are the high-zinc rice, the high-iron beans, the orange sweet potato, and the famous Golden rice (Meenakshi *et al.,* 2010). Biofortified crops have benefits in the communities between 20-200 times higher than the costs. However, these crops have difficulties like acceptance from the consumers and low dissemination caused by poor infrastructure in these regions (Horton *et al*., 2008). The primary energy source in diets (90% of the world's energy intake) comes from staple crops, which are mainly seeds such as rice, maize, wheat, and legumes (Su *et al.,* 2017). It is essential to develop new crops to study how seeds accumulate different micronutrients, such as iron. Understanding how iron is uptake, distribute,

and finally accumulate in the seed will give new possibilities to develop new varieties of biofortified crops.

1.1. Iron in plants

Iron is the chemical element most abundant on the earth, followed by oxygen (Morgan and Anders, 1980). As a transition metal, iron can change its oxidation states between Fe^{3+} and Fe^{2+}. This capability is exploited by cells in order to carry out many biochemical reactions. For instance, iron is a crucial component of the respiratory chain (forming complex with different respiratory chain proteins).

However, excess iron causes an increase of reactive oxygen species (ROS) through the Fenton reaction (In which iron catalyzes the conversion of peroxide to free-radical that can damage membranes, proteins, and DNA). This increase in free radicals has a cytotoxic effect on the cell. As a consequence, iron homeostasis is a highly regulated process.

Although iron is one of the most abundant elements in the earth's crust, it is the least bioavailable, especially in calcareous soils or alkaline soils, around 30% of the earth's land (Maeschner, 1995). Plants exposed to calcareous soils usually develop interveinal chlorosis, described by a yellowing of leaves around the veins.

The chloroplasts use 80% of the iron in leaves. It is calculated that 22 atoms of iron are required per photosynthetic electron respiratory chain, iron is used as a ligand like Fe^{2+}, heme, or Fe-Sulfur clusters in different proteins of photosystem II and photosystem I (Schmidt *et al.*, 2020). Furthermore, iron helps catalyze superoxide radicals' dismutation to hydrogen peroxide in the stroma when it is part of the FeSOD (Fe-containing superoxide dismutase) (Pilon *et al.*, 2011).

Moreover, iron is also necessary for chlorophyll production, forming part of at least four chlorophyll synthesis steps (Briat *et al*., 2015).

Because of its pivotal role in photosynthesis, iron is a limiting factor in biomass production. This role has been shown in different species, such as; Arabidopsis (*Arabidopsis thaliana*), tomato (*Solanum licopersicum*), spinach (*Spinacia oleracea*), and rice (*Oryza sativa*) (Briat *et al*., 2015). The losses in production oscillate depending on the severity of the deficiency. It has been calculated that 47% of lentil production can be lost by iron deficiency (Erskine *et al*., 1993). Just in North Central U.S., iron deficiency has caused US$120 million of loss annually in soybean production (Hansen *et al*., 2004). Fruit yield also decreased in iron-deficient tomatoes (72% decrease in fruit per plant), pears (72% decrease in fruit per tree), and peach (66-71% decrease in fruit per tree) (Álvarez-Fernández *et al*., 2006).

Interveinal chlorosis is associated with decreased fruit yields; a reduction in chloroplast function will reduce carbon fixation. Finally, this will affect plant development (Álvarez-Fernández *et al*., 2006).

The reduction in vegetative growth and fruit yields in plants affected by iron deficiency is connected to iron's vital role in photosynthesis and respiration in plant cells.

1.2. Role of iron in the plant cell

Iron deficiency has been mainly studied in the chloroplast and mitochondria due to iron's pivotal role in the photosynthetic electron chain and the respiratory electron chain. As explained above, chloroplasts have a high demand for iron. Iron enters into the stroma, following a path similar to the plasma membrane. First is reduced by FRO7 and then uptake by the PERMEASE IN CHLOROPLASTS 1 (PIC1) transporter. Once inside the stroma, iron can be used in the

different photosynthetic complexes on the thylakoid membrane. At least fourteen proteins of the photosynthetic electron transport chain need iron for function (Schmidt *et al*., 2020). Under iron deficiency, cells suffer from chloroplast and thylakoid membrane disorganization. *pic1* mutants have an impaired chloroplast development, meaning fewer and smaller chloroplasts (Duy *et al*., 2007). Iron deficiency also decreased transcripts accumulation of different photosynthetic electron chain proteins (Rodríguez-Celma *et al*., 2013). It also reduces Fe-dependent enzyme levels (Hantzis *et al*., 2018).

Mitochondria is also an iron high demand organelle, but it has been less studied than the chloroplast. The first mitochondrial iron transporter in the plant was found in rice. MITOCHONDRIAL IRON TRANSPORTER 1 (MIT1) knock-out mutant has a lethal phenotype. *mit1* knock-down has reduced mitochondrial activity, reduced fertility, and yield (g of rice per plant) (Bashir *et al*., 2011). Recently, two mitochondrial iron transporters in Arabidopsis have been described, MIT1 and MIT2. Double *mit1mit2* mutant has a lethal phenotype. *mit1--/mit2*+- mutant shows a decrease in iron content in the mitochondria and a decreased mitochondrial function (Jain *et al*., 2019). Under iron deficiency, cucumber (*Cucumis sativus*) roots suffer from reduced respiratory complex activities, and mitochondria undergo structural changes (Vigani *et al*., 2009).

Iron uptake from the soil is tricky, causing iron deficiency and all the different effects discussed above. To solve this, plants possess two strategies to uptake iron from the soil.

1.3. Iron acquisition and distribution in plants.

There are two different strategies to acquire iron in higher plants: Strategy I and strategy II. The first has been characterized in dicotyledons plants and consist of acidification, reduction, and transport. On the other hand, Strategy II relies on phytosiderophores and has been described in monocotyledons (Fig.I-1).

1.3.1. Strategy I

The strategy I have involved three essential processes; (i) acidification of the rhizosphere (Fig. I-1b), (ii) reduction of insoluble Fe^{3+} to soluble Fe^{2+} (Fig.I-1e), and (iii) transport of Fe^{2+} (Fig. I-1f) (Hell and Stephan, 2002). In the soil, iron is generally insoluble in a Fe^{3+} state, forming hydroxyls (Fig. I-1a). An H^{+}-ATPase in Arabidopsis (AHA2) increases iron solubility through proton secretion to the rhizosphere in iron deficiency conditions, lowering the pH helps to solubilized iron in the soil (Santi and Schmidt, 2009). *aha2* loss of function mutant seedling have a low proton flux to the rhizosphere under iron-sufficiency and iron-deficiency conditions compared to wild-type (Santi and Schmidt, 2009). In the second step, FRO2 (FERRIC-CHELATE REDUCTASE OXIDASE 2) reduces the ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) (Fig. I-1e). The plant then absorbs these through the iron transporter IRT1 (IRON-REGULATED TRANSPORTER1) (Fig. I-1f). These genes were first cloned from Arabidopsis (Eide et al., 1996; Robinson et al., 1999). Homologs of *FRO2* and *IRT1* genes have been cloned from different plant species, such as rice (*Oryza sativa)* (Bughio *et al*., 2002), tomato (*Lycopersicon esculentum*) (Eckhardt *et al*., 2001), cucumber (Li *et al*., 2004) and green peas (*Pisum sativum*) (Waters *et al*., 2002). Mutant plants for *FRO2* have a reduced reductase activity, a chlorotic phenotype, and a slow growth without an elevated iron supply (Robinson *et al.,* 1999). Knock-out *irt1* mutant is not viable unless rescued with high iron concentrations

(Varotto *et al*., 2002). Recently, the interactome of IRT1 has been established, showing that IRT1, AHA2, and FRO2 interact in a protein complex in the root membrane (Martín-Barranco *et al*., 2020) (Fig. I-1b, 1e, 1f). Iron uptake studies have been mainly focused on the three parts of strategy I (acidification, reduction, and transport); however, some reports have shown the role of coumarins (aromatic organic compounds) in the tolerance to iron deficiency in alkaline soils (Clemens and Weber, 2016; SisóTerraza *et al*., 2016) (Fig. I-1c).

1.3.2. Strategy II

Strategy II depends mainly on phytosiderophores' secretion, particularly the mugineic acid family (MA). These molecules can solubilize Fe^{3+}. Biosynthesis of MAs is induced by iron deficiency in the roots of gramineous (Takagi, 1976). Rice plants carrying a mutation in the nicotinamide aminotransferase (*NAAT1*) failed to produce one type of MA, deoxymugineic acid (DMA); this decrease the efficiency in Fe^{3+} absorption (Cheng *et al*., 2007). Then, MAs are transported to the rhizosphere from the root cells by TOM1 (TRANSPORTER OF MUGINEIC ACID FAMILY PHYTOSIDEROPHORES1) (Nozoye *et al*., 2011) (Fig. I-1g). Rice plants overexpressing *TOM1* secrete higher concentrations of DMAs from roots and can accumulate higher iron levels in seeds than wild-type plants. On the contrary, *TOM1* silenced plants using RNAi secreted lower concentrations of DMAs, and seeds have lower iron levels than wild-type (Nozoye *et al*., 2011). Once in the rhizosphere, secreted MAs solubilize Fe^{3+}, forming complexes Fe^{3+}-MA (Fig. I-1h). These complexes are later transported to the roots by YS1 (YELLOW STRIPE1) and YSL (YELLOW STRIPE1- LIKE) (Kobayashi and Nishizawa, 2012) (Fig. I-1i). YS1 was first described in maize (Curie *et al*., 2001). *ys1* mutants have interveinal chlorosis reported as "yellow stripes" because of monocotyledon veins parallel

orientation. When *fet3fet4* yeast mutant (deficient in iron uptake systems and unable to grow on an iron-limited soil) were complemented with the *YS1* gene were able to grow on a medium only supplemented with Fe-DMA (Curie *et al*., 2001). Searching for *YS1* homologs, the family of *YS1L* genes appears (Koike *et al*., 2004). This family mainly transports iron bonded to nicotianamine (NA). However, some members can transport Fe^{3+}-DMA, such as YSL15 (Inoue *et al*., 2008).

Once in the root cell, plant iron is transported in association with chelating molecules. These associations are due to low iron solubility and high reactivity. The main chelator molecules in the plant are citrate, nicotianamine (NA), and MAs (Kobayashi and Nishizawa, 2012). At the xylem, iron is chelated mainly by citrate (Brown and Chaney, 1971). Citrate is loaded to the xylem by the FERRIC REDUCTASE DEFECTIVE3 (FRD3). *frd3* mutant roots exudate have lower amounts of citrate than wild-type. Also, *frd3* mutant plants' chlorotic phenotype is rescued by supplementation with citrate (Durrett *et al.,* 2007).

From the xylem, iron can be used in the cell or is translocated to the phloem, where NA chelates Fe^{3+} and Fe^{2+}. In fact, if there is a lack of NA, it will cause interveinal chlorosis, which has been observed in tomato and tobacco (Ling *et al*., 1999; Takahashi *et al*., 2003). In tomato, *NICOTIANAMINE SYNTHASE* (*NAS*) mutation disrupts NA synthesis at the final step (Ling *et al*., 1999). In tobacco, transgenic plants overexpressing *NICOTIANAMINE AMINOTRANSFERASE* (*NAAT*), an enzyme necessary to produce DMA in gramineous plants but not present in non-graminaceous, suffer from chlorosis. This phenotype occurs because all NA is used to produce DMA, depleting NA for iron transport (Takahashi et al., 2003). In Arabidopsis, four NAS genes have been described (*NAS1*, *NAS2*, *NAS3,* and *NAS4*. Only

quadruple homozygous mutants showed a chlorosis phenotype (*nas4x-2* mutant). This lack of phenotype in single mutants demonstrates that NAS genes are redundant in Arabidopsis (Klatte *et al.*, 2009).

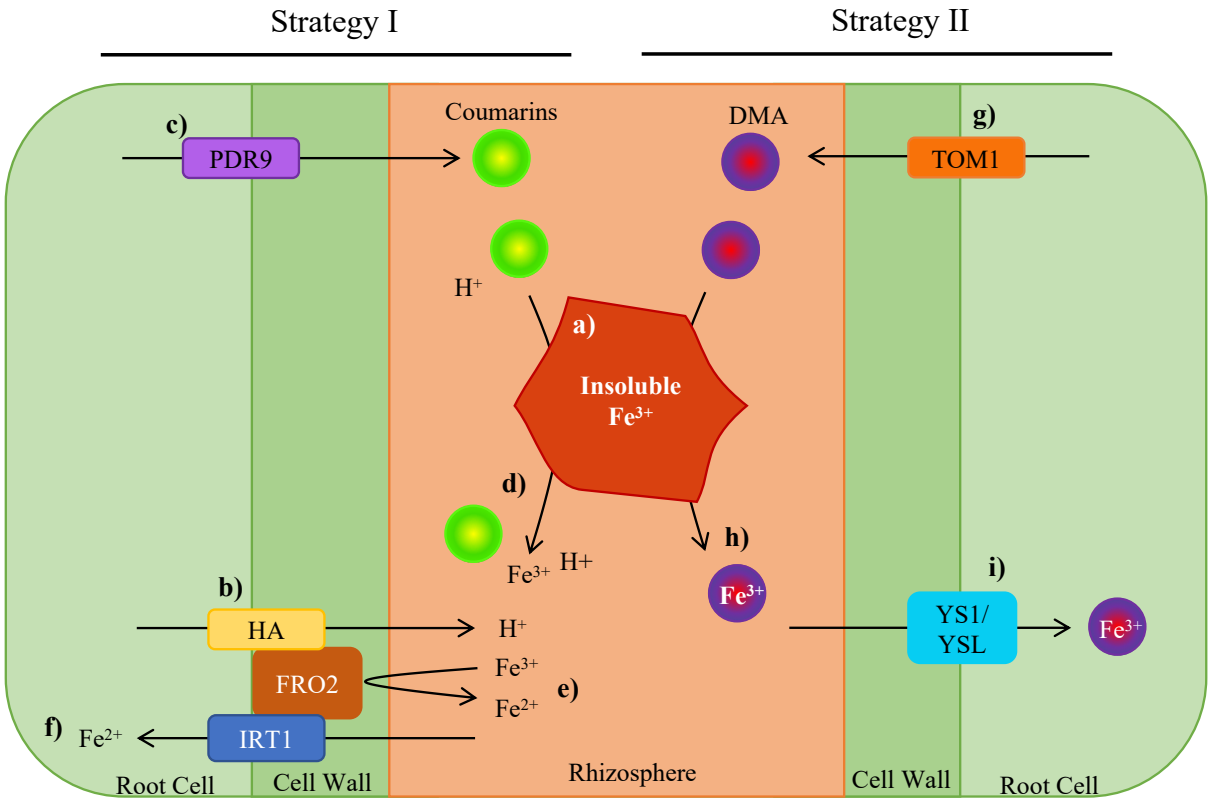

Figure I-1: Iron acquisition in plants. a) Insoluble iron, forming hydroxyls. In strategy I, iron is first solubilized by **b)** increasing pH (AHA secretes H^{+}) and **c)** the coumarins secretion (PDR9 transporter). Then, **d)** the solubilized Fe^{3+} is reduced by **e)** FRO2. **f)** IRT1 uptake the iron Fe^{+2} into the root cells. In strategy II, **g)** TOM1 secretes MAs from the root cells, like DMA, **h)** solubilized and ligated to Fe^{+3}, and its uptake by the **i)** YS1/YSL transporters. The image was modified from Kobayashi and Nishizawa, 2012.

Different transporters have been described to uptake iron from xylem and phloem to leaf and sink organs (Jeong *et al*., 2017). The *YSL* family has an important role in iron-NA transport. *YSL2* has been described as a transporter of iron-NA from the xylem to other neighboring cells and phloem. However, due to the *YSL* family's redundancy in Arabidopsis, no chlorotic phenotype has been found in the *ysl2* mutant (DiDonato *et al*., 2004). *YSL1* and *YSL3* have proved to be necessary for the correct reproduction in Arabidopsis. Double mutant *ysl1ysl3* suffer from a decrease in seed per silique, seed weight, and germination (Fig. I-2b) (Chu *et al*., 2010).

Another gene related to iron transport from source to sink tissue is *OPT3* (*OLIGOPEPTIDE TRANSPORTER 3*) (Fig.I-2c). In *opt3* mutants, both leaves and xylem contain more iron than wild-type plants, and phloem contain less iron than wild-type (Zhai *et al*., 2014). This hyperaccumulation of iron in *opt3* leaves is also observed in other tissues such as inflorescences, stems, and siliques. However, total iron content and seed yield (mg of seeds per plant) of *opt3* seeds are lower than in wild types (Stacey *et al*., 2008).

Iron is distributed through different transporters on the plant; however, it is still unclear how iron is transported and distributed to the whole plant. An example is the little amount of knowledge we have about the mechanisms in which iron is load into the embryos.

1.4. Iron distribution and accumulation in embryos

All iron content in the seeds is provided from xylem and phloem, and the percentage provided for each of them may vary depending on the species. In Arabidopsis is estimated that 60-70% comes from xylem and 30-40% from phloem sap (Grillet *et al*., 2014). However, little is known

about how iron is transported inside of the seed. In Arabidopsis, two genes could be related to this process, *OPT3* (Fig.I-2c) and *FERRIC REDUCTASE DEFECTIVE 3* (*FRD3*) (Fig. I-2d). Knock-out *opt3* mutants suffer embryo lethality (Stacey *et al*., 2002), and knock-down *opt3* mutants show a reduced iron content in the embryos (Stacey *et al*., 2008). *FRD3*, a citrate efflux transporter gene, has been shown to participate in iron transport between symplastically disconnected tissues (Fig. I-2d)**.** It has been reported that in seeds, *FDR3* is predominantly expressed in endodermal cell layers (Fig. I-3) and aleurone, as shown by *the b-Glucuronidase* (*GUS*) reporter gene expressed under *the FRD3* promoter. Moreover, *frd3* mutants produce fewer seeds but with the same iron content as wild-type seeds (Roschzttardtz *et al*., 2011). In *Arabidopsis thaliana*, iron is accumulated in the embryo during maturation (Ravet *et al*., 2009; Roschzttardtz *et al*., 2009), specifically in vacuoles of the endodermis in the hypocotyl (Fig. I-2e), the mesophyll cells surrounding the provasculature in cotyledons (Fig. I-3a and 3b) (Kim *et al*., 2006; Roschzttardtz *et al*., 2009). Transcripts of *VIT1* iron transporter are accumulated during embryo maturation and then decrease in post-mature embryos (Kim *et al*., 2006) (Fig. I-2f). *vit1* mutant seeds accumulate iron in the abaxial subepidermal cells cotyledons (Roschzttardtz *et al*., 2009). Also, *vit1* grows poorly compared to wild-type seeds in alkaline soil (Kim *et al*., 2006; Mary *et al*., 2015). MTP8 (METAL TOLERANCE PROTEIN8) is a manganese tonoplast transporter, which uptake iron in the subepidermal cells' vacuoles in *vit1* mutants (Eroglu *et al*., 2017). Iron distribution change in *mtp8vit1* double mutant, iron is distributed in all the different cell types in the embryo (Eroglu *et al*., 2017).

During seed germination, transcripts encoding *NRAMP3* and *NRAMP4* transporters (NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN3 and 4) accumulate, which are responsible for iron release from the vacuoles of endodermis cells (Fig. I-2g). Iron from endodermis vacuoles is remobilized during the post-germinative process (Laquar *et al*., 2005). Along with this, *nramp3/nramp4* double mutant plants cannot remobilize the embryo's iron, causing post-germinative development arrests (Laquar *et al*., 2005; Roschzttardtz *et al*., 2009).

It has been proposed that FERRITIN2 buffers around 5 % of total iron content in seed (Briat *et al*., 2009). The FERRITIN family has four members in Arabidopsis; FER1, FER3, and FER4 are mainly associated with vegetative and reproductive tissues. At first, FERRITINs were described as an iron storage protein. Currently, FERRITINs are described as protection against oxidative stress (Briat *et al*., 2009). *fer2* mutant seeds have a lower germination rate than wild-type and complemented mutants in high oxidative stress (Ravet *et al*., 2008).

We know the basis of iron distribution and accumulation in Arabidopsis seeds. Iron is accumulated during seed maturation (Ravet *et al*., 2009), stored into endodermis vacuoles by VIT (Kim *et al*., 2006; Roschzttardtz *et al*., 2009; Mary *et al*., 2015), and then relocated by NRAMP3 and NRAMP4 (Laquar *et al*., 2005; Roschzttardtz *et al*., 2009). However, how iron distribution and accumulation in the seed is regulated is still elusive.

Seed maturation is a highly regulated process where we can find clues on how iron load and distribution are regulated in the embryo.

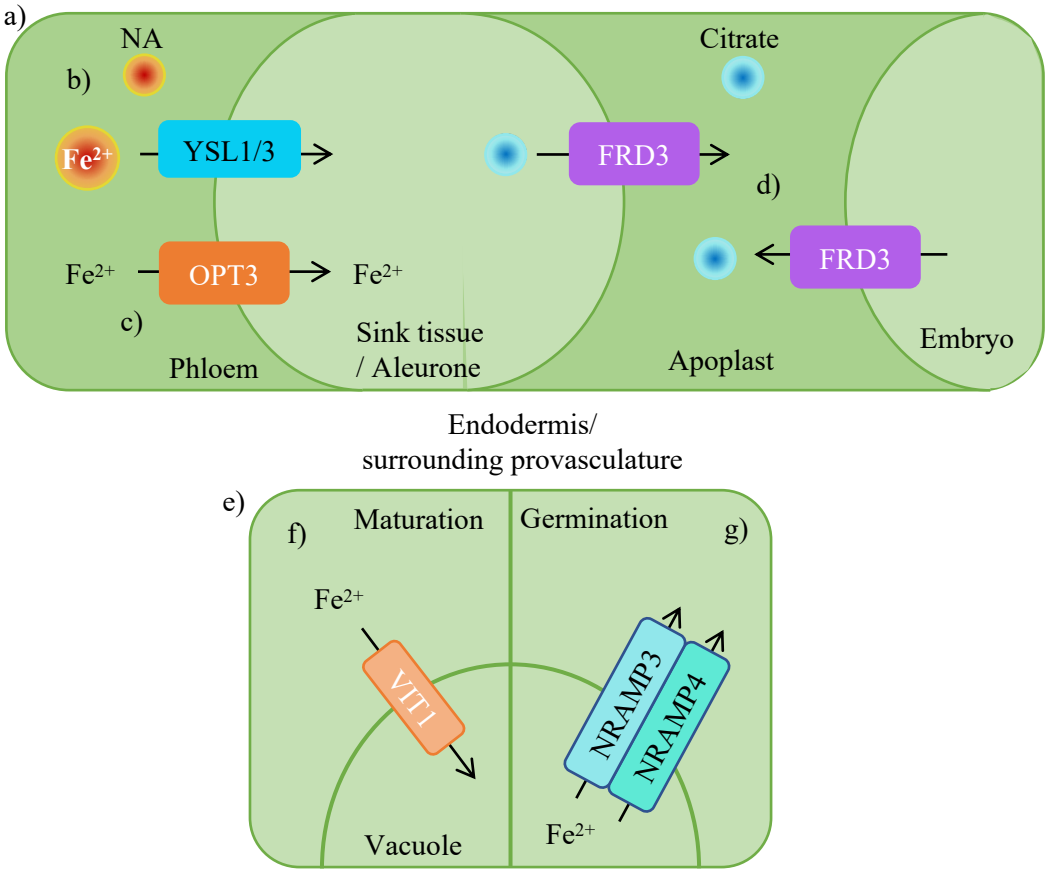

Figure I-2: Iron in the seed. a) Known transporters to be related to iron load into the cells. **b)** The complex form by iron and NA is transported from the phloem to different sink tissues such as the aleurone by YSL transporters. **c)** OPT3 also load iron into the sink tissues. **d)** FRD3 expression facilitated the iron movement of these two symplastically separated tissues, apoplast and embryo. **e)** Iron distribution in the embryo. **f)** During maturation, iron accumulates in the vacuoles of; the endodermis or mesophyll cells that surround the provasculature. VIT1 uptake the iron into the vacuoles. **g)** Then, NRAMP3 and NRAMP4 transport iron outside of the vacuole during germination and post-germinative process.

1.5. Embryo Maturation

Iron is accumulated during maturation to be used during germination, as well as different storage compounds.

In Arabidopsis, the seed embryogenesis process has two parts; morphogenesis and maturation. Embryogenesis starts with double fertilization of the embryo sac, forming the endosperm and the zygote. After this, several divisions occur, allowing the formation of various organs and tissues (meristems, cotyledons (Fig. I-2a), and axis (Fig.I-2b). Once the embryo reaches the heart stage, most differentiation events already occurred (Santos-Mendoza *et al*., 2008). At the torpedo stage, maturation starts. In this process, the embryo acquires all the necessary capacities to resist adverse conditions, such as tolerance to desiccation, accumulation of reserve compounds, and dormancy (Vicente-Carbajosa and Carbonero, 2005).

During embryo maturation, the embryo goes from cell cycle activities to a period of cellular expansion, together with the accumulation of lipids and proteins. Lipids represent approximately 60% of the cell volume in mature embryo cotyledons. Lipids accumulate, forming oil bodies in the cytosol in the form of triacylglycerols (Santos-Mendoza *et al*., 2008). Protein bodies are formed in the endoplasmic reticulum and then stored in protein storage vacuoles. 7S and 11S globulins are principally stored in these compartments (Herman and Larkins, 1999).

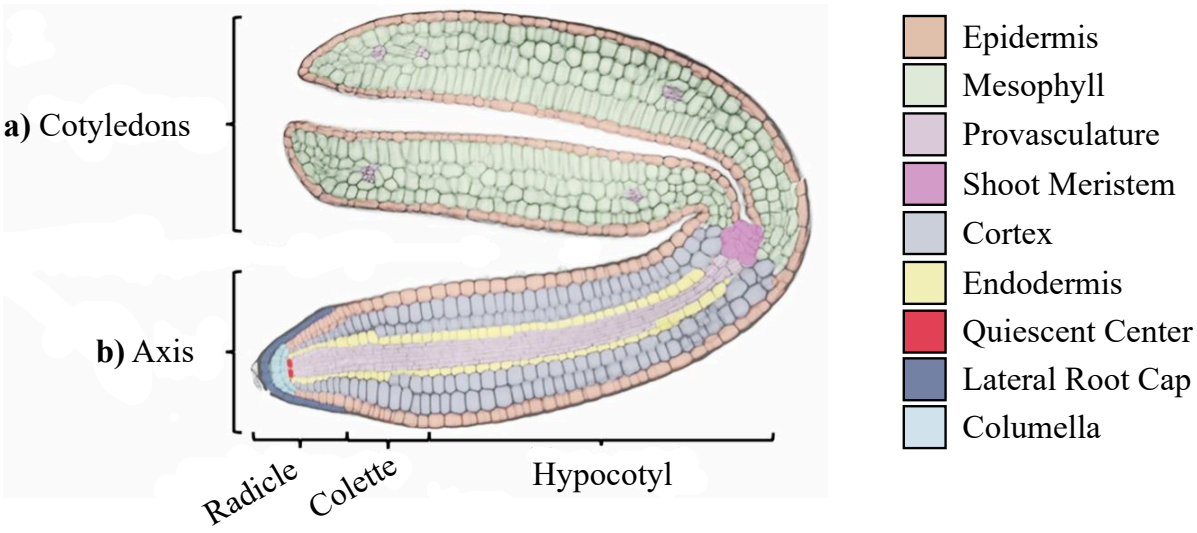

Figure I-3: Embryo structure. Arabidopsis embryo has two main sections, **a)** Cotyledons and **b)** axis. Cotyledons contain an epidermal layer, mesophyll cells (which accumulate storage compounds during maturation), and the provasculature. The hypocotyl, collet, and radicle are part of the axis. Each has different cell layers. First, the hypocotyl is formed by the epidermis, two layers of cortex, endodermis, and the provasculature. The collet is considered a transition zone because a second layer of the cortex is formed there. Finally, the radicle contains the quiescent center, the columella, and the lateral root cap. Figure modified from Bassel *et al.,* 2014.

Two hormones are essential for seed maturation; Abscisic Acid (ABA) and Gibberellin (GA) act antagonistically during seed maturation (Feurtado *et al*., 2018) (Fig. I-4a and I-4b). ABA has a role in different maturation processes, such as the production of storage compounds, acquisition of desiccation, and dormancy. Two peaks of ABA occur during maturation; the first one from the maternal origin is required to end the cell division from morphogenesis, and the second peak from an embryonic origin is essential for dormancy induction (Yamaguchi *et al.*, 2018). On the other hand, GA has several roles in seed development, participated in fertilization, embryo growth, and prevention of seed abortion. GA also has two peaks during embryo development, the first at three days after pollination (DAP) and the second one at the middle of maturation, at the bend cotyledon stage (9DAP) (Hu et al., 2018). However, the most significant increase in GA levels is during germination; simultaneously, ABA concentration decreases. The accumulation of these two hormones seems to be antagonistic during maturation but is significantly stronger during germination. The regulation between these hormones occurs, which correlated negatively, thanks to the B3 transcription factors (Gazzarrini *et al*., 2004; Yamaguchi *et al*., 2018; Yamaguchi and Nambara, 2018).

In the '90s, FUSCA3 (FUS3), LEAFY COTYLEDON2 (LEC2), ABSCISIC ACID INSENSITIVE3 (ABI3), and LEAFY COTYLEDON1 (LEC1) were characterized as master transcription factors in seed development due to pleiotropic phenotypes of their mutants (Ooms et al., 1993; Meinke et al., 1994; Kroj et al., 2003; Santos-Mendoza et al., 2008). Mutants for these transcription factors shared phenotypes such as low accumulation of storage protein and affected seed maturation. Nevertheless, each of these mutants has specific phenotypes; ectopic

trichomes and accumulation of anthocyanins in cotyledons (*fus3*, *lec1*, and *lec2*), the inability to degrade chlorophyll (*abi3*, *lec1*, and *lec2*), intolerance to desiccation (*abi3*, *lec1*, and *fus3*) (To et al., 2006).

All these characteristics are associated with heterochrony, which means that two developmental programs overlap (Keith et al.1994). Recently the heterochrony of the B3 transcription factors mutants has been demonstrated at a molecular level. PYK10, an endoplasmatic reticulum protein that has been used as a molecular marker for post-germinative development. PYK10 has promoter activity in all B3 transcription factor mutants during embryo maturation, demonstrating that mutant embryos of B3 transcription factors contain two different developmental processes simultaneously (Yamamoto *et al*., 2014).

ABI3, *FUS3*, and *LEC2* belong to the B3 transcription factor family, which can bind at the RY motif (CATGCA) present in promoters of seed genes involved in maturation (Fig. I-4c, Fig. I-4d, and Fig. I-4e), like seed storage protein (SSP) genes (Verdier and Thompson, 2008; Carbonero *et al*., 2017), and fatty acid biosynthesis (Baud et al., 2016). Furthermore, the ectopic expression of *ABI3* and *FUS3* under the control of the *35S* promoter stimulates the transcription of seed proteins (At2S1 and CRU3) in vegetative tissues (Carbonero et al., 2017). *LEC2* regulates the expression of *WRINKLED1*, an essential transcription factor that regulates fatty acid biosynthesis upon seed maturation (Baud et al., 2016).

As seed storage proteins (SSP) or lipids bodies (LB) (Fig. I-4f and Fig. I-4g), iron is also a storage compound needed for proper seedling growth (Laquar et al., 2005; Kim et al., 2006; Roschzttardtz et al., 2009). As indicated above, iron is accumulated during embryo maturation, and *VIT1* transcripts accumulated in this developmental stage (Kim et al., 2006; Roschzttardtz

et al., 2009). B3 transcription factors are known for regulating embryo maturation and also the biosynthesis of storage compounds. These processes occur by B3 transcription factors binding to promoters of genes that belong to a biosynthesis pathway or indirectly by repressing processes like differentiation (To et al., 2006; Carbonero et al., 2017).

Due to iron accumulation and distribution occur in concomitance with the synthesis and accumulation of other storage compounds (Fig. I-4h) regulated by B3 transcription factors, it is probable that other storage compounds, like iron, be regulated by B3 transcription factors as well.

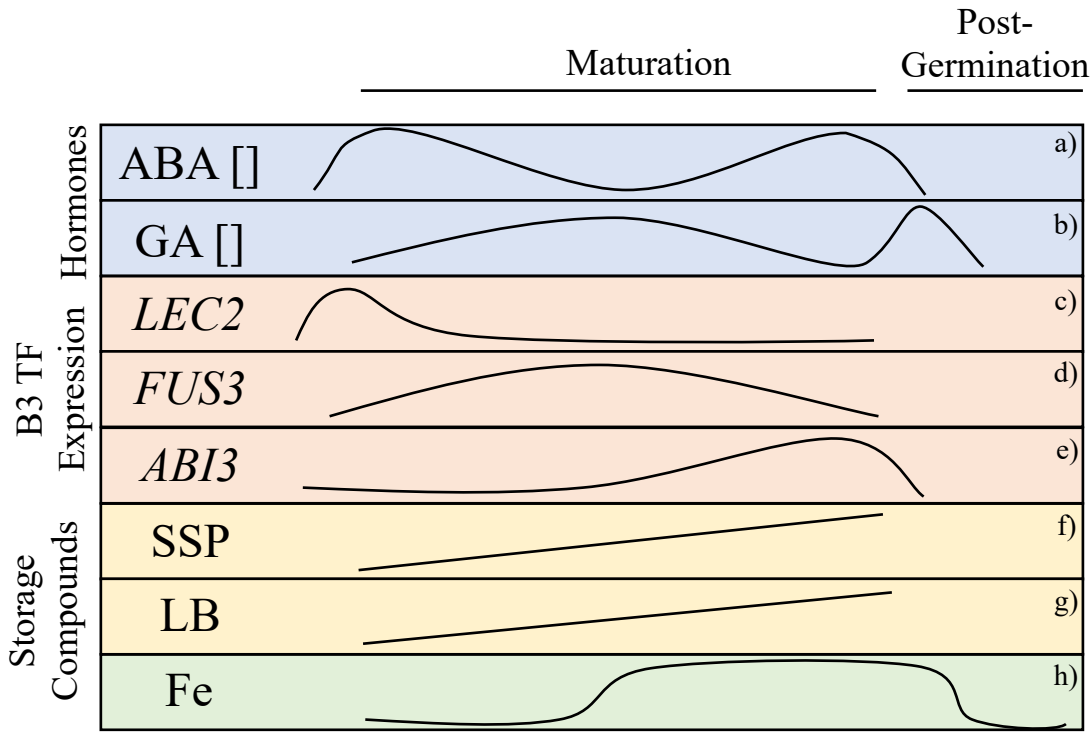

Figure I-4: Main players in maturation and iron. a-b) The two antagonist hormones; ABA and GA accumulation, are inversely correlated. To maintain dormancy, the ratio ABA/GA have to be kept. When the ratio is inverted, germination occurs. B3 transcription factors have a sequential expression. First, **c)** LEC2 expression peak is just before maturation, then **d)** FUS3, and finally **e)** ABI3 has a peak associated with ABA to the dormancy acquisition. Storage compounds (**f)** SSP and **g)** LB) are synthesized and accumulated during maturation and used during the post-germinative process. **h)** Iron (Fe) also accumulated in the seed during maturation and then re-distributed in the post-germinative process.

1.6. Hypothesis

Genes regulated by B3 transcription factors participate in the accumulation and distribution of iron in *Arabidopsis thaliana* embryos.

1.7. General Aim

To determine if the genes regulated by B3 transcription factors participate in the accumulation and distribution of iron in Arabidopsis thaliana embryos.

1.8. Specific Aims

- To evaluate accumulation and distribution of iron in mutants for B3 transcription factors genes during development of Arabidopsis embryos.
- To determine if B3 transcription factors regulate genes related to iron homeostasis in seeds.
- To evaluate B3 transcription factors' role in regulating genes related to iron homeostasis in seeds in A. thaliana embryos.

1.9. Materials and Methods

1.9.1. Arabidopsis Growth Conditions

In this work, *Arabidopsis thaliana* was used. Seeds for the following genotypes were obtain from *Arabidopsis Biological Resource Center* (ABRC, Ohio State, Ohio, USA): *ein3-3* (SALK_208607C), *ein3-4* (SAIL_894_B07), *abi3-6* (SALK_138922), *wrky9-1* (SALK_067122C), *eil1-1* (SALK_042113C), *eil1-3* (SALK_049679C). Homozygous mutants were selected by PCR (Tabla).

The following seeds were kindly provided by Dr. Xavier Jordana, Biological Faculty, Pontificia Universidad Católica de Chile: Columbia-0 (Col-0), *fus3-3* (CS8014), Wassilewskija (Ws), *lec2-1* (CS2728), Landsberg (Ler), *abi3-1* (CS24), *abi3-5* (CS6131).

All these genotypes were grown in soil and grown in a growth chamber (21 ºC, 16 hr light / 8 hr dark). For the *fus3-3* and *abi3-6* mutants, we used embryos in the green cotyledon stage to sow. The plants were irrigated with water as needed.

1.9.2. Histochemical Procedures

Histological sections were conducted as follows, embryos from dry seeds were dissected and then vacuumed infiltrated with a solution with 4% w/v of formaldehyde (Sigma) in 0,1 M Na-phosphate buffer pH 7,1 for 20 minutes. Then, embryos were incubated overnight at room temperature. Fixated embryos were dehydrated with a serial bath of ethanol (Winkler), 50%, 60%, 70%, 80%, 90%, and 95% v/v for 1 hour per bath. Dehydrated embryos were incubated with ethanol/butanol (Sigma) overnight and then incubated again in butanol 100% overnight. A final incubation with butanol/resin overnight at room temperature was performed.

Then, embryos were embedded in Technovit 7100 resin (Kulzer) according to the manufacturer's recommendation. Sections of 2-3 μm were obtained using a microtome.

To perform immuno-detection, dry seeds were fixated with paraformaldehyde(Sigma) 4% in 1X PBS pH 7,4 and Tween 80 (Merck)(one drop) and vacuum applied for one hour (3 to 4 times vacuum were broken), then samples were leaving at 4° on a rotary shaker overnight. Two washes of 15 min with glycine in PBS, and then two washes with only PBS were performed next. Dehydration was performed using subsequential baths of ethanol 50%, 70%, ethanol/butanol, butanol, butanol/paraffine in the Histos 5 Microwave Tissue Processor (Rankin Biomedical). Sections of 8 μm were obtained. Paraffin was removed from the samples using SafeSolv and rehydrated by subsequential baths of Ethanol 100%, 70%, 50%, and 1X PBS. Samples were treated with trypsin 0,1% for 8 min, and the reaction was stopped using PMSF 0,05% for 5 min. Then, samples were saturated with FBS (Fetal Bovine Serum) 6% overnight at 4ºC. Purified anti-FERRITINs (rabbit) 8 μg/mL in FBS were incubated overnight at 4ºC (Purified anti-FERRITINs was kindly provided by Geneviève Conéjéro, Montpellier Pessources Imagerie, CIRAD, Montpellier). Before the incubation with the secondary antibody, three washes with PBS were made. Alexa488 anti-rabbit 1/500 (Molecular Probes) in 3% FBS were used to incubate for 1,5 hours at room temperature, then three washes with PBS for 10 min were performed. The slices were mounted with Mowiol (ROTH, prepared following manufacturer instructions) and analyzed using an epifluorescence or confocal microscopy the next day.

1.9.3. Perls stain and DAB/H2O2 intensification

Perls staining was performed as described by Roschzttardtz et al., 2009. Briefly, embryos were dissected from silique or seed depending on whether we analyzed maturation (torpedo, bent,

curled, or green cotyledon) or seed. Seeds were imbibed in distilled water before dissecting (no more than 2 hours). The embryos were vacuum infiltrated at room temperature for 20-40 minutes with Perls stain solution. Embryos were maintained in Perls solution until the image was taken.

After Perls staining, for DAB/H2O2 intensification, embryos were incubated for 15 minutes with a methanol solution that contained 0.01 w/v NaN3 and 0,03% v/v H2O2. Finally, after a wash with Na-phosphate buffer 0.1 M pH 7.4, intensification was performed. Embryos were incubated with an intensification solution that contained phosphate buffer, 0.005 w/v DAB, 0.005 vv/v H2O2 and 0.005 w/v CoCl2 for 15 minutes. Embryos were washed and maintained in distilled water to stop the reaction. Images were acquired using SMZ800 zoom stereomicroscope (Nikon). Sections were observed with a microscope Eclipse 80i (Nikon). In both cases, images were acquired with the camera Nikon Digital Sight DS-5M.

1.9.4. Iron quantification

For each genotype, 20-22 mg of dry seeds were digested with 3 mL of HNO3 bi-distilled (Sigma) 0.5 M and 1 mL H2O2 (Sigma). Then, samples were taken to 10 mL with HNO3 0.5 M and analyzed in an Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Thermo Fisher Scientific Finnigan X series 2). Each replicate corresponds to seeds from individual plants.

1.9.5. RNA isolation and cDNA synthesis

RNA was obtained from 5 – 6 siliques that contain mature green cotyledons or 20-25 mg of dry seeds. Siliques or dry seeds were frozen with liquid nitrogen and maintained at -80 ºC until required. The tissue was ground with the FastPrep-24 instrument (MP Biomedicals) and metal

(2.8mm stainless steel beads), ceramic beads (1.4mm zirconium oxide beads) following the manufacturer's recommendation.

According to the manufacturer's instructions, the powder was used to isolate RNA using Spectrum Plant Total RNA kit (Sigma).

After obtaining the RNA, 1 µg was used to digest the DNA residual with RQ1 RNase-Free DNase (Promega) according to the manufacturer's instruction. The cDNA synthesis was then performed with the First Strand cDNA Synthesis kit (Thermo) following manufacturer recommendation.

1.9.6. qPCR analysis

Primers were designed using AmplifiX 1.6.3 by Nicolas Jullien, CNRS, Aix-Marseille Université (*http://crn2m.univ-mrs.fr/pub/amplifx-dist*). Primers were designed to have a Tm of 60 ºC and obtain amplicons between 100 and 150 bp long. Then, primers were used to make a standard curve (Supplementary Table 1). StepOnePlus Real-Time PCR System (Thermo), and Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent)we used for qPCR procedures and standard curve, according to manufacturer's instruction. 1/10 dilution of cDNA was used for the qPCR reaction, and as a housekeeping gene TIP41-like (AT3G54000).

1.9.7. Western blot

Total proteins of dry seeds were extracted in the following manner. First, dry seeds (20mg) were frozen with liquid nitrogen and ground using the FastPrep-24 instrument (MP Biomedicals) and metal (2.8mm stainless steel beads), ceramic beads (1.4mm zirconium oxide beads) following the manufacturer's recommendation. Then, homogenized seeds were resuspended in 500 µL of Urea/Thiourea Buffer (7 M Urea, 2 M thiourea, 4% CHAMPS, 1% DTT (Invitrogen) in 30 mM

Tris–HCl solution (pH 8.5)(Winkler)), and vortexed for 30 min at 4ºC, centrifuged and the supernatant recover. Total proteins were measured using the Bradford (Thermo Scientific) method. All the following procedures were performed as Tissot *et al.,* 2019. Immunodetection was performed using SUPER SIGNAL WEST PICO PLUS (Thermo Scientific).

1.9.8. Gene Regulatory Network

First, Biological Processes related to "iron" were searched in the Gene Ontology database, 165 processes were found, and 590 Arabidopsis genes were associated with those Biological processes. Using microarray data from GEO (GSE61686), genes differentially expressed between B3 transcription factors and wild-type were determined in two different stages (early and late maturation) using a two-way ANOVA was performed, using a p-value <= 0.005. Genes differently expressed were intersected by the 590 genes associated with the term "iron". Then, the interactions between these genes were searched in the following databases: AGRIS (Yilmaz *et al.,* 2011), DAP-Seq (O'Malley *et al*., 2016), PBMs (Weirauch *et al.,* 2014), and ANAP (Wang *et al.,* 2012). Correlation between pairs of genes was made using AtGenExpress: Developmental Series (GSE5634) (Schmid *et al*., 2005). Genes with a correlation >0.8 (or -0.8), and p-value <= 0.01 were kept. Also, genes abundant in seed were searched in the AtGenExpress: Developmental series (Schmid *et al*., 2005), siliques and seeds samples were compared to Shoots & Stems, Toots, and Leaves, using the Pavlidis Template Matching (MeV). Finally, the network visualization was made in Cytoscape using the data obtained in Gene Ontology and the different microarrays, together with interaction databases (AGRIS, DAP-seq, PBMs, ANAP) and known regulatory relationships in the literature of B3 transcription factor.

Chapter II: Iron distribution and accumulation in B3 transcription factor mutant embryos

1. Introduction

LEC2, FUS3, and ABI3 belong to the plant-specific B3 superfamily. All the 118 members of this family have a ~110 amino acid region called the B3 domain, which encodes a sequence-specific DNA binding activity. The B3 superfamily is divided into subfamilies; LEAFY COTYLEDON2-ABSCISIC ACID INSENSITIVE3-VAL (LAV), AUXIN RESPONSE FACTOR (ARF), RELATED TO ABI3 AND VP1 (RAV), and REPRODUCTIVE MERISTEM (REM) families (Fig. II-1)(Swaminathan *et al*., 2008). The LAV family is formed by six genes in Arabidopsis divided into two groups; the LEC2-ABI3 group and the VAL group. The LEC2-ABI3 group function in seed development; meanwhile, the VAL group function in different organs during plant development (Swaminathan *et al*., 2008). ARF1 bound to a DNA element present in auxin response genes, the 22 genes members of the ARF group have been identified by the similitude with ARF1. ARF group participate in different processes of plant life. RAV genes have been associated with leaves and flower development. REM group has been poorly characterized. However, some subgroups have been studied, suggesting a role in shoot meristems function and flower development (Swaminathan *et al*., 2008).

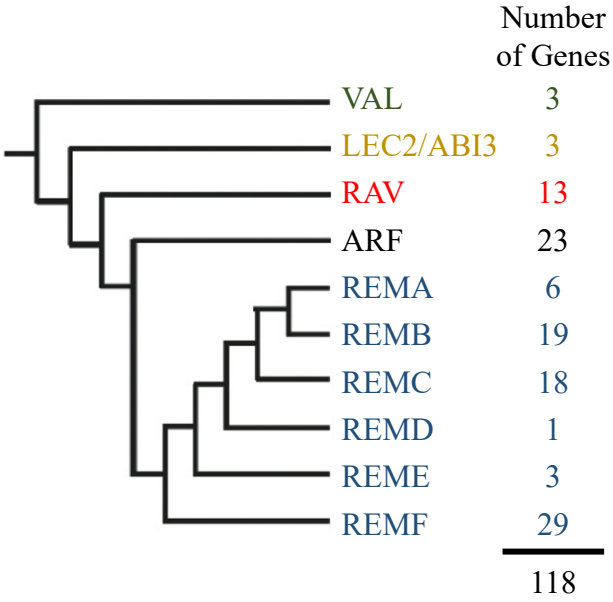

Figure II-1: The B3 superfamily in Arabidopsis. One hundred eighteen members are in the B3 transcription factor family. The VAL and LEC2/ABI3 sub-groups form the LAV group that has six genes. The RAV and ARF have thirteen and twenty-three genes, respectively. In the REM group, six subgroups are formed by phylogenic relatedness, including seventy-six genes. Figure modified from Swaminathan *et al.*, 2008.

1.1.LEC2

LEC2 gene encodes a 363 polypeptide expressed primarily during seed development (Stone *et al*., 2001). *LEC2* promoter is principally activated in the axis and only follows the cotyledons' provasculature pattern (To *et al*., 2006). By screening a collection of putative embryo-defective mutants, Meinke *et al.* (1994) *found* one line with a phenotype similar to the LEC1 (*LEAFY COTYLEDON 1*) phenotype. *lec2* morphology has some similarities with *lec1* and *fus3* mutants, such as trichomes on the cotyledons, expansion of the shoot apex, and stomata formation. *lec2* phenotype was described as highly pigmented seeds due to their accumulation of anthocyanins in the top half of the cotyledons. *lec2* mutant seeds are tolerant to desiccation (Meinke *et al*., 1994). *lec2* mutant seeds have 15% less protein, 30% fewer lipids than WT. However, *lec2* seeds accumulated 140% more sucrose and 5-fold more starch than WT. It is hypothesized that an impairment in oil synthesis in seeds could cause this increase in sucrose and starch (Angeles-Núñez and Tiessen, 2011). LEC2 has an essential role in lipids biosynthesis, binding directly to different the promoter regions of genes related to lipid biosynthesis such as; WRINKLE1, a transcription factor necessary for the accumulation of triacylglycerols, or OLEOSIN, a structural protein for lipids bodies (Baud *et al*., 2007; Che *et al*., 2009). *LEC2* ectopic expression in leaf triggers the accumulation of lipids and seed-specific gene transcripts, and *LEC2* overexpression produces somatic embryos (Santos-Mendoza et al., 2005; Stone et al., 2001).

1.2.FUS3

FUSCA3 gene was first isolated in 1998, described as a gene composed of six exon, five introns, and a conserved B3 domain (Luerben *et al.,* 1998). However, the *fusca* phenotype was described earlier.

According to Wold of Dictionary (https://worldofdictionary.com/dict/latin-english/meaning/fuscus), the word fusca derives from the Latin word fuscus, which means dark or swarthy, and was used as a nickname for people with a dark complexion. In 1994, more than 200 *fusca* mutants were described by their main characteristic; darker seeds caused by anthocyanins accumulation (Miséra and Müller, 1994).

Other phenotypical characteristics, along with anthocyanins accumulation, are the presence of trichomes in cotyledons, the appearance of leaf primordia before seed desiccation (Keith et al., 1994). *fus3* cotyledons contain little to no storage proteins and lipids, and cotyledon cells' appearance is similar to developing leaves (Keith et al., 1994). Earlier studies on proteins and lipids composition of *fus3* mutants shown that cruciferin and napin (the more abundant seed storage proteins) are almost absent, and storage lipids are reduced, suffering a change in their composition (Bäumlein *et al*., 1994). These phenotypical changes are the outcome of an intense alteration in late embryo development. *fusca3* mutants have been described as heterochronic mutants, meaning that two developmental programs overlap in *fus3* late embryo maturation and vegetative germination (Keith et al., 1994). At a molecular level, FUS3 repressed *the MYB13* gene. MYB13 is a transcription factor active in the shoot meristem region, the axillary buds, and the basis of flowers. In *fus3* mutants, *MYB13*, which is generally repressed in wild-type seed, is activated (Kirik *et al*., 1998). Another example is the previously described (section

Chapter I-1.5.) expression of the post-germinative marker PYK10 in *fus3* (Yamamoto *et al*., 2014).

Besides FUS3 role in maturation and embryo identity, FUS3 is part of hormone regulation. *fus3* mutants are sensitive to ABA, and also *fus3* embryo mutants produce one-third of ABA than wild-type (Nambara *et al*., 2000). *fus3* can germinate during maturation; however, in the presence of external ABA, *fus3* is unable to germinate. When *fus3* is treated with GA inhibitor, germination was not affected (Keith et al., 1994).

Moreover, ectopic FUS3 expression increased ABA production in 5 and 9 days-old seedlings (Gazzarrini *et al.,* 2004). Contrary to ABA, GA synthesis is repressed by FUS3. Genes like GIBBERELLIN 3-BETA-DIOXYGENASE 1 (GA3ox1) are repressed by ectopic FUS3(Gazzarrini *et al.,* 2004). Furthermore, FUS3 has been related to ethylene biosynthesis by repressing the ethylene biosynthesis's transcription factors and enzymes. This repression prevents premature development of the seedlings (Lumba *et al*., 2012).

1.3.ABI3

The first three loci described as ABA insensitive (ABI) were *abi1*, *abi2*, and *abi3* (Koornneef *et al.,* 1984). The same criteria to characterize the ABA deficient (*aba*) mutants were used; changes in the water relations, reduce ABA content, and seed dormancy. However, the *abi* mutants had more ABA than wild-type and *aba* mutants and were from 5 to 20 times less sensitive to exogen ABA than wild-type (Koornneef *et al.,* 1984). *abi3* mutant phenotype differs from *abi1* and *abi2* because *abi3* does not show disturbed water relations (Koornneef *et al.,* 1984). Also, *the abi3* phenotype mutant was compared to the *viviparous1 (Zmvp1)* maize

mutant, which germinates precociously in the ear (or female inflorescence) and ABA insensibility besides producing the same amounts of endogenous ABA (Robichaud and Sussex, 1986; Robichaud and Sussex, 1987). Later, after Zm*VP1* and *ABI3* genes were sequenced, their sequences' similarity will reveal that Zm*VP1* and *ABI3* were orthologs (McCarty *et al*., 1991; Giraudat *et al.,* 1992). ABI3 protein contains the same three basic domains described first in ZmVP1 (Giraudat *et al.,* 1992). In addition to ABA insensitivity, *abi3* mutants have reduced accumulation of storage compounds such as CRUCIFERINs and NAPIN (Nambara *et al.*, 1992; Nambara *et al*., 2005), do not tolerate desiccation (Ooms *et al*., 1993), also do not degrade chlorophyll at the end of maturation (Nambara *et al*., 1992).

1.4. B3 regulatory network

LEC2, FUS3, and ABI3 are part of a complex regulatory network in which every transcription factor plays a role in regulating another. LEC2 is the first B3 to be expressed during maturation (Santos-Mendoza *et al*., 2008; Carbonero *et al*., 2016). LEC2 positively regulates the expression of FUS3 and ABI3 (Santos-Mendoza *et al*., 2005; To *et al*., 2006) (Fig. II-2a), especially in cotyledons. FUS3 and ABI3 positively regulate each other and positively regulate themselves (To *et al*., 2006) (Fig. II-2a). These first connexions were made first by indirect approaches like GUS messenger, *in situ* hybridization, or ectopic gene expression. Later, CHIP-seq or CHIP-on-chip techniques allow the researchers to unveil if the B3 transcription factors bind directly to the other promoters. There is no information about LEC2 binding directly to any regulatory regions of either *FUS3* or *ABI3*. However, FUS3 binds directly to *the ABI3* regulatory region

and its regulatory region (Wang and Perry *et al*., 2013). The same is true for ABI3(Tian *et al*., 2020) (Fig. II-2a).

Besides the intrinsic relations between the B3 transcription factors, B3 transcription factors also have a closed relationship with hormones, such as ABA, GA, and ETHYLENE.

ABA has an essential role in seed development, and its presence or absence produces substantial changes in different development points. ABA is positively regulated by FUS3 and ABI3 (Gazzarinni *et al*., 2004) (Fig. II-2b). On the contrary, FUS3 and ABI3 have the contrary role with GA. (Gazzarinni *et al*., 2004) (Fig. II-2b). LEC2 and FUS3 also inhibit GA biosynthesis by repressing genes that belong to the biosynthesis pathway (Curaba *et al*., 2004). FUS3 represses genes of ethylene's biosynthesis, which finally inhibits the following ethylene response pathway (Lumba *et al*., 2012) (Fig. II-2b).

Altogether, the B3 transcription factor regulates maturation and avoids precocious germination and maintains the temporality of the processes necessary for maturation.

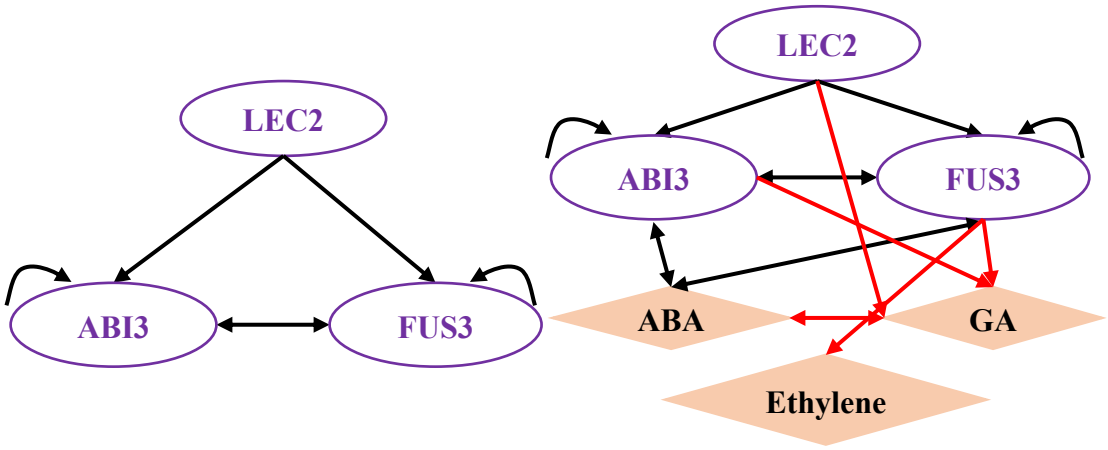

Figure II-2: B3 transcription factor regulatory network. a) Only B3 transcription factor regulation. b) B3 transcription factors regulation, plus interaction with hormones. Black arrows represent positive regulation, red arrow negative regulation. Curaba *et al.*, 2004, Gazzarinni *et al.*, 2004, Lumba et al., 2012, To et al., 2006, Yamaguchi *et al.*, 2007, and others within.

B3 transcription factors have a significant role in embryo development, affecting the accumulation of nutrients, dormancy, and desiccation tolerance. Overall, the B3 transcription factor gives the embryo the identity necessary to fulfill its development to a seedling.

B3 transcription factor mutants were used to understand if they also regulated iron accumulation in embryos during maturation. These results will help to elucidate if the iron is also regulated as a storage nutrient by the seeds.

2. Results

B3 transcription factors affect the accumulation of different nutrients in seed during maturation; iron is one of these nutrients. B3 transcription factor mutant embryos were analyzed during maturation to determine if B3 transcription factors affect iron accumulation in Arabidopsis seeds. Perls/DAB staining was performed on isolated mutant embryos from four stages of embryo maturation; torpedo (T), bend (B), curled (C), and green cotyledon (G) (Figure II-3).

No differences in iron distribution were observed for WT embryos from two different analyzed ecotypes, Col-0 and WS (Fig. II-3 to II-7), where iron is detected in cotyledons and hypocotyl following the provasculature patterning (Figure II-3). *lec2-1* embryos show no differences in the iron staining pattern in the hypocotyl. However, differences with WT embryos are observed in cotyledons for three maturation stages (Bend, Curled, and Green cotyledon) studied. Iron is not detectable in the sections affected by the leafy cotyledon phenotype (Figure II-4A). Iron was poorly detected in *fus3-3* embryos at all seed maturation stages compared to Col-0 (Figure II-3B). It is important to point out that *fus3-3* embryos have variations in the phenotype (Kroj *et al.,* 2003); iron detection reflected this variation. Finally, in *abi3-6* mutant embryos, iron is

detected following the provasculature like WT embryos and in the rest of the embryo, suggesting that iron accumulates in different embryonic cell layers (Figure II-3C).

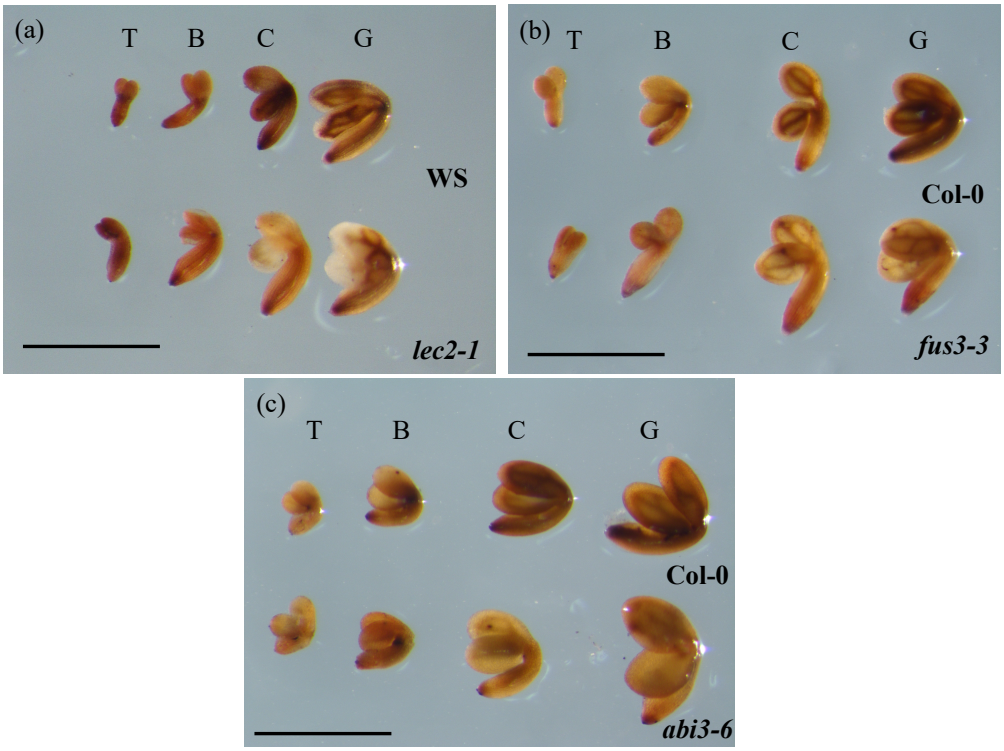

Figure II-3: Iron distribution during embryo development in B3 transcription factors mutants and wild-type. Perls/DAB staining was performed in embryos from torpedo to green cotyledon stage. The upper rows show wild-type embryos in each image, and the lower rows represent B3 transcription tractor mutants. (a) to (c) show lec2-1, fus3-3 and abi3-6 respectively. Bar = 0,5 mm. T: torpedo, B: Bend cotyledon, C: Curled cotyledon, G: Green Cotyledon.

Changes in iron distribution were observed in all three B3 transcription factor mutants during maturation. However, these changes were observed on a big scale. To determine if these changes are reflected at a cellular level, histological sections of dry seeds were stained with Perls/DAB to detect iron. Additionally, B3 transcription factor mutant seeds and wild-type were analyzed to determine total iron content.

Wild-type embryos accumulate iron in vacuoles of the endodermis and mesophylls surrounding provasculature (Figure II-3A, II-4A, and II-5A) as it has been described previously (Kim et al., 2006; Roschzttardtz et al., 2009). In *lec2-1* dry seed sections, iron was detected in vacuoles of endodermis cells in the hypocotyl. However, no iron was detected in leafy cotyledons (Figure II-4B). Perls staining on isolated dry seed embryos showed that *lec2-1* cotyledons seem to have less iron than WT (Figure II-4G). However, total iron content is not affected in lec*2-1* dry seeds (Figure II-4H). In *fus3-3* seed sections, iron was detected in several cell layers, including mesophyll (Fig.II-5D), cortex, endodermal (Fig.II-4F), and provasculature (Fig.II-5D,5F). Perls/DAB staining was less intense than Col-0 embryos stained sections, and the iron seems to be detected in subcellular structures different from vacuoles (Figure II-5A, 5B). Perls/DAB staining intensity varies from embryo to embryo; nevertheless, low intensity and homogeneous distribution were observed in all *fus3-3* embryos studied. Perls staining indicated that *fus3-3* dry seed embryos had less iron than WT (Figure II-5G). However, like *lec2-1, fus3-3* dry seeds had similar total iron content to WT (Figure II-5H).

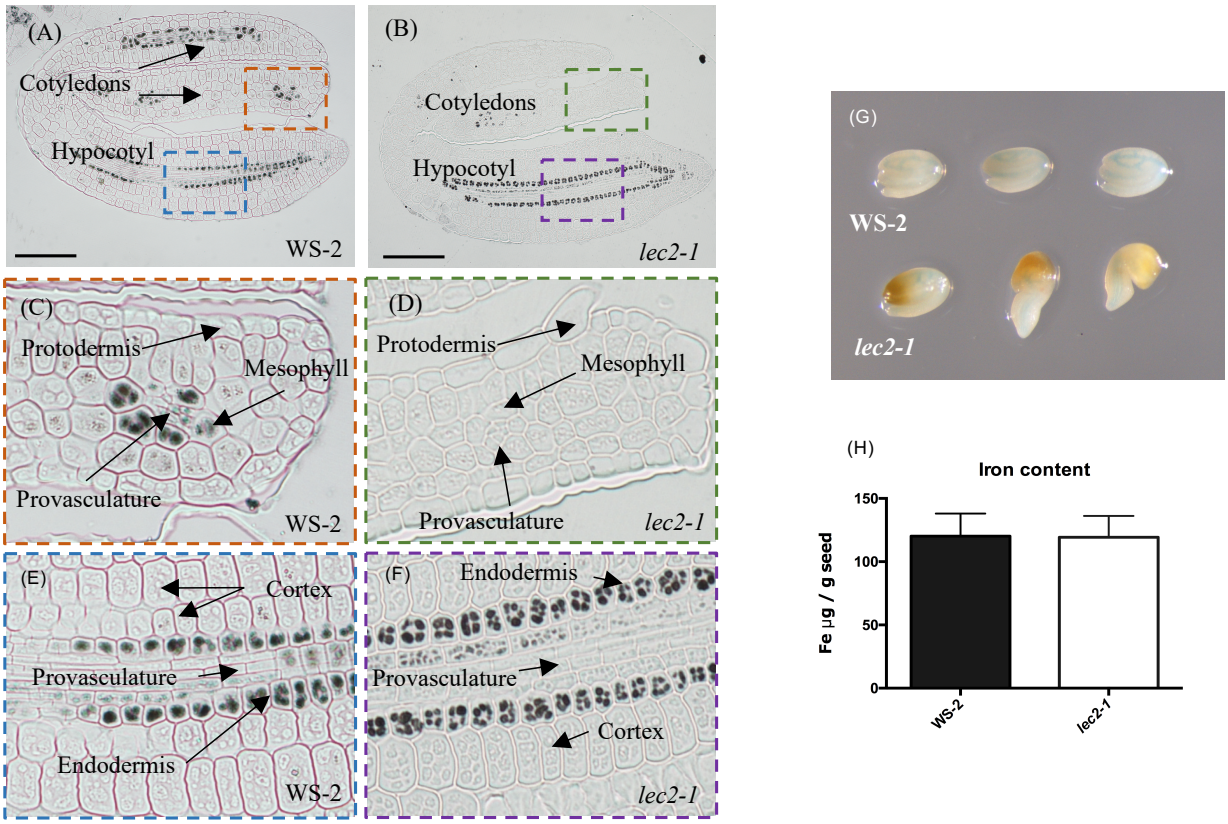

Figure II-4: Iron distribution and accumulation in lec2-1 mutants. Histological section of dry seeds of *Arabidopsis thaliana* WS-2 (A, C, E) and *lec2-1* (B, D, F) stained with Perls/DAB. (C)-(D) Cotyledons and (E-F) hypocotyl close-up. Bars = 100 µm. (G) Perls staining in whole embryos, in the top row three WS-2 embryos, in the bottom row three *lec2-1*. (H) Iron content in dry seeds of WS-2 and *lec2-1*. Four samples were analyzed for each genotype. T-test was performed. P value< 0.05

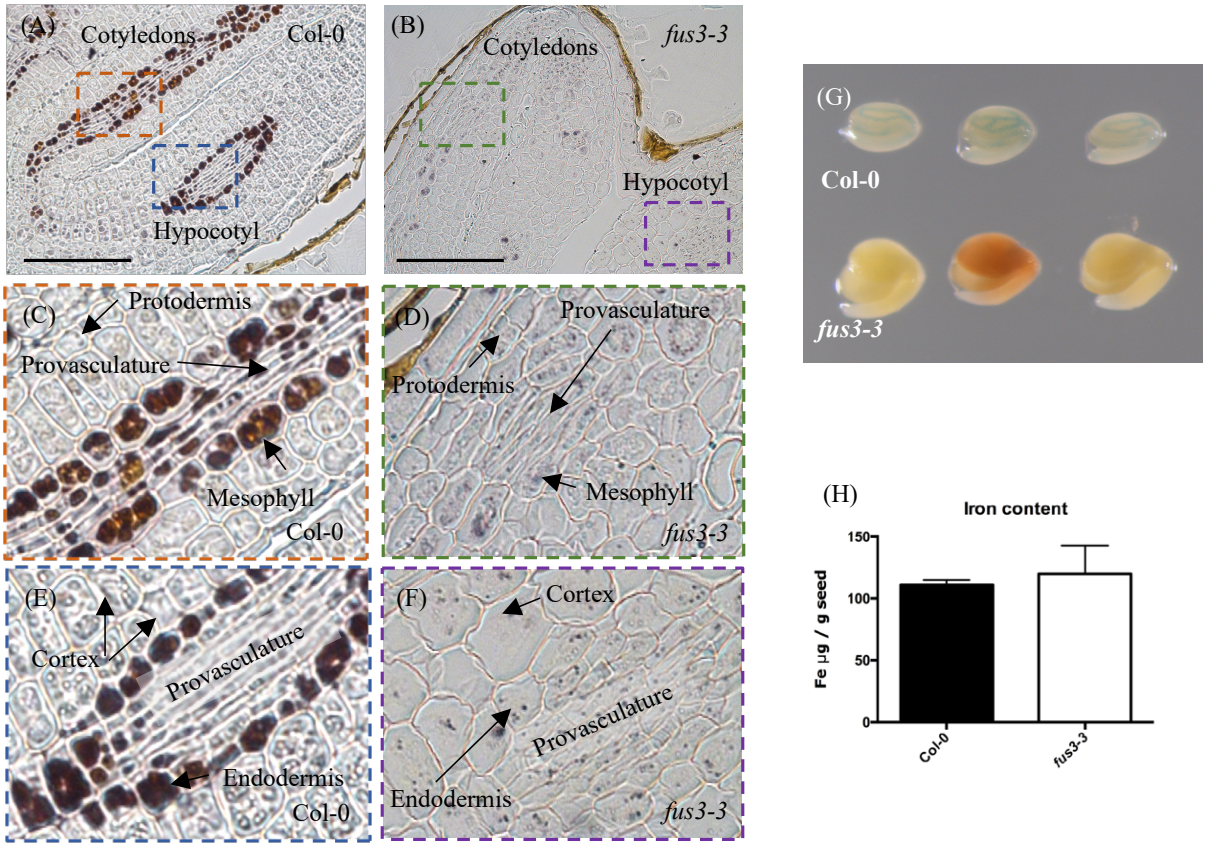

Figure II-5: Iron distribution and accumulation in fus3-3 mutants. Histological section of dry seeds of Arabidopsis thaliana Col-0 (A, C, E) and fus3-3 (B, D, F) stained with Perls/DAB. (C)-(D) Cotyledons and (E-F) hypocotyl close-up. Bars = 100 µm. (G) Perls staining in whole embryos, in the top row three Col-0 embryos, in the bottom row three fus3-3. (H) Iron content in dry seeds of Col-0 and fus3-3. Four samples were analyzed for each genotype. T-test was performed. P value< 0.05

Different *abi3* mutant alleles were analyzed in this study (Fig. II-6 and II-7). First, abi3-1 and abi3-5 were analyzed. The phenotypical difference between them is too hard (Fig. II-6J). We observed that the *abi3-1* mutant has a discontinuous iron accumulation pattern in the endodermis (Fig. II-6B, 6E, 6H). On the other hand, the *abi3-5* phenotype presents iron detection in all cell layers (Fig. II-6C, 6F, 6I). It is almost like were mutants of different genes. Thus, the *abi3-6* allele was chosen to perform the following experiment because it represents the three alleles phenotype (Figure II-7). In Perls/DAB stained *abi3-6* dry seed embryo sections, iron is less concentrated in the vacuoles of cells surrounding provasculature similar to *abi3-1* (Fig. II-7B). Some iron is detected in other cells as mesophyll, protodermis and cortex cells (Figure II-7B, 7D, 7F). Like *fus3-3* mutants, *abi3-6* embryos stained with Perls seem to contain less iron than wild-type (Fig. II-7G). However, there is no difference in iron content between *abi3-6* and WT in dry seed (Figure II-7H).

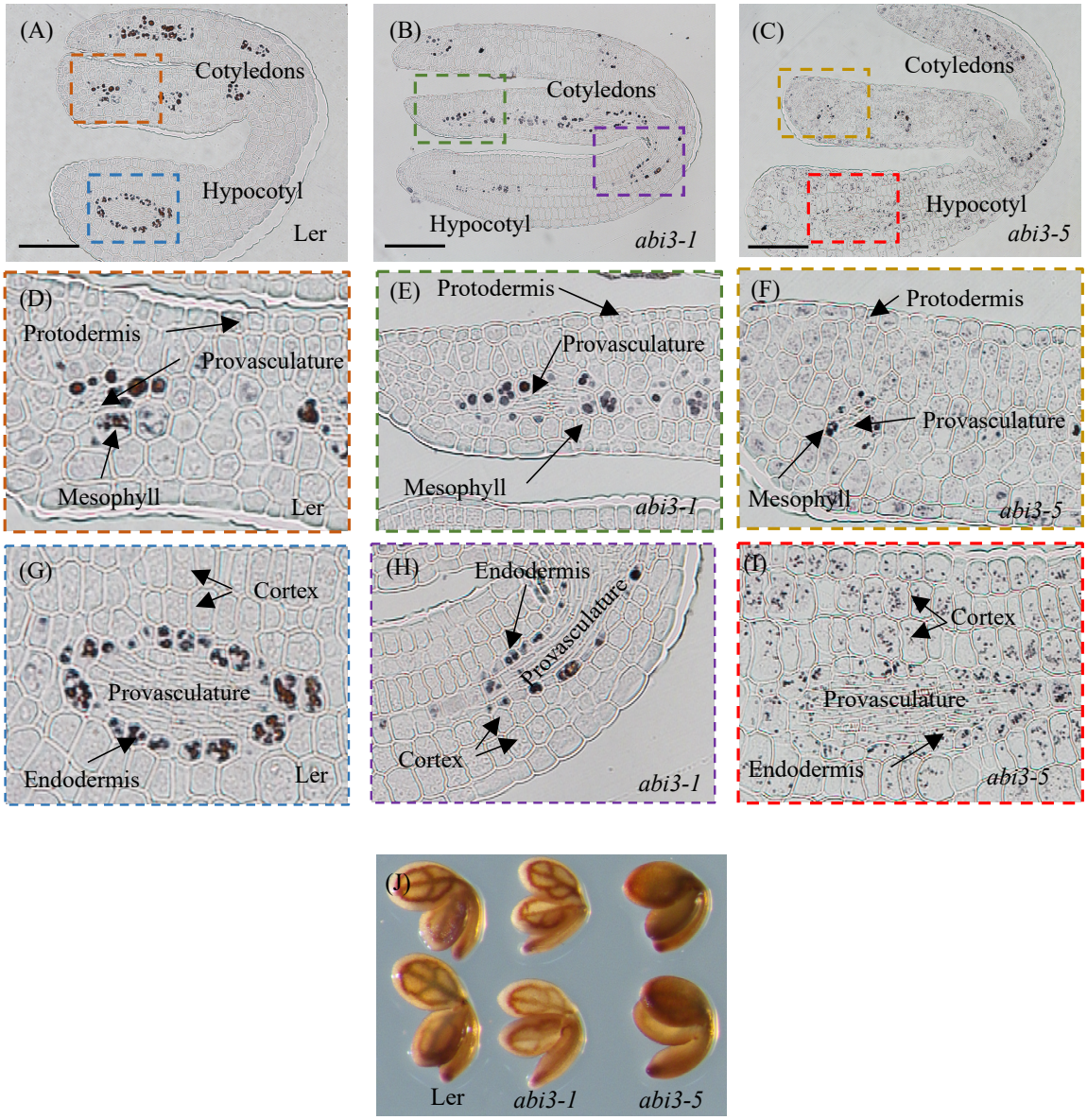

Figure II-6: Iron distribution and accumulation in abi3 mutants. Histological section of dry seed of Arabidopsis thaliana Ler (A, B, C), *abi3-1* (D, E, F) and *abi3-5* (G, H, I) stained with Perls/DAB. (D)-(F) Cotyledons and (G-I) hypocotyl close-up. Bars = 100 µm. (D) Perls/DAB staining in whole embryo, in the first column two Ler embryos, in the second column *abi3-1*, in the last *abi3-5* embryos.

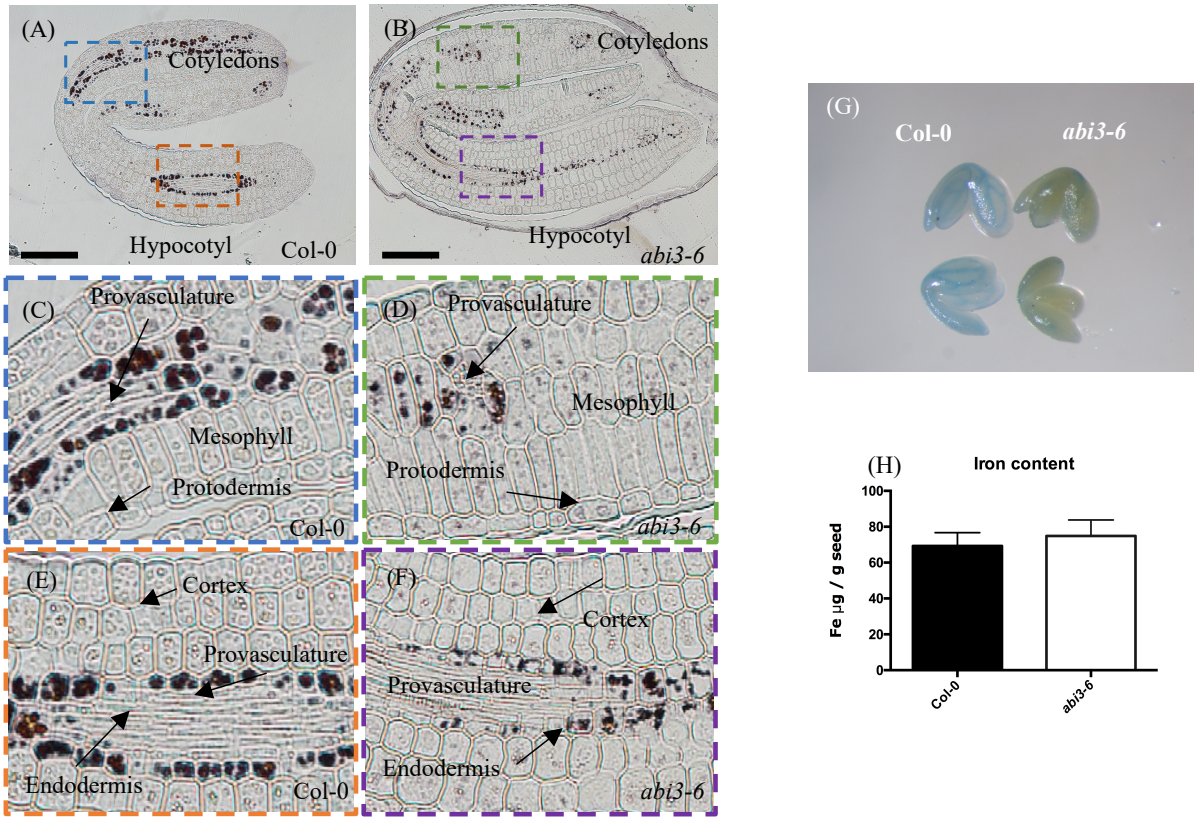

Figure II-7: Iron distribution and accumulation in abi3-6 mutants. Histological section of dry seed of *Arabidopsis thaliana* Col-0 **(A, C, E)** and *abi3-6* **(B, D, F)** stained with Perls/DAB. **(C)-(D)** Cotyledons and **(E-F)** hypocotyl close-up. Bars = 50 µm. **(G)** Perls staining in whole embryo, in the top row three Col-0 embryos, in the bottom row three *abi3-6*. **(H)** Iron content in dry seed of Col-0 and *abi3-6*. Four samples were analyzed for each genotype. T-test was performed. P value< 0.05

3. Discussion

There is shallow knowledge about iron in Arabidopsis seeds. There is only one gene reported in which a mutation causes a change in iron distribution. *vit1* mutant seeds accumulated iron in the subepidermal cell layer, in the abaxial side of cotyledons, without changing seed iron content (Kim *et al.*, 2001). Besides *VIT1*, there is no other gene related to iron distribution in Arabidopsis embryos.

As a gene candidate approach, we focus our study on mutants of the B3 transcription factors known as master regulators of embryo maturation expression genes like LEC2, FUS3, and ABI3 (Santos-Mendoza et al., 2008). We determined that LEC2, FUS3, and ABI3 have a crucial role in iron distribution (Fig. II-3, II- 4, II-5, II-6, and II-7). In the same way that each B3 transcriptions factor mutant has its phenotype, each has a particular iron distribution pattern. It is crucial to notice that total iron content is not affected in any of the B3 transcription factor mutants studied (Fig. II- 4H, II-5H, and II-7H).

Unlike other nutrients that are synthesized and accumulated by the seeds (such as storage protein and lipids), iron is transported into the seed. 54% of iron accumulated in seeds comes from other tissues' remobilization (Pottier *et al.*, 2018). And when remobilization is affected, like in *opt3* mutants, the seed's total iron content decreased (Stayce *et al.*, 2008). These indicated the possibility that iron remobilization in the plant determines the amount of iron accumulated in the seed. In our case, due to B3 transcription factors are expressed and regulated mainly embryo development, they do not have a participation in iron remobilization from other tissues into the seeds.

To understand LEC2, FUS3, and ABI3 iron distribution phenotypes, we must understand the complex regulation between them. The lack of any B3 transcription factor affects the regulation of the others. In *lec2-1,* iron detection is lost in the leafy cotyledons (Fig. II-3 and Fig. II-4). It has been reported that *lec2* leafy cotyledons lack promoter activity of *FUS3* and *ABI3*. However, in cotyledons not affected by the leafy cotyledon phenotype and the hypocotyl, this decrease in promoter activity is not observed (To *et al.*, 2006). Our results show a loss of iron detection in leafy cotyledons but no change in iron distribution in the rest of the embryo in *lec2.* Unlike *fus3* and *abi3*, *lec2* lose their identity only in the leafy cotyledon, allowing iron to be store in the rest of the embryo that it is not affected by the change of identity.

fus3-3 and *abi3-6* have a similar iron distribution, and this similarity may be given by the closed relationship between FUS3 and ABI3. Iron is located in different cell layers in *fus3-3,* and it is not located in the endodermis vacuole (Fig. II-5). FUS3 increased its accumulation at early maturation, in contrast to ABI3, which increased at late maturation. FUS3 accumulation happens in concomitance with iron uptake to the embryo (Ravet *et al.,* 2009). FUS3 lack could be regulating the process in which iron is compartmentalized in the vacuole, increasing the amount of iron available in the embryo.

On the other hand, *abi3-6* also has iron distributed in other cell layers, but iron is detected in the endodermis (Fig. II-7). This phenotype is more pronounced in the stronger allele *abi3-5* (Fig. II-6C, 6F, 6I). The difference can be generated by the different temporality in which FUS3 and ABI3 accumulate during embryo development (Winter *et al.,* 2007; Carbonero *et al.,* 2016). ABI3 lack probably also affects the same process; however, this happens later during

development, allowing iron to compartmentalize. Finally, *lec2, fus3,* and *abi3* are the only mutants together with *vit1* (Kim *et al.,* 2006) with a phenotype for iron distribution in seeds.

The heterochronic phenotype that has been shown in the B3 transcription factor mutants caused them to lose their identity as embryonary tissue. In the leaf, iron is transported into the chloroplasts and mitochondria, and when it is in excess is capture by FERRITINs. Suppose this is the case for heterochronic embryos. In that case, iron remobilized from the vacuoles could be tampon by FERRITINs in the other cell layers of the embryo.

With these results, we could presume that B3 transcription factors regulated iron homeostasis during embryo maturation without affecting the load and total content of iron in seeds.

Chapter III: Genes related to iron homeostasis in seeds appears to be regulated by B3 transcription factors

1. Introduction

Early experiments show that iron distribution is altered in the B3 transcription factor mutants; however, total iron content did not change (Fig. II-4, 5, and 7). These results show that iron load is not affected, but iron distribution in embryos is. Few genes are related to iron distribution in embryos; VIT1, NRAMP3, NRAMP4, and FER2.

1.1.VIT1

Kim *et al.* (2006) searching for the orthologous in Arabidopsis of the yeast CCC1, an iron/manganese transporter found VIT1. VIT1 shared 62% of amino acid similarity with CCC1. *VIT1* also complements the yeast *ccc1* mutant. The authors were also able to determine VIT1 subcellular localization in the vacuolar membrane (Kim *et al.*, 2006). Metal content (Fe, Mn, Zn) was analyzed using an X-ray fluorescence microtomography in *vit1* mutants. Iron distribution change in *vit1* mutant embryo, Kim *et al*. (2006) detect iron diffusely in the hypocotyl and radicle and in the abaxial epidermis. This change in iron distribution affects the *vit1* mutant growth in alkaline soil. *vit1* mutants grow similar to wt seeds in acidic soil, and in alkaline soil, growth is impaired in *vit1* mutants because alkaline soil decreased iron availability (Kim *et al*., 2006).

Later, Roschzttardtz *et al.* (2009) show iron accumulated in the cells that surround de provasculature, in the hypocotyl in the endodermis, and in cotyledons in the mesophylls cells that are around the provasculature. In *vit1* mutant, accumulated iron in the hypocotyls' cortex

cells and in the subepidermal cell layer in the cotyledons. MTP8 (METAL TOLERANCE PROTEIN 8) is responsible for iron distribution in *vit1* mutants. MTP8 is a tonoplast transporter of Mn in the subepidermal cell layer and cortex cells. The *mtp8/vit1* double mutants iron is distributed in all embryo cell layers (Eroglu *et al*., 2017).

1.2.NRAMP3 and NRAMP4

The first *NRAMP (NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN)* gene identified was *NRAMP1* in mouse (*Mus musculus*), where plays a role in intracellular bacterial pathogen sensitivity (Supek *et al*., 1996). Six *MmNRAMP1* homologs in Arabidopsis were found. Arabidopsis NRAMP3 and NRAMP4 can complement the yeast mutant *fet3fet4*. The *fet3fet4* is defective in the uptake of iron. *NRAMP3* and *NRAMP4* expression are similar in leaves and roots of Arabidopsis seedlings. However, when seedlings are grown in an iron chelated medium, NRAMP3 increases its expression mainly in roots; meanwhile, NRAMP4 expression increases in roots and shoots (Thomine *et al*., 2000). NRAMP3 localization was pointed to the vacuoles of the endodermis (Thomine *et al*., 2003). Laquar *et al.* (2005) found something similar for NRAMP4, GUS reporter assay performed in iron starvation showed similar expression between NRAMP3 and NRAMP4. However, the *nramp4* knockout mutant showed no obvious phenotype in iron-deficient conditions (Laquar *et al*., 2005). Due to the similarities between *NRAMP3* and *NRAMP4* expression and the fact that the *NRAMP3* and *NRAMP4* loci are duplicated in the Arabidopsis genome, Laquar et al. (2005) proposed that NRAMP3 and NRAMP4 have redundant functions. Effectively, low iron medium seedling arrested the *nramp3/nramp4* double mutant growth. Furthermore,

nramp3/nramp4 hypersensitivity of iron starvation provokes lethality in nramp3/nramp4 mutants sow in calcareous soil (Laquar *et al*., 2005). The inability to remobilize the iron store in the embryo endodermis vacuoles cause the lethality of the nramp3/nramp4 mutants (Roschzttardtz *et al.*, 2009).

1.3.FERRITINs

FERRITINs are found in bacteria, plants, and animals. In the cell, FERRITINs primary role is to accumulate iron, preventing iron toxicity by Fenton reaction (Andrews, 2010). FERRITINs are composed of 24 subunits that assemble in a spherical protein shell. FERRITINs can store between 2000 and 4000 ferric iron atoms. The sequestered iron is bioavailable, and it is not reactive to oxygen (Briat *et al.*, 2010). In plants, FERRITINs localize in plastids and mitochondria, unlike mammal FERRITINs that are primarily found in the cytoplasm. Another difference between mammal and plants FERRITINs is them regulation. Meanwhile, plant FERRITINs are regulated at a transcriptional level; mammal FERRITINs are regulated at a translational level by an IRE/IRP complex that prevents ribosome translation (Briat *et al*., 2010). In plants, FERRITINs' primary function has been associated with the defense machinery against oxidative stress (Ravet *et al*., 2009; Briat *et al.,* 2010; Bottchet and Mazzafera, 2012). Due to the high sequence identity between FERRITINs, researchers have used Arabidopsis that only has four *FERRITIN*s copies to analyze the biological function of plant FERRITINs (Briat *et al*., 2010).

Petit *et al.* (2001) performed a robust analysis of FERRITINs expression in Arabidopsis at different developmental stages and in the presence of different conditions. Researchers treated

6-week old plants with an excess of iron and studied mRNA accumulation of FERRITINs in roots and leaves. They found that FER1 and FER3 increased their expression in root. In leaves, FER1, FER3, and FER4 also increase their mRNA accumulation. FER2 does not show changes in leaves and roots. However, *FER2* mRNA accumulated mainly in mature siliques and dry seeds (Petit *et al*., 2001). Seedling of 8-week treated with H_2O_2 increased the accumulation only of *FER1* mRNA, neither of the others FERRINTS show difference. The authors also treated 2-weeks old seedlings with ABA. Only FER2 increased its accumulation. This research shows the expression of FERRITINS across the development of Arabidopsis. Besides the fact that FERRITINs are duplicates, each has a particular role during development and different conditions.

In Arabidopsis, seed iron is stored primarily in the endodermis vacuoles, or provasculature surrounding cells, and only 5% of total iron is accumulated in FERRITINs, particularly FER2 (Ravet *et al*., 2009). FER2 has a protective role against oxidative stress in the seed. *fer2* mutants seed treated with methyl viologen, a pro-oxidative agent, suffer from poor germination. The ectopic expression of FER1 reverts this phenotype (Ravet *et al*., 2009).

There are two reasons for studied the genes above; first, iron is mislocated in the three B3 transcription factor mutants perhaps genes related to iron homeostasis, like VIT1 and NRAMPs, are affected in B3 transcription factor; second, the pattern of the Perls/DAB staining in fus3 and abi3 mutants is similar to FERRITINs immunodetection in leaves (Annexed Figure 1). Different analyses were performed to determine if genes related to iron homeostasis are affected by the B3 transcription factor. qRT-PCR was performed in to analyze the change in transcript

accumulation in B3 transcription factor embryo mutants at different developmental stages. Due to access to anti-FERRITINs antibodies, western blot and immunodetection in embryos were performed to described FERRITINs accumulation and localization in B3 mutant embryos.

2. Results

2.1. Genes related to iron homeostasis are affected in B3 transcription factor mutants.

Iron distribution is altered in B3 transcription factor mutant embryos. These results imply that B3 transcription factors regulated the process of iron distribution during maturation. Only three genes have been related to iron distribution in the Arabidopsis embryos; *VIT1*, *NRAMP3,* and *NRAMP4* (Laquar *et al*., 2005, Kim *et al*., 2006, Roschzttardtz *et al.*, 2009, Thomine *et al*., 2000). Therefore, to study *VIT1*, *NRAMP3*, and *NRAMP4* expression during maturation in the B3 transcription factors mutants will provide information about which part of the process is affected.

We analyzed embryos at a curled-green cotyledon stage (Mature) or seeds (Dry). Entire siliques were used for RNA extraction of the mature stage. In *lec2-1,* we observed a decrease in the *VIT1* transcripts accumulation in a mature stage but no significant difference in dry seed (Fig. III-1A). *NRAMP3* showed increased transcript accumulation in both mature and dry seed (Fig. III-1B). Following this pattern, *NRAMP4* transcripts also increase but only in dry seed (Fig. III-1C).

VIT1 transcripts accumulation is lower in the mature stage in *fus3-3* mutants. However, this is reversed in dry seed where *VIT1* transcripts accumulation is higher in *fus3-3* than wild-type (Fig. III-2A). *NRAMP3* expression did not show a significant difference in *fus3-3* embryos (Fig.

III-2B). A significant increase in *NRAMP4* transcripts accumulation was found in dry seeds of *fus3-3* (Fig. III-2C). Finally, until now, we have only performed the qPCR analysis to *abi3-1*. In this weak allele, we did not find a significant difference in transcripts accumulation of *VIT1* and *NRAMP3*(Fig. III-3A, 3B). *NRAMP4* expression increased in *abi3-1* mature embryos compared to WT, but no change was observed in dry seed (Fig. III-3C). It was planned to perform this experiment in the *abi3*-6 allele; however, we could not do it due to the current situation. In general, we could observe that *VIT1* expression in B3 transcription factors decreases in mature embryos compared to WT. Furthermore, *NRAMP3* and *NRAMP4* transcripts accumulation is higher in B3 transcription mutants than in wild-type embryos. These results suggest that the lower iron accumulation in vacuoles of the endodermis present in the B3 transcription mutants could be caused by a deregulation of *VIT1, NRAMP3,* and *NRAMP4.*

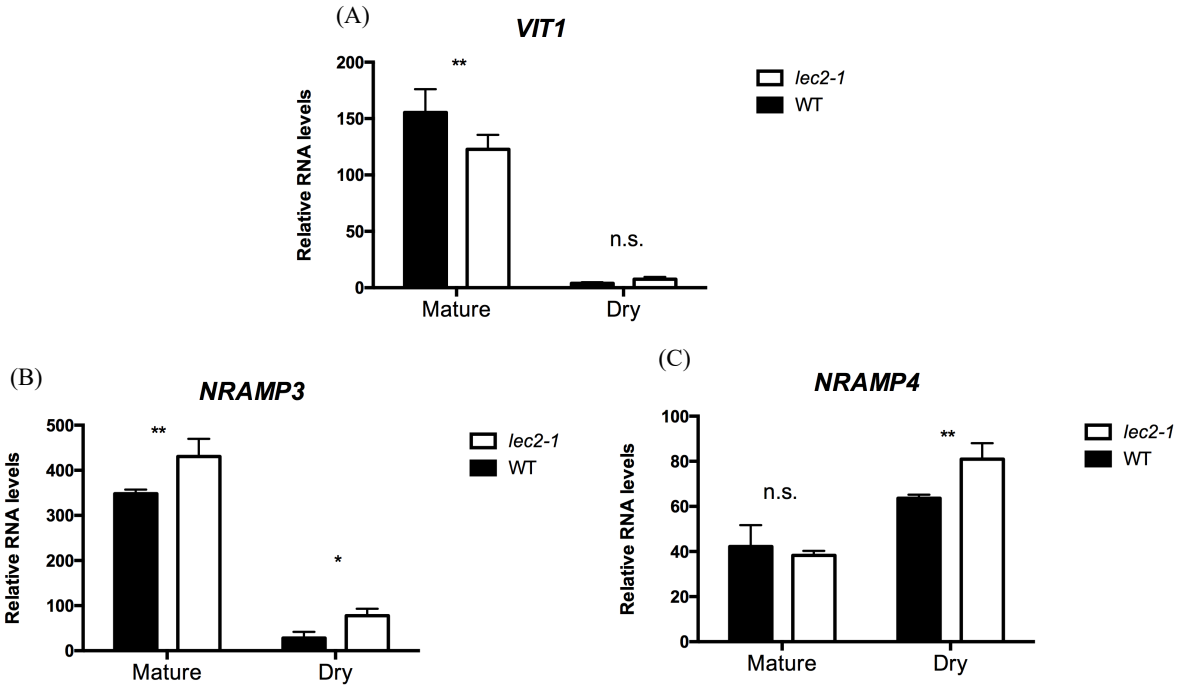

Figure III-1: Gene related to iron homeostasis qRT-PCR analysis in *lec2-1* seeds. qRT-PCR were performed with two maturation stages; green cotyledon (Mature) and dry seed (Dry). WT corresponds to WS-2. (A) *VIT1*. (B) *NRAMP3*. (C) *NRAMP4*. (D) *FER4*. A two-way ANOVA was performed with P-value<0.05.

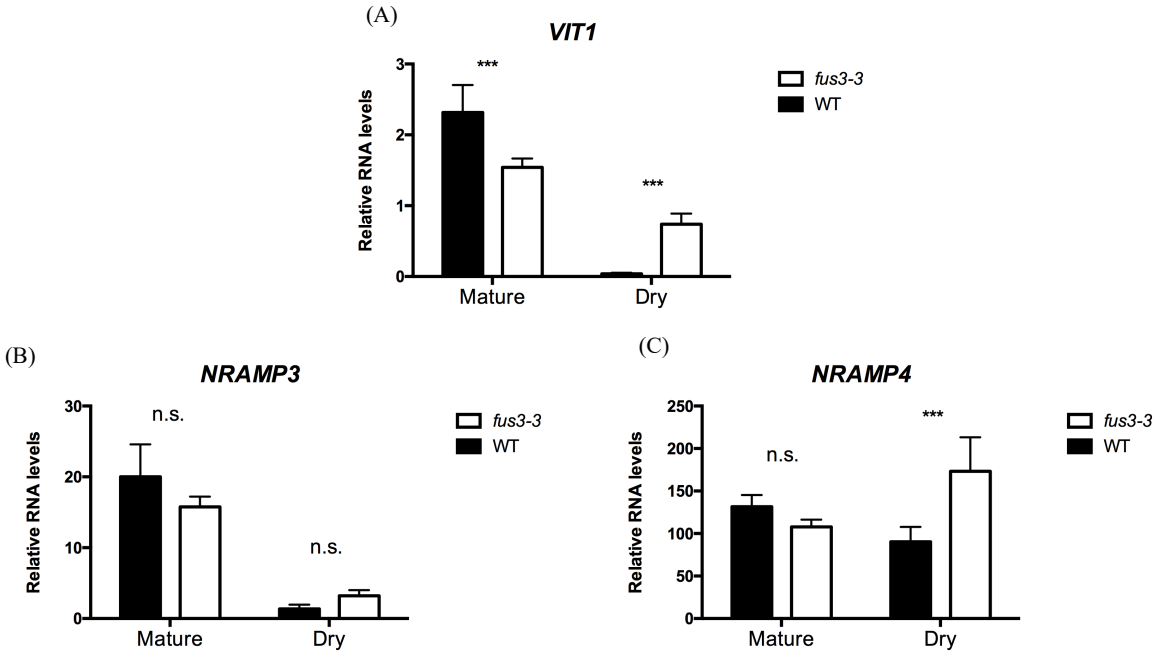

Figure III-2: Gene related to iron homeostasis qRT-PCR analysis in fus3-3 seeds. qRT-PCR were performed with two maturation stages; green cotyledon (Mature) and dry seed (Dry). WT corresponds to WS-2. (A) *VIT1*. (B) *NRAMP3*. (C) *NRAMP4*. A two-way ANOVA was performed with P-value<0.05.

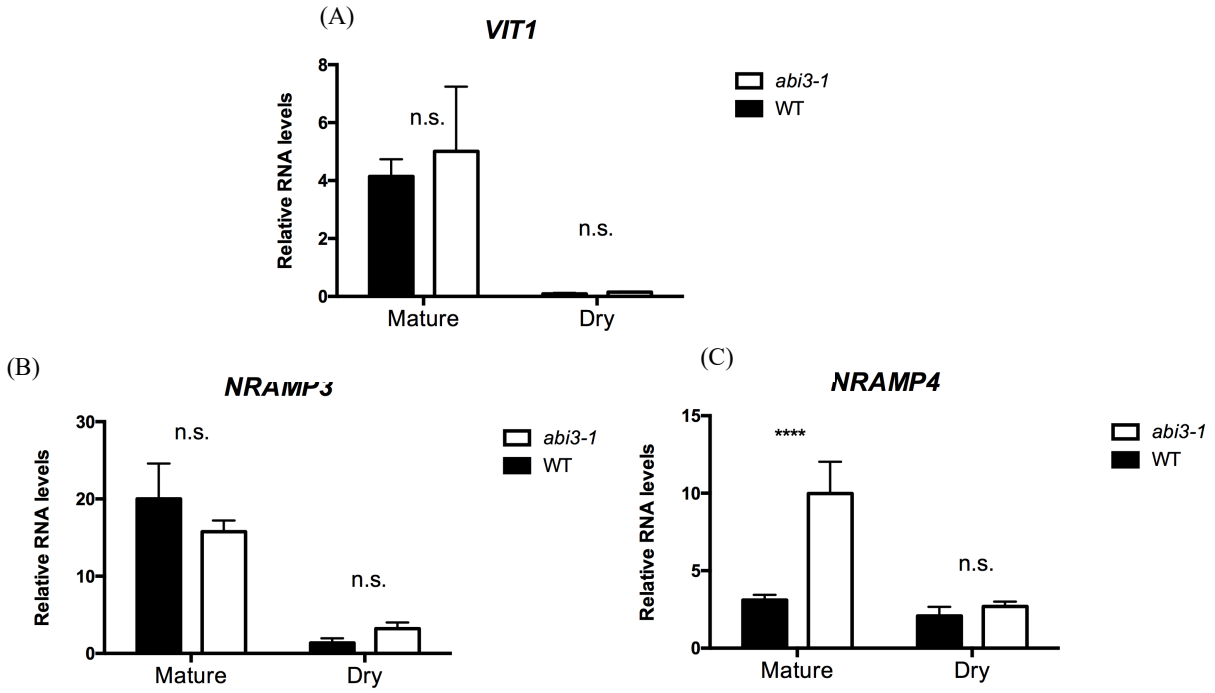

Figure III-3: Gene related to iron homeostasis qRT-PCR analysis in abi3-1 seeds. qRT-PCR were performed with two maturation stages; green cotyledon (Mature) and dry seed (Dry). WT corresponds to WS-2. (A) *VIT1*. (B) *NRAMP3*. (C) *NRAMP4*. A two-way ANOVA was performed with P-value<0.05.

2.2. FERRITIN encoding genes are deregulated in B3 transcription factor seed mutants

The analysis of *fus3-3* and *abi3-6* dry seed embryo sections stained with Perls/DAB reveals that iron accumulates in dots that resemble iron-FERRITIN complex (Fig. II-5, II-5). These complexes were detected previously using leaves from iron excess treated plants (Divol *et al*., 2013, Roschzttardtz *et al*., 2013). Ravet et al., (2009) studied the FERRITIN expression pattern during seed development for the four genes encoding FERRITINs in Arabidopsis. *FER1*, *FER3*, and *FER4* accumulate during morphogenesis stages and are not detected during seed maturation. In contrast, *FER2* is the only *FERRITIN* detected in mature seeds. To evaluate if FERRITIN encoding genes are deregulated in *lec2-1*, *fus3-3*, and *abi3-6* mutants, we performed qRT-PCR analysis. Total RNA from two stages of seed maturation, curled-green cotyledon as the mature stage, and dry seeds were used.

In *lec2-1* mutants, *FER1* transcripts were more accumulated in mature and dry seed than wild-type (Fig. III-4a). On the other hand, *FER2* transcripts accumulation shows no significant difference between wild type and *lec2-1* (Fig. III-4b). *FER3* transcript only shown an increase in accumulation at the mature stage. No difference was found in dry seed (Fig. III-4c). Finally, an increment in *FER4* transcripts was observed in *the lec2-1* mutant dry seed; however, no difference was found at the mature stage (Fig. III-4d).

In *fus3-3*, similarly to *lec2-1, FER1* transcripts were highly accumulated at mature and dry stages compare to the wild-type (Fig. III-5a). On the contrary, *FER2* transcripts decrease their accumulation compared to wild-type in mature and dry seeds (Fig. III-5b). At the mature stage, *FER3* transcripts accumulation is higher in *fus3-3* mutants than wild-type. Conversely, at the dry seed, *FER3* accumulation in *fus3-3* is lower than wild-type (Fig. III-5c). No significant

difference was observed in *FER4* transcript accumulation at the mature stage; however, *FER4* accumulation in *fus3-3* dry seeds was lower than wild-type (Fig. III-5d).

Finally, due to the current situation, only two *abi3* alleles (*abi3-1* and *abi3-6*) were analyzed. In *abi3-1, FER1* transcripts accumulation was higher in dry seeds than wild-type, and no significant difference was found at the mature stage (Fig. III-6a). *FER2* transcripts accumulation in *abi3-1* was lower in dry seed than wild-type. No significant difference was found between *abi3-1* and wild-type at the mature stage (Fig. III-6b). No difference was found in *FER3* transcripts accumulation between *fus3-3* and wild-type in any stage (Fig. III-6c). In *abi3-1*, *FER4* transcripts accumulation in dry seed was lower than wild-type. No significant difference was observed in *FER4* transcript accumulation at the mature stage in *abi3-1* (Fig. III-6d). For *abi3-6*, only dry seeds were analyzed. The results obtained were similar to *abi3-1* dry seeds. A high accumulation of FER1 transcripts was observed in *abi3-6* dry seeds than wild-type (Fig. III-7a). Conversely to *FER1*, *FER2* transcripts accumulation was lower in *abi3-6* than wild-type (Fig. III-7b). No difference was found in *FER3* transcripts accumulation between *abi3-6* and wild-type in dry seed (Fig. III-7c). *FER4* transcripts accumulation was lower in *abi3-6* than wild-type (Fig. III-7d).

Overall, all four *FERRITINs* were deregulated in the mutants, except for *FER2* in *lec2-1* (Fig. III-4B).

Interestingly, *FER1* transcript accumulation has similar behavior in all three B3 transcription factor mutants. A significant enhancement in transcript accumulation was observed in mature and dry seed in *lec2-1, fus3-3, abi3-1,* and *abi3-6* (Figure III-4A, 5A, 6A, and 7A). To determine if the changes observed in the accumulation of *FERRITINs* transcripts are correlated

with changes in FERRITINs protein level, we performed a western blot analysis using an antiserum against plant FERRITINs.

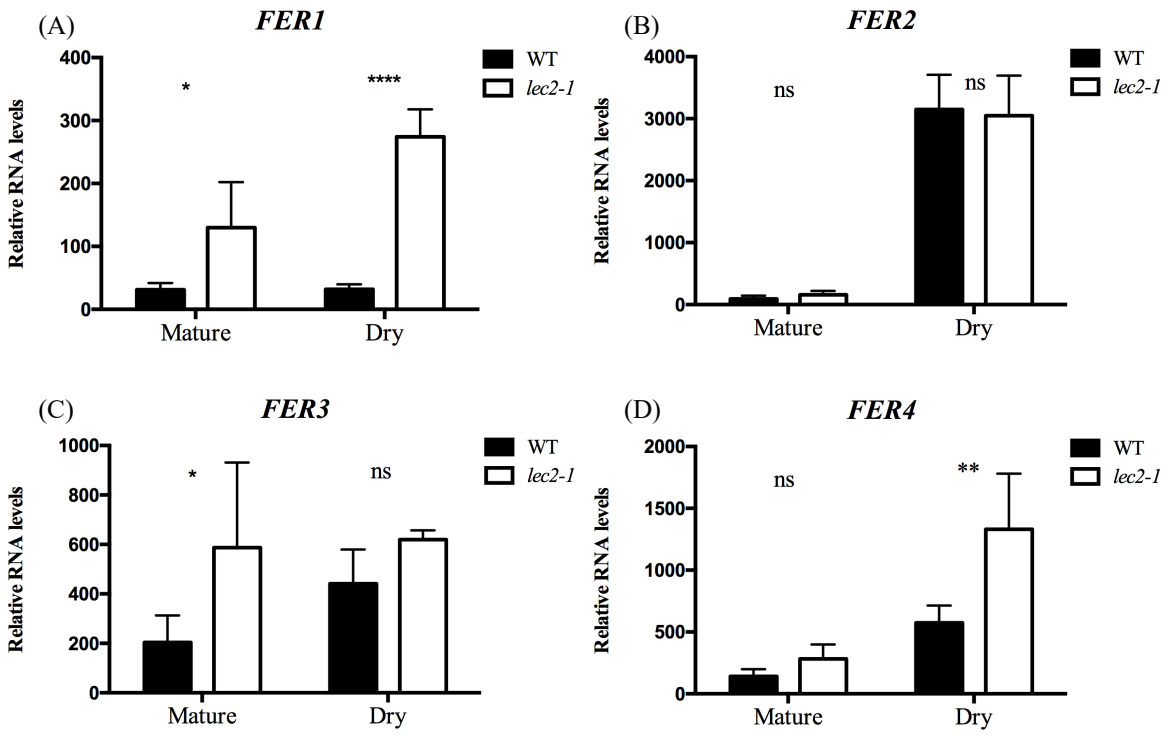

Figure III-4: FERRITINs expression in lec2-1 mutants. qRT-PCR were performed with RNA from Arabidopsis seeds in two maturation stages; green cotyledon (Mature) and dry seed (Dry). WT corresponds to WS-2. (A) *FER1*. (B) *FER2*. (C) *FER3*. (D) *FER4*. A two-way ANOVA was performed with P-value<0.05.

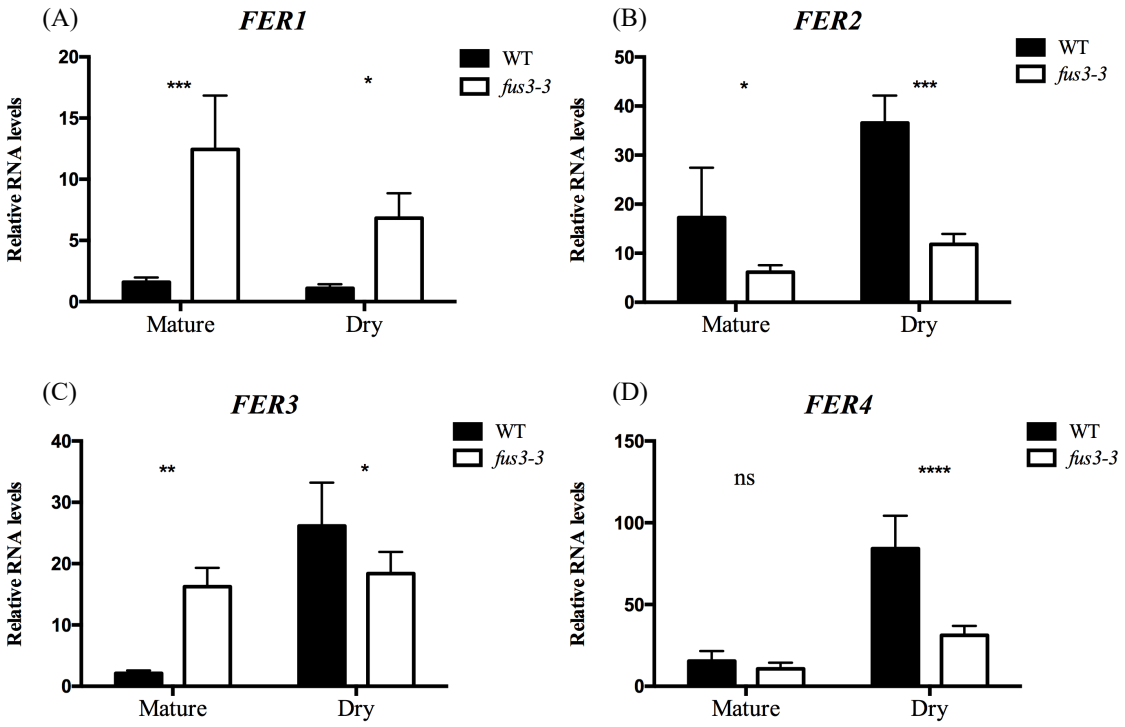

Figure III-5: FERRITINs expression in fus3-3 mutants. qRT-PCR were performed with RNA from Arabidopsis seeds in two maturation stages; green cotyledon (Mature) and dry seed (Dry). WT corresponds to Col-0. (A) *FER1*. (B) *FER2*. (C) *FER3*. (D) *FER4*. A two-way ANOVA was performed with P-value<0.05.

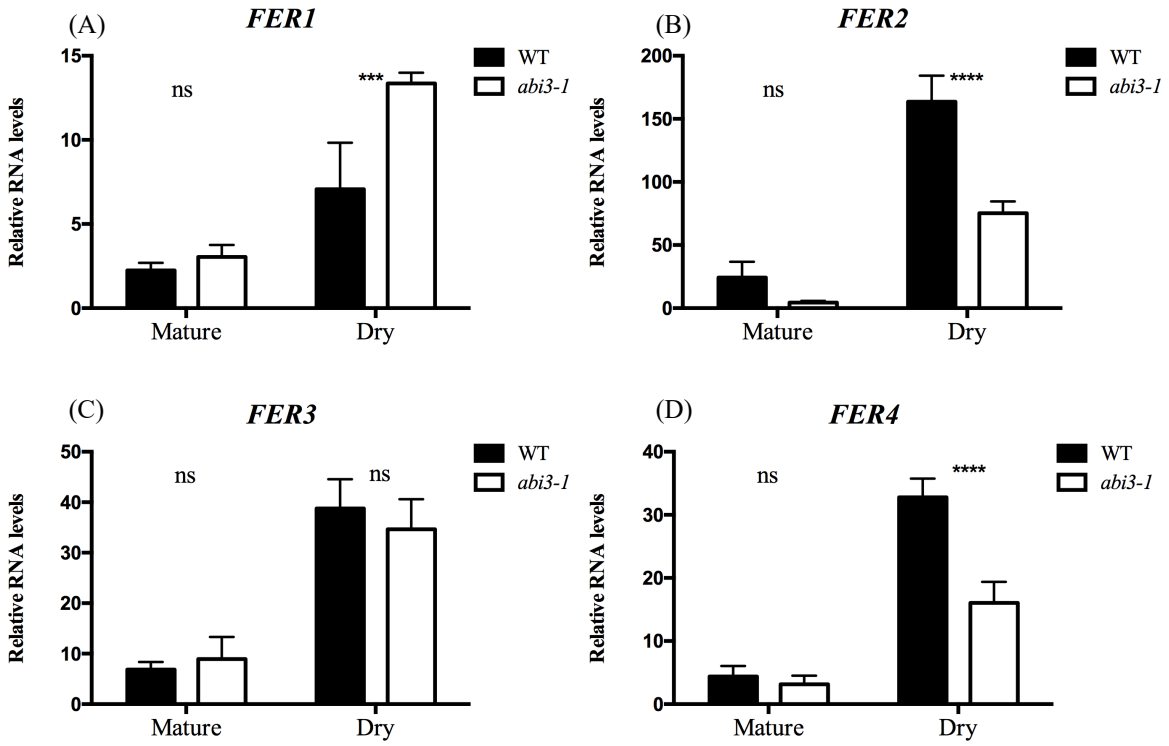

Figure III-6: FERRITINs expression in abi3-1 mutants. qRT-PCR were performed with RNA from Arabidopsis seeds in two maturation stages; green cotyledon (Mature) and dry seed (Dry). WT corresponds to Ler. (A) *FER1*. (B) *FER2*. (C) *FER3*. (D) *FER4*. A two-way ANOVA was performed with P-value<0.05.

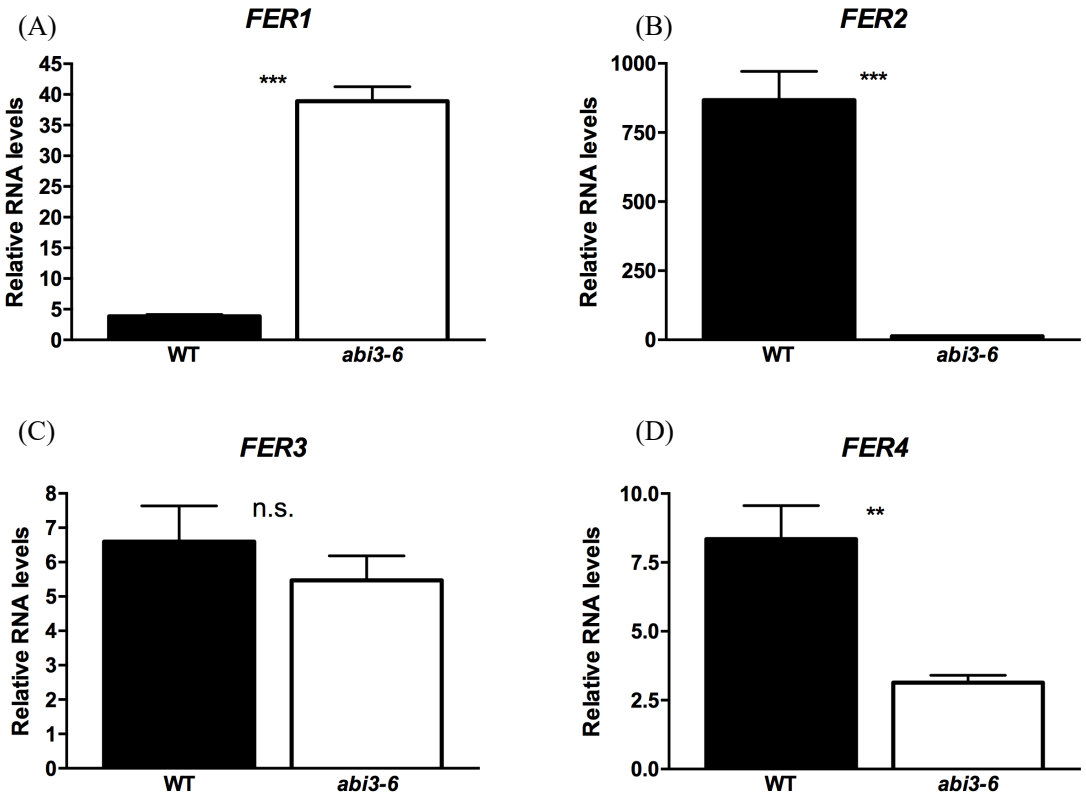

Figure III-7: FERRITINs expression in abi3-6 mutants seeds. qRT-PCR was performed with RNA from Arabidopsis dry seeds. WT corresponds to Col-0. (A) *FER1*. (B) *FER2*. (C) *FER3*. (D) *FER4*. A T-test was performed, P-value < 0.05.

2.3. FERRITINs expression and localization in dry embryos of Arabidopsis thaliana

FERRITINs gene expression is altered in all the B3 transcription factor mutants; this change in transcript levels varies for each *FERRITIN.* However, *FER1* expression has the same increased transcripts accumulation pattern in all B3 transcription factor mutants (Figure III-4A, 5A, 6A, and 7A). A Western blot was used to evaluate if the *FERRITINs* transcripts accumulation is reflected on FERRITINs protein levels.

Total proteins from *lec2-1*, *fus3-3*, and *abi3-6* dry seeds were analyzed together with *fer2* (negative control to the presence of FER2 in seeds) and *fer134* (positive control for FER2 presence) mutants (Figure III-8). We were able to detect FERRITINs in all the samples, except in *fer2* dry seeds. An increase in FERRITINs was observed in all B3 transcription factor mutants. Moreover, in *fus3-3* and *abi3-6* total protein, FERRITINs detection was higher than *lec2-1* and Col-0 total protein (Figure III-8).

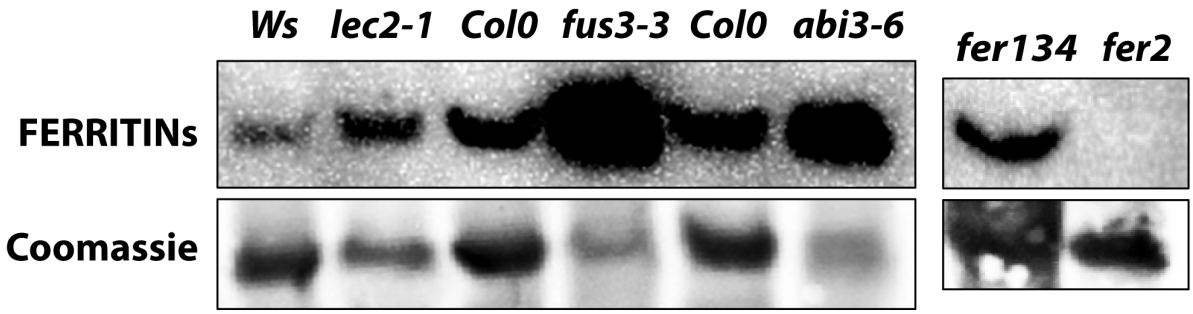

Figure III-8: FERRITINs expression in B3 transcription factors mutant seeds. Protein accumulation of FERRITINs in dry seeds. 10 µg of proteins were loaded in each well. As a loading control, the membrane dye with Coomassie Blue is shown.

To elucidate if the iron pattern observed previously corresponds to FERRITINs in the embryos, immunodetection of FERRITINs was performed. Unfortunately, due to time-limiting circumstances, the wild-type control sample could not be analyzed. Despite *fer2* and *fer134* were analyzed, this does not allow to give a complete result. However, this unfinished result gives us a slight gleam of who FERRITINs distribute in the B3 transcription factors mutant embryos. Briefly, FERRITINs were detected in similar patterns that iron in the B3 transcription factors mutants (*fus3-3* and *abi3-6*) except for *lec2-1* (Annexed Figure 1). Surprisingly, FERRITINs were detected in the leafy cotyledons of *lec2-1,* where the Perls/DAB staining does not detect iron (Fig. II-2d).

3. Discussion

3.1. Genes related to iron homeostasis in seeds expression change in B3 transcription factor mutants

Due to the change of iron distribution in B3 transcription factors, we analyzed three genes that play an essential role in iron homeostasis in seeds. First, *VIT1* is the only gene known for the uptake of iron into the endodermis vacuoles during embryo maturation (Kim *et al.,* 2006). We determined that *VIT1* expression is affected in *lec2-1* and *fus3-3*, indicating a possible change in how and where iron stores in mutant seeds. However, *VIT1* transcripts decreased their accumulation at the mature stage, and it is impossible to say if these change at a transcript level repeats at a translational level. If VIT1 protein accumulation decreased, we would expect to observe a phenotype similar to the *vit1* mutant where iron accumulated in the subepidermal layer's vacuoles. MTP8, which primary function is to uptake manganese into the subepidermal layer's vacuoles, captures iron into these vacuoles in the *vit1* mutants. Double mutants of *vit1/mpt8* have a similar phenotype regarding iron distribution to *fus3-3* and *abi3-6*. *vit1/mpt8* distributes iron ubiquitously in the different embryo cell layers. Like the B3 transcription factor mutants, iron concentration in seeds shows no significant difference between wild-type, *vit1, mpt8,* and *vit1/mpt8* (Eroglu et al., 2017). The similarity between B3 transcription factor mutant phenotypes, specially *fus3-3 and abi3-6,* and double mutant *vit1/mtp8,* could be given by two conditions. First, B3 transcription factors regulated these genes' expression during development, allowing iron and other metals to store correctly. Secondly, the correct expression could be affected by the loss of identity and the mutants' heterochronic phenotypes.

Our experiments clearly show that *fus3* and *abi3* embryos suffer a change in iron homeostasis in maturation, and this change is more substantial in *fus3* than *abi3. FUS3* and *ABI3* activate genes associated with maturation and inhibit genes associated with germination (Nambara *et al.,* 2000). The lack of FUS3 and ABI3 changes embryo development in different ways. *fus3-3* was first described as a heterochronic mutation, in which two developmental programs overlap; leaf development and late maturation development (Keith *et al*., 1994). ABI3 mutants get ahead in the germination showing a viviparous phenotype (Raz *et al.,* 2001). There is a change in the embryo's behavior or physiology in both circumstances, from embryonic tissue to an "adult" plant behavior. For *VIT1*, *NRAMP3,* and *NRAMP4,* this change in the embryo's physiology may cause deregulation in these genes expression. However, it is still necessary to comprehend which transcription factors could be regulating VIT1, NRAMP3, and NRAMP4 directly and how the B3 transcription factors are interacting in the process of maintaining the correct iron distribution in seeds.

3.1.FERRITINs accumulate in B3 transcription factor mutants

Our results showed a dotted pattern for iron detection, at least in *fus3-3* and *abi3-6* (Fig.II-3 and II-4). This pattern is similar to that found in *A. thaliana* leaves treated with iron, associated with iron binding to FERRITINs (Divol *et al*., 2013; Roschzttardtz *et al*., 2013). We showed deregulation in FERRITINs at a transcripts level, increased FERRITINs protein in dry seed, and FERRITINs detection in different cell layers in B3 mutant dry seeds (Fig. III-4 to III-8 and Annexed Fig. 1). There is a clear difference between the *lec2* and the *fus3-abi3* phenotypes.

For *lec2,* we were able to detect FERRITINs mainly in the leafy cotyledon (Annexed Fig. 1), but not iron (Fig. II-2). These results contradict previous reports indicating that FER2 needs to be bound to iron to be stable (Ravet *et al.,* 2009a). We could imply that another FERRITIN is accumulating in leafy cotyledons, such as FER1. *lec2* accumulates more FER1 transcripts than wild-type seeds (Fig. III-4A), and *lec2* also accumulates more FERRITINs (Fig. III-8). Despite this, we were not able to determine which FERRITIN is accumulated in leafy cotyledons. However, FER1 could be a candidate.

fus3 and *abi3* phenotypes are similar, but *fus3* has higher iron and FERRITINs detection than *abi3* (Fig. II-3, II-4, III-8, and Annexed Fig. 1). Contrary to the *lec2* phenotype, FERRITINs detection does correlate with iron detection in *fus3* and *abi3.* These results indicate that *fus3* and *abi3* embryos have more iron out of the endodermis vacuoles than *lec2.* FUS3 and ABI3 have a synergic and additive role in late maturation and germination, activating SSP and LEA genes (LATE EMBRYOGENESIS ABUNDANT) and inhibiting genes related to germination (Nambara *et al.,* 2000). Furthermore, *fus3* siliques contain one-third less of ABA (Nambara *et al.,* 2000), and *abi3,* as its name indicates, is ABA insensitive. This relationship with ABA could be the reason why *FER2* expression decreases in *fus3* and *abi3*. *FER2* expression is activated by ABA (Petit *et al*., 2001), and lately has been discovered that ABI3 binds to the regulatory region of *FER2,* although *FER2* does not have an RY-site (Tian *et al.,* 2020). It seems that ABA has a significant role in association with FUS3 and ABI3 regulating *FER2*, which LEC2 does not have.

FER1 was the only FERRITIN that expressed similarly in all the three B3 transcription factor mutants. *FER1* has been reported to be regulated principally by iron, ROS, and circadian

rhythms (Briat *et al*., 2009). However, *FER1* is mainly expressed in leaves and reproductive tissues, not in seeds (Petit *et al*., 2001, Briat *et al.,* 2010*)*. *lec2* (in the leafy cotyledon section), *fus3,* and *abi3* show the loss of embryonic identity (Nambara *et al.,* 2000). This loss of identity and overlapping with the "adult" identity could allow the expression of *FER1* in embryos that have an altered iron distribution. It is unclear if there is a direct link between the B3 transcription factors and *FER1* repression in embryos. However, the loss of correct iron distribution could be one factor. Still, this does not explain why in leafy cotyledons where no iron was detected, FERRITINs are detected.

Chapter IV: B3 transcription factors regulated indirectly genes related to iron homeostasis.

1. Introduction

The earlier result showed that B3 transcription factors regulate the iron distribution (Fig. II-3 to II-7) and genes related to iron homeostasis in embryos (Fig. III-1 to III-8). However, how this regulation occurs is not clear. A Gene Regulatory Network (GRN) was performed to understand how iron homeostasis in seeds is regulated. Different public access data was obtained; a) gene expression analysis of B3 transcription factor mutants of two different embryo developmental stages(GEO accession GSE61686), b) genes that in their description has the term iron in Gene Ontology, c) DAP-seq data(O'Malley *et al.,* 2016), d) DNA-Binding Domains (DBD) (Weirauch *et al*., 2014), e) ANAP (Wang *et al*., 2012), and f) AGRIS (Davuluri *et al*., 2003). To understand how B3 transcription factors could be involved in iron distribution regulation, all these interactions were analyzed.

It is expected that the Gene Regulatory Network shows how the B3 transcription factors regulate iron distribution in seeds and found candidate genes for iron homeostasis regulators.

2. Results

Although B3 transcription factors are well known for controlling maturation, research on B3 transcription factors mainly focuses on general processes rather than specific gene regulation. A Gene Regulatory Network (GRN) was performed using public databases containing; gene expression levels and predicted or inferred gene interactions to search possible interactions between B3 transcription factors and genes related to iron.

At first, the GRN was planned to find candidates genes that regulate iron homeostasis in seeds. With this in mind, we selected genes with a higher amount of interaction following the next

criteria; the gene has the most interaction in a cluster, has a high Outdegree value and, has a high Betweenness Centrality value.

Six different clusters were formed: EIN3-EIL3-WRKY9 cluster (Fig. IV-1a), ABI3-FUS3 cluster (Fig. IV-1b), EIL3 cluster (Fig. IV-1c), PDF2 cluster (Fig. IV-1d), LEC2 cluster (Fig. IV-1e), and WRKY65 cluster (Fig. IV-1f).

These genes form clusters due to a large number of interactions with other genes. All these genes are part of the teen genes with more interaction with other genes (Outdegree) (Table IV-1), and also are genes that have interaction with genes from another cluster (Betweenness Centrality) (Table IV-2). With this information, we select eight candidate genes that have an important role in the Gene Regulatory Network (Table IV-3). Mutants for the candidate genes were requested from ABRC; for some, we could obtain homozygote embryos. To observe if the iron distribution was affected in the mutants, preliminary analysis of iron detection was performed. No change in iron distribution was observed (Table IV-3).

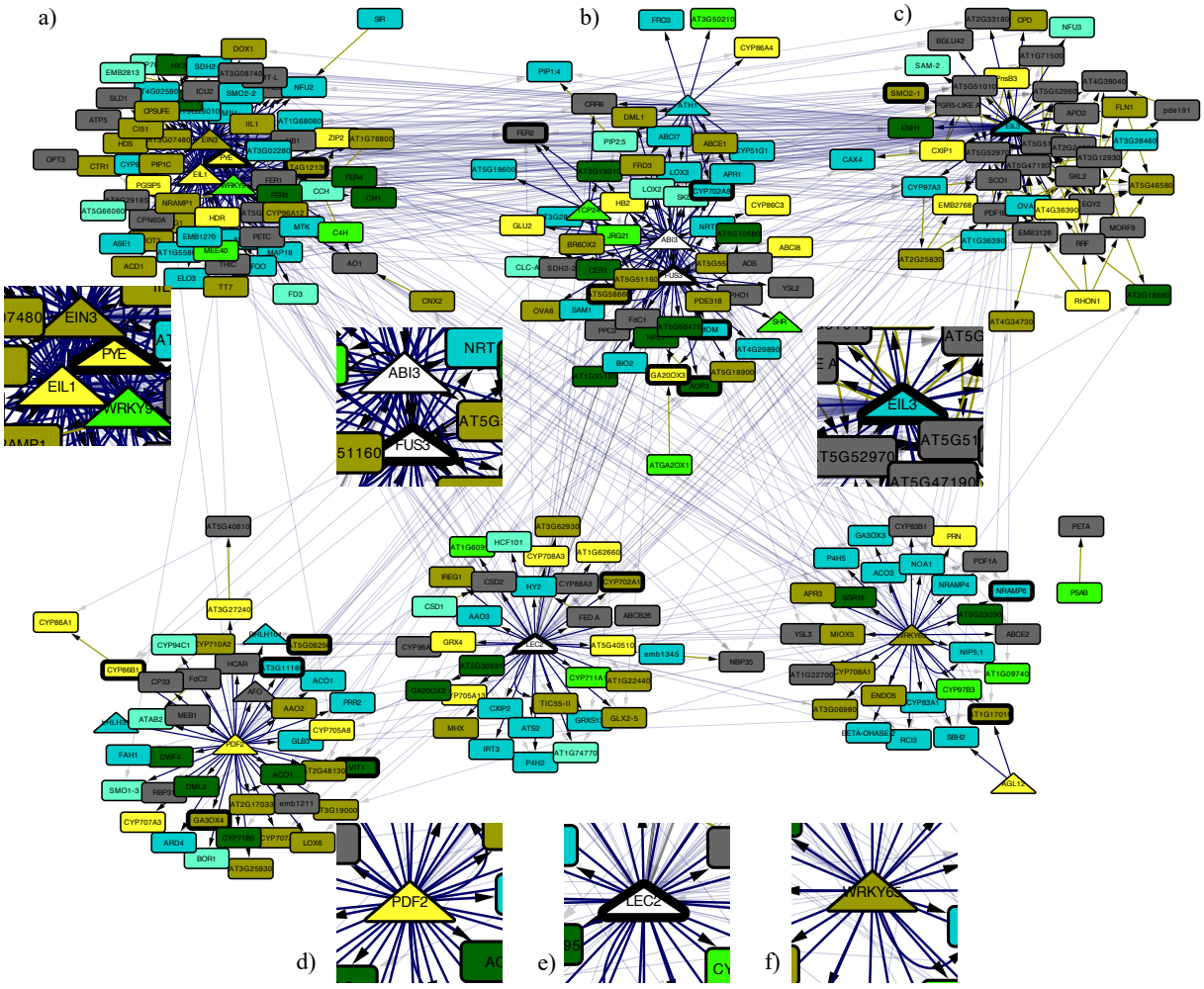

Figure IV-1: B3 transcription factors regulate different genes related to iron homeostasis in seed embryos. (a) Gene Regulatory network of genes directed regulated by B3 transcription factors. (b) ABI3-FUS3 cluster. (c) LEC2 cluster. (d) EIL1-EIN3-WRKY9 cluster. Triangles represent transcription factors; Boxes represent other genes. Bold borders are genes enriched in embryos. Colors represent a change in transcript accumulation in a specific B3 transcription factor mutant. Example: Light green are genes that change the transcript accumulation in lec2. Dark green: lec2 and fus3. Light yellow: fus3. Dark yellow: fus3 and abi3. Dark emerald: abi3. Emerald: abi3 and lec2. Gray: lec2, fus3, and, abi3.

Table IV-1: Top 10 Outdegree

Gene	TAIR	Outdegree
LEC2	AT1G28300	88
ABI3	AT3G24650	75
FUS3	AT3G26790	72
EIL3	AT1G73730	56
EIN3	AT3G20770	50
EIL1	AT2G27050	36
WRKY65	AT1G29280	36
WRKY9	AT1G68150	32
TCP24	AT1G30210	12
DG238	AT3G12930	7

Table IV-2: Top 10 Betweenness Centrality

Gene	TAIR	Betweenness Centrality
LEC2	AT1G28300	0.023
WRKY9	AT1G68150	0.012
FUS3	AT3G26790	0.010
ABI3	AT3G24650	0.005
EIL3	AT1G73730	0.002
EIL1	AT2G27050	0.001
TCP24	AT1G30210	0.001
EIN3	AT3G20770	0.0007
DG238	AT3G12930	0.0006
PDF1B	AT5G14660	0.0004

Table IV-3: Advance of iron distribution analysis in mutants for candidates genes.

Candidate genes	Accession number	Mutant	Mutant selection	Iron distribution phenotype
EIL3	AT1G73730	SALK_138152	-	-
EIN3	AT3G20770	SALK_208607C	Homozygote	X
EIL1	AT2G27050	SALK_042113C, SALK_049679C	Homozygote	X
WRKY65	AT1G29280	CS311467, SALK_050247C	Heterozygote	-
WRKY9	AT1G68150	SALK_067122C	Homozygote	X
TCP24	AT1G30210	SALK_077675	-	-
DG238	AT3G12930	SALK_121803	-	-
PDF1B	AT5G14660	SAIL825_D08	-	-

After obtaining negative results, the GRN was used to look closer to how B3 transcription factors could be regulating VIT1, NRAMP3, NRAMP4, and FERRITINs. To observe possible regulators for the genes related to iron homeostasis, we look for the genes directly connected (first neighbors) to the genes related to iron homeostasis. *VIT1* has three neighbors; *LEC2*, *FUS3*, and *PDF2* (Fig. IV-2a). The binding site RY motive for B3 transcription factors is CAT GCA, but it was not found in previous *in silico* analysis. The interaction shown in the network for *LEC2* is a prediction. In the case of FUS3 is from a DAP-seq database in which the preferred motive was also CAT GCA; however, this was in the coding region and not in the regulatory region. *PROTODERMAL FACTOR2* (*PDF2*) belongs to a class IV homeodomain-leucine zipper gene family, which "are functionally significant in epidermal development" (Kamata et al., 2013). PDF2 binds to an L1 box (TAAATGCA) (Abe et al. 2003); however, the promoter region of *VIT1* does not possess this box. The interaction between VIT1 and PDF2 is inferred (Weirauch et al., 2014). *NRAMP4* only has two neighbors, *WRKY65* and *ABI3*. WRKY65 belongs to a superfamily of transcription factors with more than 100 members in Arabidopsis (Eulgem *et al*., 2000). NRAMP4 regulatory region possesses a W-box motif, the binding site of the WRKY family. Even though *ABI3* interacts with *NRAMP4*, this interaction is inferred, and no RY-motif was found in the *NRAMP4* sequence. *NRAMP3* does not have any correlation higher than 0.8; hence it does not appear in the network. NRAMP3 has two predicted protein-protein interactions with FER1 and FER2, but these correlations are lower than 0.2 and, for this reason, do not appear in the network that only contain interactions >0.8 or <-0.8.

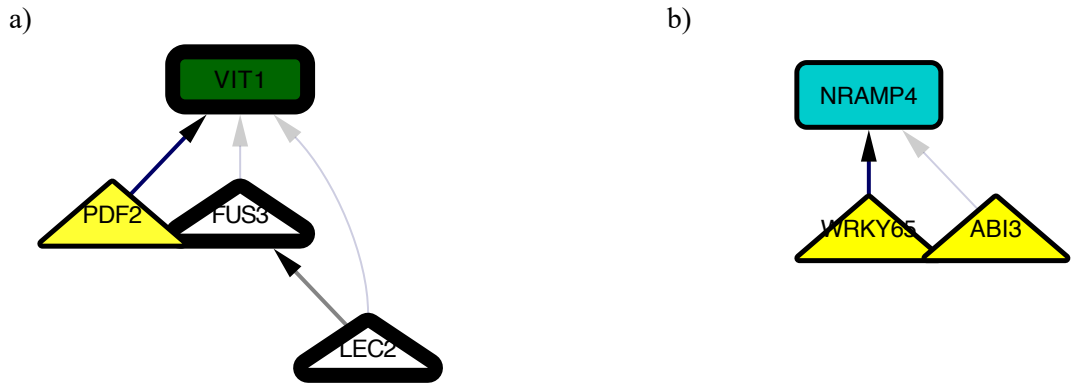

Figure IV-2: VIT1 and NRAMP4 firsts neighbors in B3 transcription factors regulatory network. (a) VIT1 first neighbors. (b) NRAMP4 first neighbors. Triangles represent transcription factors; Boxes represent other genes. Bold borders are genes enriched in embryos. Colors represent a change in transcript accumulation in a specific B3 transcription factor mutant. Example: Light green are genes that change the transcript accumulation in lec2. Dark green: lec2 and fus3. Light yellow: fus3. Dark yellow: fus3 and abi3. Dark emerald: abi3. Emerald: abi3 and lec2. Gray: lec2, fus3, and abi3.

*FERRITIN*s neighbors are more complex. *FERRITINs* showed possible interaction with different protein and transcription factors. The most common interactions are with; *ETHYLENE INSENSITIVE3* (*EIN3*), *ETHYLENE INSENSITIVE3-LIKE1* (*EIL1*), and *ETHYLENE INSENSITIVE3-LIKE3* (*EIL3*). These transcription factors belong to the *EIN3/EIL* gene family that has six members in Arabidopsis. EIN3 and EIL1 are transcription factors that participate in the canonical ethylene signaling and are the most closely related proteins from the EIN3/EIL family (Binder, 2020; Guo & Ecker, 2004). A common neighbor of *FERRITINs* is *EIL3*, which belongs to the *EIN3/EIL* family; besides its similarities in sequences to other family members, *EIL3* does not complement the *ein3* mutant, and neither recognized the binding side of EIN3 (Solano *et al*., 1998). Also, *EIL3* has a particular role in sulfur response regulation (Maruyama-Nakashita *et al*., 2006).

FER1 has predicted protein-protein interaction with FER4 and NRAMP1. Additionally, it has interactions with EIN3, EIL1, and EIL3 (Fig. IV-3a).

FER2 has only transcription factors as neighbors. LEC2, ABI3, and WRKY65, interaction with FER2 is a DNA-binding prediction. Similar to FER1, FER2 also interacts with EIN3, EIL1, and EIL3. Finally, there is another predicted interaction between FER2 and TCP24 (Fig. IV-3b). TCP24 belongs to the TEOSINTE BRANCHED1, GYGLOIDEA, and PCF (TCP) family. This family of transcription factors contains a conserved bHLH motif that concedes DNA binding and protein-protein interactions (Wang *et al*., 2015). The TCP family is divided into two groups, and it is thought that class I promote cell division. Meanwhile, class II inhibits cell division. TPC24 has been associated with cell wall thickening and anther development (Wang *et al*., 2015).

FER3, as well as FER1 and FER2, share interactions with EIN3, EIL1, and EIL3. FER4 interacts with FER3; however, this interaction is due to the co-expression of these two genes (Fig. IV-3c). Finally, FER4, together with the interactions already show in Fig. IV-3a and 3c, also has interactions with FUS3 and ABI3. Similar to FER1, FER2, and FER3, FER4 also has interactions with EIN3/EIL genes; however, it does not share an interaction with EIL1 (Fig. IV-3d). EIN3 and EIL1 correlate positively with FER1, FER3, and FER4, meaning that their expression is similar during embryo development. On the contrary, FER2 correlates negatively with EIN3 and EIL1.

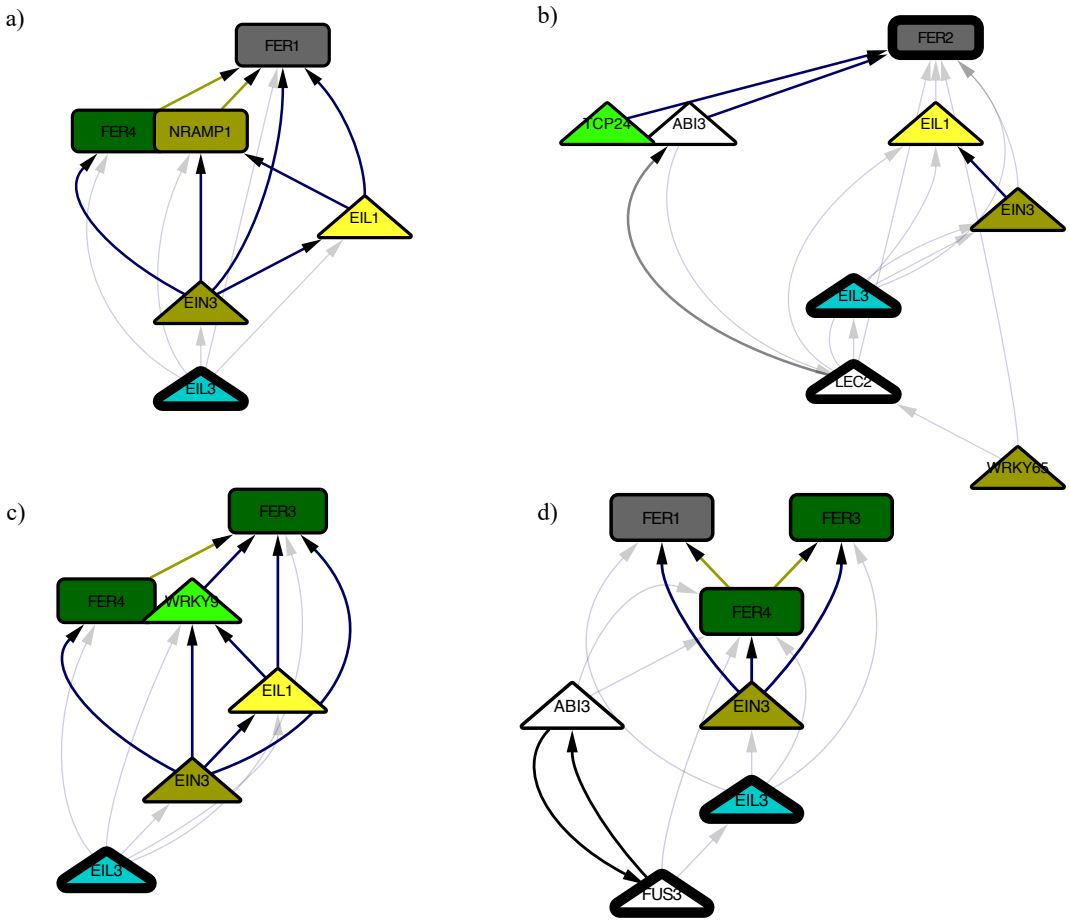

Figure IV-3: FERRITINs firsts neighbors in B3 transcription factors regulatory network. (a) FERRITIN1 first neighbors. (b) FERRITIN2 first neighbors. (c) FERRITIN3 first neighbors. (d) FERRITIN4 first neighbors. Triangles represent transcription factors; Boxes represent other genes. Bold borders are genes enriched in embryos. Colors represent a change in transcript accumulation in a specific B3 transcription factor mutant. Example: Light green represents genes that change the transcript accumulation in lec2. Dark green: *lec2* and fus3. Light yellow: *fus3*. Dark yellow: *fus3* and abi3. Dark emerald: *abi3*. Emerald: abi3 and lec2. Gray: *lec2*, fus3, and *abi3*.

3. Discussion

Earlier, Chapter II described that B3 transcription factors have a role in the iron distribution in Arabidopsis embryos. Chapter III showed that the B3 transcription factors control the proper expression of genes related to iron distribution in embryos. This Chapter tries to propose how B3 transcription factors regulate the expression of the genes related to iron homeostasis in the seed. Candidate genes were searched that could be associated with the iron distribution phenotype of the B3 transcription factor mutants, using a system biology approach. Although the search for candidate genes did not go as expected, the Gene Regulatory Network unveils a new regulator of iron distribution in Arabidopsis seeds.

One of the more exciting findings in the network was the importance of three genes related to ethylene response (Fig. IV-1a and 1c). Ethylene is known in general as a ripening and senesces hormone (Schaller, 2012). Also, ethylene has a role in dormancy and seed germination, even though its exact role in this process is not well understood (Corbineau *et al*., 2014). A peak of ethylene is reached in germination, and treatments with cold and GA, that break the dormancy provoke an increase in ethylene (Arc *et al.*, 2013).

How does ethylene connect to iron and the B3 transcription factors?

First, it has been shown ethylene has a role in response to iron deficiency (Romera *et al*., 1999). Plants that suffer from iron deficiency showed an increase in ethylene production in the roots. The ethylene increase occurs before interveinal chlorosis and at concomitance with the iron deficiency response (Romera *et al*., 1999). Second, Lumba *et al*. (2012) show the connection between FUS3 and ethylene. Ectopic expression of *FUS3* causes repression of the genes

involved in the ethylene pathway. Along with the above, *fus3* mutants show an increased expression of the same genes involved in the ethylene pathway. Furthermore, EIN3, a transcription factor fundamental for the ethylene response, shows high stability in the emerging root cells in the *fus3* mutant (Lumba *et al*., 2012).

EIN3 and EIL1 are known for having a central role in ethylene response (Solano *et al.,* 1998). It has also been studied that EIN3 and EIL1 interact with FIT (FER-LIKE FE DEFICIENCY-INDUCED TRANSCRIPTION FACTOR), which regulated iron uptake under iron deficiency condition (Lingam *et al*., 2011). *ein3/eil1* double mutant has an impaired response to iron deficiency, which is shown by a decrease in *IRT1*, *FRO2*, and *FIT* (Lingam *et al*., 2011). EIL3 is another EIN3-like transcription factor. EIL3 or SLIM1 (SULFUR LIMITATION1) was first described as a central transcriptional regulator in the acquisition of sulfur in Arabidopsis (Maruyama-Nakashita *et al.*, 2006). In the preliminary analysis, *ein3* and *eil1* mutant did not show a phenotype in iron distribution (Table IV-3). It is necessary to highlight that EIN3, EIL1, and even EIL3 interact with each other to regulate genes at a transcriptional and post-translational level (An *et al.,* 2010; Wawrzynska and Sirko, 2016). Also, in the case of EIN3 and EIL1, overexpression of any can restore wild-type phenotype of *ein3* and *eil1* mutants (Chao *et al*., 1997). Hence experiments with double mutants have to be performed to observe a change in the phenotype.

Two *WRKYs* genes show to influence the network (Fig. IV-1a, 1f). However, these genes, *WRKY65* and *WRKY9*, have not been studied so far. *wrky9* mutant shows no iron distribution phenotype in Arabidopsis embryos (Table IV-3). The WRKYs superfamily is an abundant and redundant family of genes (Eulgem *et al*., 2000). WRKYs genes are linked to

abiotic and biotic stresses and hormone response (Bakshi and Oelmüller, 2014). Due to this redundancy, *WRKY* genes are frequently studied using multiple mutants to observe a phenotype (Chen *et al.*, 2010; Skibbe *et al.*, 2008; Gao *et al.,* 2011; Arraño-Salinas *et al.*, 2018). If *WRKY9* and *WRKY65* regulate iron distribution in seeds, their role could be redundant to each other or, in the case of *WRKY9*, to the other central nodes in their clusters (*EIN3* and *EIL3*) (Table IV-3).

Nevertheless, candidate genes analyzed have no iron distribution in Arabidopsis embryos. The GRN shows a link between iron distribution, ethylene, and B3 transcription factors. New experiments should be performed to reveal ethylene's complete role and the ethylene response pathway in iron distribution. A first approach could be to determine the transcripts levels of *EIN3* and *EIN3-like* genes in the B3 transcription factors mutants. Also, ethylene application during embryo development may show if ethylene modulates iron distribution during maturation.

The GRN results showed the possible participation of ethylene in the iron distribution in embryos by the relevance of *EIN3* and *EIN3-like* genes in the network. The WRKY gene family had relevance in the GRN, which could influence B3 transcription factor mutants' iron pattern phenotype. These results indicated that B3 transcription factors do not regulate iron distribution in embryos directly. But instead, B3 transcription factors could be regulating iron homeostasis through other transcription factors.

Chapter V: General discussion and Conclusions

Seeds are the primary source of energy in human diets (Su *et al.,* 2017). Thus, how seeds accumulate the nutrients is the most important because it determines the nutrients humans consume. Anemia is a disease mainly caused by a deficiency of iron in the diet (Stevens *et al*., 2013). It has been proposed that the biofortification of crops such as seeds can help reduce anemia (Horton *et al*., 2008). To develop new strategies for biofortification, it is necessary to understand better how iron accumulates in seeds.

Little is known about how iron accumulates in seeds. In Arabidopsis, VIT1 plays a pivotal role in the correct storage of iron in the vacuoles of cells surrounding the provasculature (Kim *et al*., 2006; Roschzttardtz *et al*., 2009), and NRAMP3 and NRAMP4 redistributed this iron during post germinative growth (Laquar *et al*., 2005; Roschzttardtz *et al*., 2009). The load and storage of iron in seeds happen during maturation (Ravet *et al*., 2009). For this reason, mutants for the master regulators of the B3 transcription factors were used to understand better how iron accumulates in seeds. It was studied if B3 transcription factors have a role in iron homeostasis in seeds. If B3 transcription factors regulate genes associated with iron homeostasis in seeds. And if B3 transcription factors regulate genes related to iron directly or indirectly.

The results showed that B3 transcription factors regulated iron distribution (Fig. II-3 to II-7) by regulating the iron homeostasis genes (Fig. III-1 to III-8). Also, the results showed that the ethylene pathway could be another player in the regulation of iron distribution in seeds.

These results indicated that the lack of a proper maturation stage in the B3 transcription factors mutants affects iron distribution in seeds. The heterochronic phenotype that causes the overlapping of two developmental programs is responsible for bringing an early adult program

development (Keith *et al*., 1994). On the one hand, embryos are trying to store iron, and on the other are trying to germinate and develop. The transcripts accumulation of *VIT1*, *NRAMP3*, *NRAMP4*, and *FERRITINs* showed this. A decrease of accumulation of *VIT1* and *FER2(*Fig. III-1 to III-7*),* genes that have to accumulate during maturation. And an increase in NRAMP3, NRAMP4, and FER1 (Fig. III-1 to III-7), genes associated with germination and adult development. Although B3 transcription factors seem not to regulate the iron homeostasis genes directly, the GRN showed that the ethylene pathway could be involved in regulating iron homeostasis in seeds (Fig. IV-1). These results indicate that B3 transcription factors probably regulate iron homeostasis indirectly by maintaining maturation. Ethylene has a role in germination. Perhaps the overlapping of germination and maturation in the B3 transcription factors cause the earlier activation of the ethylene pathway, causing some of the phenotypes of B3 transcription factors mutants, including the change in iron distribution.

There are unsolved questions that are very interesting to answer from this work. The results showed an increase in FERRITINs in the B3 transcription factors. That correlated with an increased in FER1 transcript accumulation. However, it is not clear that the FERRITINs protein accumulation in seed corresponds uniquely to FER1. This determination could be determined using the molecular weight of the different FERRINTINs, previously performed by Ravet *et al*. (2009). Crosses between the B3 transcription factor mutants and plants containing the promoter region of FER1 fused with GUS were performed in the laboratory. This experiment will show us if the promoter region of FER1 is active in the B3 transcription factor mutants and in which part of the embryo. The results also show the importance of EIN3, EIL1, and EIL3 in the GRN (Fig. IV-1). It would be attractive to analyze the transcript levels of these genes in the B3

transcription factor mutants, in addition to study the effect of ethylene application on wild-type embryos. These results would show if the ethylene pathway is activated in the B3 transcription factor mutants and if the ethylene pathway can provoke the same phenotype as the mutation of any B3 transcription factor.

This work gives more knowledge about iron accumulation. However, there is a lot to comprehend as the direct regulators of the genes associated with iron homeostasis. Another point that this work did not study is how iron total content in seeds is regulated. According to these results, B3 transcription factors do not regulate iron accumulation in seeds. This result could mean that iron accumulation in seed does not depend on the embryo but depends on the adult tissue that nurtures the embryo and is not affected by the B3 transcription factor mutants.

In this work was determined that B3 transcription factors regulate iron distribution in Arabidopsis embryos. Also, it determines that genes related to iron homeostasis, like *VIT1*, *NRAMPs,* and *FERRITINs,* change their expression in B3 transcription factor mutants. And that this change in expression matches the heterochronic phenotype of the B3 transcription factor mutants. Finally, in this work, a new role for ethylene response in iron homeostasis in Arabidopsis embryos was found.

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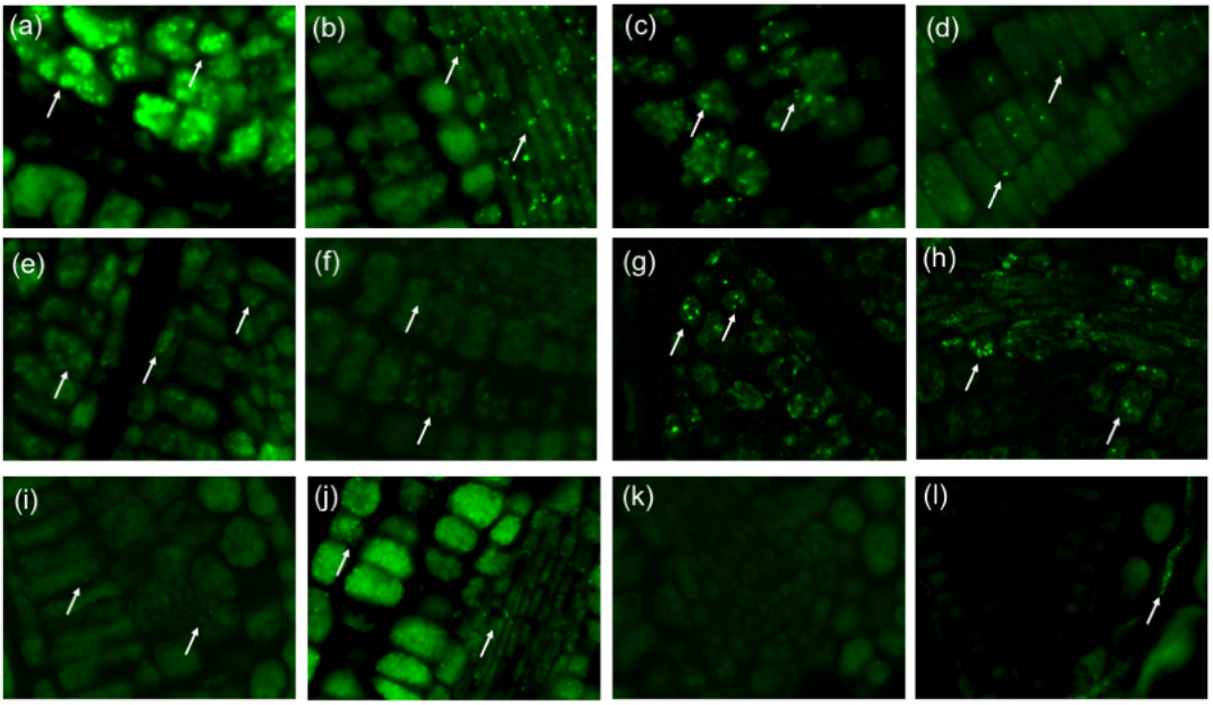

Annexed Figure 1: Immunodeteccion of FERRITINs in *Arabidopsis thaliana* dry seeds. Sections embedded in paraffin were incubated with anti-FERRITINs serum. Images were acquired in an epifluorescence microscope, except for (g-h) acquired using a confocal microscope. For the following images, the first letter represents a cotyledon and the second a hypocotyl. (a)-(b) *lec2-1*. (c)-(d) *fus3-3.* (e)-(f) *abi3-6* with an epifluorescence microscope. (g)-(h) *abi3-6* with a confocal microscope. (i)-(j) *fer134*. (k)-(l) *fer2*. White arrows indicate FERRITINs detection.

Annexed Table 1: qRT-PCR primers

Name	Accession number	Sequence 5'- 3'	Calibration Curve
qVIT1F	AT2G01770	CGCTTATGTTCTTGGCGGTT	y= -3,661 + 14,229
qVIT1R		GGCGGTTTCAAACGCACTTC	
qFER1 F	AT5G01600	TCCAACGATGGCCTCAAACG	y = -3,4x + 14,818
qFER1 R		GAGAAACCGACGGAGAAGCA	
qFER2 F	AT3G11050	ATGTCGGCTTGAAAGGTTTCGC	y= -3,587 + 14,304
qFER2 R		TCAAACTCAGAGACGGGCATCA	
qFER3 F	AT3G56090	AGCTGTAATCTCGCAGTTTGGT	y= -3,446 + 16,492
qFER3 R		CCCAAGGAGCGTAATAGCTTGT	
qFER4 F	AT2G40300	GCATGGAGCTTGCTCTGTCACT	y=-3,828x + 16,415
qFER4 R		TGATTGCTTCCACCTGCTCTGT	
qNRAMP3 F	AT2G23150	TGCTGCGCTCGTGATAATGA	y= -3,451 + 16,333
qNRAMP3 R		ATGCCACGAGCAATGAGGTA	
qNRAMP4 F	AT3G54000	ACAATGATCGTGGCGCTTGTCT	y= -3,514x + 13,031
qNRAMP4 R		AGTGGGATCACAGCGAAAGGGA	
qTIP41-like F	AT3G54000	TCATGCCAAGCTCATGGTTCCT	y= -3,414x + 11,952
qTIP41-like R		TTGGTGCCTCATCTTCGCCAAA	