

**THE Zn-FINGER TRANSCRIPTION FACTOR ZAT12:  
A MOLECULAR LINK BETWEEN IRON DEFICIENCY  
RESPONSES AND OXIDATIVE STRESS**

Dissertation

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## Summary

Plants grown under conditions where iron is poorly available induce a set of genes which enhance the efficiency of its uptake by the roots. A central regulator of this response in the model plant *Arabidopsis* (*Arabidopsis thaliana*) is the basic helix-loop-helix transcription factor FIT whose activity is tightly regulated. We identified the oxidative stress-responsive zinc finger transcription factor ZAT12 in an attempt to find a link between iron deficiency responses and oxidative stress. ZAT12, whose expression is upregulated by several stresses like oxidative, cold and heat stresses etc., contains an EAR motif and is thought to function as a repressor of gene expression. Using yeast-two-hybrid assay, we demonstrate that the EAR motif is necessary for the interaction between FIT and ZAT12. Expression of the *FIT* gene was upregulated in *zat12* loss-of-function plants. In addition, these plants accumulated higher amount of iron compared to the wild type. We generated transgenic plants expressing ZAT12 protein fused to GFP driven by the *ZAT12* upstream DNA sequence. GFP fluorescence was detected in *Arabidopsis* roots, where it could be observed in the nuclei. Abundance of this protein was elevated upon iron deficiency, H<sub>2</sub>O<sub>2</sub> and MG132 treatment. We conclude that ZAT12 links iron deficiency and oxidative stress responses through direct interaction with and negative regulation of FIT.

### Zusammenfassung

Wenn Pflanzen unter Bedingungen wachsen, bei denen Eisen nur gering verfügbar ist, induzieren sie ein Set von Genen, die die Effizienz der Eisenaufnahme durch die Wurzel steigern. Ein zentraler Regulator dieser Antwort in der Modellpflanze Ackerschmalwand (*Arabidopsis thaliana*) ist der basische Helix-Loop-Helix-Transkriptionsfaktor FIT, dessen Aktivität eng reguliert ist. Im Versuch, eine Verbindung zwischen den Antworten auf Eisenmangel und oxidativen Stress zu finden, haben wir den Zinkfinger-Transkriptionsfaktor ZAT12 identifiziert, dessen Expression durch verschiedene Stresse wie oxidativem Stress, Kälte- oder Hitzestress hochreguliert wird. ZAT12 enthält ein EAR-Motiv und wirkt vermutlich als Repressor der Genexpression. In einem Yeast-Two-Hybrid-Assay konnten wir zeigen, dass das EAR-Motiv für die Interaktion zwischen FIT und ZAT12 erforderlich ist. Die Expression des *FIT*-Gens wurde in *zat12*-Loss-of-Function-Pflanzen hochreguliert. Außerdem akkumulierten diese Pflanzen mehr Eisen als der Wildtyp. Wir haben transgene Pflanzen generiert, die unter der Kontrolle der ZAT12-Promotors ein ZAT12- GFP-Fusionsprotein exprimieren. GFP-Fluoreszenz wurde in den Zellkernen von Arabidopsis-Wurzeln detektiert. Die Menge dieses Proteins war sowohl unter Eisenmangel als auch unter H<sub>2</sub>O<sub>2</sub>- und MG132-Behandlung erhöht. Daraus folgern wir, dass ZAT12 Antworten auf Eisenmangel und oxidativen Stress durch direkte Interaktion mit FIT und dessen negative Regulation verbindet.

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**Abbreviations**

ACC	1-aminocyclopropane 1-carboxylic acid
AHA	Arabidopsis H <sup>+</sup> -ATPase
APX1	Ascorbate peroxidase
AZF	Arabidopsis zinc-finger protein
bHLH	basic Helix Loop Helix
BTS	BRUTUS
BiFC	Bimolecular Fluorescence Complementation
CHX	Cycloheximide
Col-0	Columbia-0
EAR	Ethylene responsive element binding factor-associated Amphiphilic Repression
EIL1	ETHYLENE INSENSITIVE3-LIKE1
EIN3	ETHYLENE INSENSITIVE3
Fe	Iron
FIT	FER LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR
FRO2	FERRIC REDUCTASE OXIDASE 2
GFP	GREEN FLUORESCENT PROTEIN
HA	Hemagglutinine
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HSF	Heat shock factor
HSE	Heat shock element



IRT1	IRON REGULATED TRANSPORTER 1
MG132	N-(benzyloxycarbonyl)leucinylleucinylleucinal Z-Leu-Leu-Leu-al
MA	Mugineic acid
NA	Nicotianamine
O <sub>2</sub> <sup>1</sup>	singlet oxygen
O <sub>2</sub> <sup>-</sup>	superoxide radical
HO <sup>-</sup>	hydroxyl radical
PAGE	Polyacrylamid Gelelectrophoresis
PCR	Polymerase Chain Reaction
PYE	POPEYE
RHD2	ROOT HAIR DEFECTIVE 2
PS	Phytosiderophores
RT qPCR	Reverse transcription real time quantitative PCR
RBOH	Respiratory Burst Oxidase Homolog
ROS	Reactive oxygen species
SOD	Superoxide dismutase
VIT1	VACUOLAR IRON TRANSPORTER 1
ZAT12	Zinc finger Arabidopsis thaliana 12
YS1	YELLOW STRIPE 1
YSL	YELLOW STRIPE 1-like
ZnF	Zinc finger
ZFP	Zinc Finger Protein

### 1. Introduction

#### 1.1 The physiological importance of Fe for all living organisms.

Iron (Fe) is one of the vital micronutrients for survival and proliferation of all plants. As a component in heme, Fe-sulfur cluster and other Fe binding sites, it plays a crucial role in a wide range of important processes, such as the electron- transport in the respiratory chain (cytochromes) of respiration and photosynthesis, the biosynthesis of DNA (ribonucleotide reductase), lipids (lipoxygenase), hormones (1-aminocyclopropane 1-carboxylic acid (ACC) oxidase, ACC- ethylene precursor), the detoxification of reactive oxygen species (ROS) (peroxidase, catalase), nitrogen fixation (nitrite and nitrate reductase), etc. (Marschner, 1995; Balk & Lobréaux, 2005).

Fe has two redox states, the oxidized ferric  $\text{Fe}^{3+}$  and the reduced ferrous  $\text{Fe}^{2+}$ . They are able to gain or lose an electron, respectively, within metalloproteins (e.g. ferrous ferredoxin and superoxide dismutase (SOD)), and these redox properties of Fe can generate ROS via Fenton reaction when Fe exists in a free state and large amounts. To avoid toxicity, plants tightly control the uptake, utilization and storage of Fe in coordination with its environmental availability. Despite the high abundance of Fe in soils, Fe availability to plants is low. The concentration of free Fe is limited to approximately  $10^{-17}$  M. This value is far below the requirement for the optimal growth of plants –  $10^{-9}$  to  $10^{-4}$  M (Guerinot and Yi, 1994). That is due to the insoluble state of Fe hydroxides ( $\text{FeOOH}$ ) in aerobic conditions, especially in alkaline pH and calcareous soils. Unfortunately, 30% of the world's arable lands are calcareous. Thus, plants have to develop mechanisms to acquire enough Fe, otherwise Fe deficiency occurs, mainly in plants grown on calcareous soils. This is manifested as chlorosis (yellowing) of young leaves that severely affects crop yields. However, Fe deficiency has not only impact on crop production; it also poses a threat to human health in both developing and developed countries. The World Health Organization (WHO) estimated that approximately two billion people over the world are suffering from anaemia, primarily caused by Fe insufficiency (<http://www.who.int/nutrition/topics/ida/en/index.html>). In its severe form, Fe deficiency anemia is associated with fatigue, weakness, dizziness and drowsiness. Pregnant women and children are particularly vulnerable (<http://www.who.int/topics/anaemia/en/>). The poor uptake of Fe from foods from predominantly plant sources, that represent the predominant diet of people from the developing countries, is one of the main reasons for the

high occurrence of anaemia in these regions. Therefore, efforts to develop new plant varieties with higher bioavailable Fe contents in their edible parts are important for combating this major nutritional deficiency in humans.

### 1.2 Strategies of Fe uptake

The plant's tolerance to Fe deficiency differs from species to species. It is found that in cereals the tolerance can be ranked in the following order: barley/wheat > oat/rye > maize/sorghum > rice (Römheld & Marschner, 1990; Kanazawa et al., 1994; Takahashi et al., 2001). In the 1970s, Takagi showed an increased release of mugineic acid (MA) family phytosiderophores, which are Fe<sup>3+</sup> chelators, under Fe deficiency in oat and rice (Takagi, 1976). The above alkaline tolerance order is parallel to the amount of MAs secreted into the soil (Takahashi et al., 2001). Subsequently, Römheld and Marschner (Römheld and Marschner, 1986) indicated that plants can take up Fe from the soil not only based on secreting MAs to chelate Fe<sup>3+</sup> but also by reducing Fe<sup>3+</sup>. They categorized Fe uptake in plants into 2 groups: reduction-based strategy for the most nongraminaceous plants (strategy I) and chelation-based strategy for grasses (strategy II).

#### 1.2.1 Strategy I

This mechanism is used by the dicot plants, such as the model plant *Arabidopsis thaliana*, tomato (*Solanum lycopersicum*) and tobacco (*Nicotiana tabacum*), and the monocots except those from the graminaceae family. The main steps of strategy I Fe uptake are (Fig.1.1):

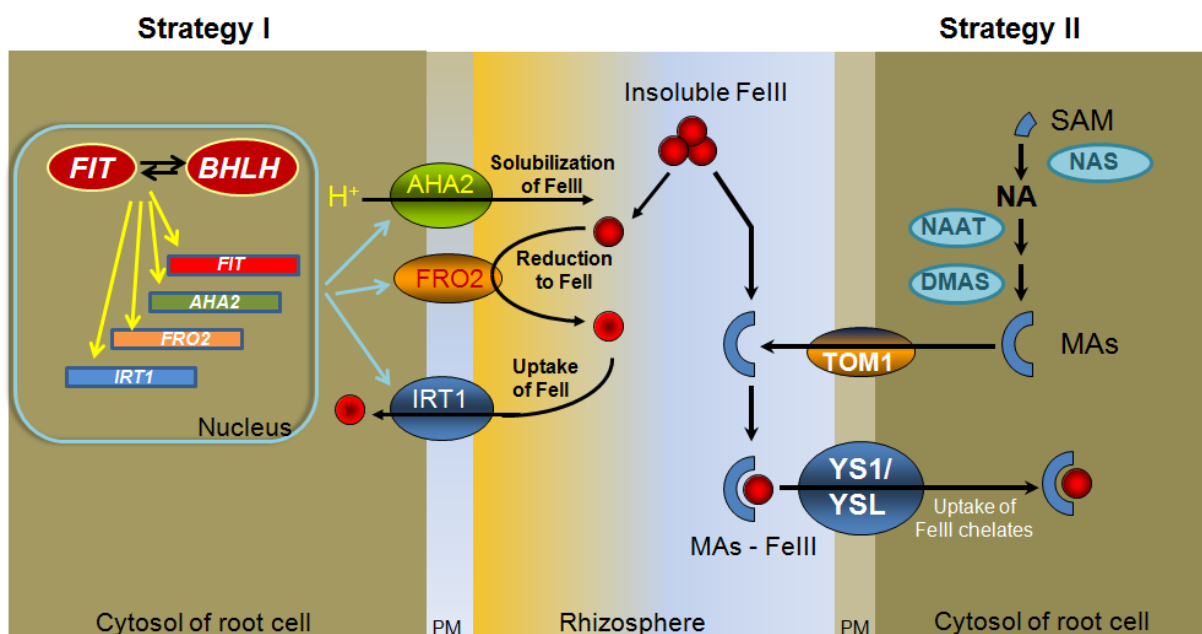
- Solubilization of Fe from soil particles through acidification of the rhizosphere by a plasma membrane (PM) proton pump;
- Reduction of Fe<sup>3+</sup> at the root surface by a membrane-bound ferric chelate reductase;
- Uptake of the generated ferrous ions into the root cells through a transmembrane divalent metal ion transporter.

In the first step of rhizosphere acidification, the pH of the soil surrounding the root is lowered by protons (H<sup>+</sup>) extruded by a root PM H<sup>+</sup>-ATPase which leads to the solubilization of Fe<sup>3+</sup>. PM H<sup>+</sup>-ATPases are a family of proteins, which extrude protons outside of the cell. In *Arabidopsis*, there are 12 H<sup>+</sup>-ATPases functioning primarily to provide an energy source for transport of nutrients into the cell (Palmgren, 2001). Recent studies have shown that *AHA2* and *AHA7* are involved in the response to low Fe conditions. *AHA2* plays a role in the soil acidification, whereas *AHA7* is involved in the rhizodermic cell differentiation (Guerinot & Yi, 1994; Santi & Schmidt, 2009; Ivanov et al., 2012).

Subsequently,  $\text{Fe}^{3+}$  is being reduced to  $\text{Fe}^{2+}$  by a membrane-bound ferric reduction oxidase 2 (FRO2). The FRO family consists of 8 members involved in metal ion homeostasis in different parts of the Arabidopsis plant (Mukherjee et al., 2006). The *frd1* mutant, which is defective in FRO2 activity, has an increased acidification of the rhizosphere, but impaired  $\text{Fe}^{3+}$  reductase activity in response to Fe deficiency (Yi and Geurinot, 1996; Robinson et al., 1999). Moreover, FRO2 transcript levels steadily increased to a maximum at 3 days after movement of plants to Fe starvation media (Connolly et al., 2003).

The reduced Fe ( $\text{Fe}^{2+}$ ), in turn, was carried into the root cells by a iron-regulated transporter (IRT1) (Eide et al., 1996; Vert et al., 2002). IRT1 belongs to the ZIP (ZRT, IRT-like Protein) family of metal transporters. Most of the members of this family are predicted to have eight transmembrane (TM) domains and have a variable length region located between TM domain III and IV. This region facing the cytoplasm of the cell contains a potential histidine rich metal-binding motif, such as the HGHGHH sequence in IRT1. Along with FRO2, IRT1 has the same pattern of expression under Fe depriving conditions. Additionally, when expressed, IRT1 is able to import Zn and Mn besides Fe (Korshunova et al., 1999).

Besides  $\text{H}^+$  extrusion into the rhizosphere, strategy I plants secrete phenolic compounds to facilitate Fe uptake (Ishimaru et al., 2011). However, the genes responsible for this process are still unknown.



**Fig. 1.1. Fe uptake strategies in higher plants.**

Strategy I in nongraminaceous plants (*left*) and Strategy II in graminaceous plants (*right*). Ovals represent the transcription factor, transporters and enzymes that play central roles in these strategies. Rectangulars represent Fe acquisition genes. Abbreviations: PM, Plasma membrane; DMAS, deoxymugineic acid synthase; FRO, ferric-

chelate reductase oxidase; AHA2, Arabidopsis H<sup>+</sup>-ATPase 2; IRT, iron-regulated transporter; MAs, mugineic acid family phytosiderophores; NA, nicotianamine; NAAT, nicotianamine aminotransferase; NAS, nicotianamine synthase; SAM, S-adenosyl-L-methionine; YS1/YSL, YELLOW STRIPE 1/YELLOW STRIPE 1-like (Romheld and Marschner, 1986; Mori & Nishizawa, 1987; Eide et al., 1996; Yi & Guerinot, 1996; Robinson et al., 1999; Curie et al., 2001; Colangelo & Guerinot, 2004; Jakoby et al., 2004; Koike et al., 2004; Yuan et al., 2005; Bauer et al., 2007; Yuan et al., 2008; Santi & Schmidt, 2009; Nozoye et al., 2011; Ivanov et al., 2012; Wang et al., 2012).

### 1.2.2 Strategy II

The strategy II of Fe uptake is used by graminaceous monocot plants, such as rice (*Oryza sativa*), barley (*Hordum vulgare*), wheat (*Triticum aestivum*) and maize (*Zea mays*). This strategy is based on chelating Fe<sup>3+</sup> into complexes which are then transported into the root cells by specific transporters (Fig.1.1). The Fe<sup>3+</sup> chelators are named phytosiderophores (PS). To date, nine types of PS, belonging to the MA family and synthesized from S-adenosyl-L-methionine (SAM), are known (Mori & Nishizawa, 1987). After chelation of Fe<sup>3+</sup> by MAs, the resulting complexes are taken up into the root cells by YELLOW STRIPE 1 (YS1) and YELLOW STRIPE 1-like (YSL) transporters. The maize *yellow stripe 1* (*ys1*) mutant is defective in Fe<sup>3+</sup>-MA uptake. YS1 contains 12 putative TM domains (Curie et al., 2001). Proteins with sequence similarity to YS1 exist in different plant species and are designated YELLOW STRIPE 1-like (YSL) transporters. For example, rice has 18 YSL genes which are expressed and function in different parts of the plant (Koike et al., 2004). Currently, only *OsYSL15* was shown to be expressed in the roots where it imports Fe<sup>3+</sup>-DMA from the rhizosphere (Inoue et al., 2009; Lee et al., 2009). *HvYS1* in barley has the same pattern of expression and function (Murata et al., 2006).

Besides the uptake of Fe<sup>3+</sup>-phytosiderophores from the rhizosphere, rice has also mechanisms to take up Fe<sup>2+</sup> through the OsIRT1 transporter when growing in submerged fields (Ishimaru et al., 2006).

Since our research was based on the model plant *Arabidopsis thaliana* which is a dicotyledonous plant, we will further concentrate on strategy I.

## 1.3 Iron mobilization

### 1.3.1 Long-distance transport of Fe

Due to the insolubility and the chemical properties of Fe, when Fe is transported into the root symplast, which transports from one cell to the next via plasmodesmata, it requires to be bound by a suitable chelator. Known Fe chelators are citrate (Brown & Chaney, 1971) and

nicotianamine (NA) (Takahashi et al., 2003). Rellán-Alvarez et al. have successfully identified a tri-Fe<sup>3+</sup> tri-citrate complex (Fe<sub>3</sub>Cit<sub>3</sub>) in the xylem sap of Fe-deficient tomato plants after short-term Fe resupply (Rellán-Alvarez et al., 2010). Members of the YSL family are considered promising candidates for xylem influx transporters of Fe. YSL is present in nongraminaceous species where MAs are not synthesized. For example Arabidopsis has eight identified YSL proteins (Curie et al., 2001). Schaaf et al. showed that Fe-NA complexes can be transported by ZmYS1 (Schaaf et al., 2004).

### 1.3.2 Subcellular transport and storage of Fe

Accumulation of Fe may be dangerous to the cells. Fe can react with H<sub>2</sub>O<sub>2</sub>, which is a normal by-product of metabolism. The resulting reactive oxygen species (ROS) can cause extensive cellular damage. Therefore, plant cells have developed mechanisms for Fe compartmentalization. The processes of photosynthesis, heme biosynthesis and Fe-S cluster formation all require Fe. Recently, the Arabidopsis PERMEASE IN CHLOROPLASTS1 (PIC1) localized in the inner envelope of the chloroplast; its expression complements the growth of metal uptake-defective yeast mutants were suggested to transport Fe into the chloroplast. (Duy et al., 2007; Duy et al., 2011). FRO7, whose mutants had significant decrease in Fe content and Fe<sup>3+</sup> chelate reductase activity and showed severe chlorosis in alkaline soil, was suggested to transport Fe into the chloroplast (Jeong et al., 2008). Loss-of-function mutant of MIT resulted in lower Fe accumulation in mitochondria which suggested that it is involved in Fe transportation in mitochondria (Bashir et al., 2011). Moreover, AtNRAMP3 and AtNRAMP4 were shown to transport Fe from the vacuole into the cytosol. They mobilize vacuolar Fe stores to support the germination of Arabidopsis. The double *nramp3nramp4* mutant was unable to retrieve Fe from the vacuolar globoids (Lanquar et al., 2005). The Arabidopsis vacuolar iron transporter 1 (VIT1) transports Fe into the vacuole. In the *vit-1* mutant Fe was mislocalized (Kim et al., 2006).

### 1.4 Fe uptake regulation by bHLHs (basic helix-loop-helix)

In order to adapt to the environmental changes, plants have developed regulatory mechanisms for inducing or repressing gene transcription. For example, under low Fe conditions, strategy I plants modulate a set of Fe acquisition genes where the bHLH transcription factor FIT (FER-LIKE FE DEFICIENCY INDUCED TRANSCRIPTION FACTOR, formerly *FIT1/FRU/AtbHLH29*) plays a central role. *FIT* is the functional ortholog of the bHLH transcription factor *LeFER* (*Lycopersicon esculentum*), which was the first identified regulator of Fe responses in plants (Ling et al., 2002; Bauer et al., 2004; Colangelo & Guerinot, 2004;

Yuan et al., 2005; Bauer et al., 2007). *fit* mutant plants also displayed chlorosis due to defect in Fe uptake and die as seedlings unless exogenously supplied with high concentrations of Fe (Colangelo & Guerinot, 2004; Jakoby et al., 2004). Both FIT and FER are regulated by the Fe status of the plant at the transcriptional and posttranscriptional levels (Jakoby et al., 2004; Brumbarova and Bauer, 2005). In addition, recent studies showed that *FIT* was also modulated at post-translational level. Using *FIT* overexpressing lines, the authors identified another Fe-dependent regulatory mechanism of FIT where its protein stability was regulated in a 26S proteasome-dependent manner (Sivitz et al., 2011). Application of NO inhibitors also led to decreases in FIT protein levels suggesting that NO may act as an enhancer of FIT protein stability (Meiser et al., 2011). In addition, an interaction between ETHYLENE INSENSITIVE3 (EIN3) or ETHYLENE INSENSITIVE3-LIKE1 (EIL1) with FIT leads to the stabilization of FIT protein levels, which shows the role of the plant hormone ethylene in controlling Fe acquisition gene responses (Lingam et al., 2011). FIT induces *FRO2* and *IRT1* upon Fe deficiency at the transcriptional level (Colangelo & Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2005; Bauer et al., 2007). However, the enhanced expression of *FIT* did not cause the increase of *IRT1* and *FRO2* in FIT overexpressing plants (Jakoby et al., 2004; Meiser et al., 2011). Therefore, other factors besides FIT may be needed to induce these genes. Among the 162 members of the bHLH family of transcription factors (Bailey et al., 2003), *POPEYE* (*PYE*) and the four members of group Ib – *bHLH038*, *bHLH039*, *bHLH100*, and *bHLH101* are induced by Fe deprivation (Wang et al., 2007; Long et al., 2010). The bHLH proteins contain a characteristic basic helix-loop-helix domain of 60 amino acids. The amino-terminal end of the bHLH structure contains the basic domain and binds hexanucleotide DNA sequences called E-boxes. The HLH region functions as a dimerization domain to form homodimers or heterodimers with other bHLH proteins (Murre et al., 1989; Ferre-D'Amar et al., 1994). Recent studies showed that FIT can interact with *bHLH038*, *bHLH039*, *bHLH100* and *bHLH101* in yeast and also after transient overexpression in Arabidopsis protoplasts. These expression of FIT and one of four members of this subgroup Ib activated GUS expression regulated by promoters of *IRT1* and *FRO2* in yeast cells (Yuan et al., 2008; Wang et al., 2012).

Another FIT independent regulatory mechanism functions through the bHLH transcription factor *PYE* (Long et al., 2010). *PYE* is highly expressed in response to Fe deprivation. The *pye* loss-function mutant has disrupted root growth and altered shoot responses at Fe deficiency. Unlike FIT, *PYE* seems to regulate Fe mobilization, rather than the uptake of Fe. Several genes involved in the long-distance Fe transport like *OPT3* and *FRD3*, or expressed in



the stele and related to Fe homeostasis such as *ZIF1*, *NAS4*, and *FRO3* are up-regulated in the *pye* mutant. The putative E3 ubiquitin-protein ligase BRUTUS (BTS) was identified in the coexpression network of PYE. Like PYE, expression of *BTS* is also induced by Fe starvation in the pericycle. By conducting yeast-two-hybrid experiment, it could be seen that PYE did not interact with BTS. This was partly reflected in the phenotype of the *bts* mutant, which has symptoms opposite to *pye* mutant. However, both of them interacted with bHLH115 and ILR3 (IAA–Leu resistant, bHLH105) (Long et al., 2010). ILR3 modulates metal transporter genes related to auxin metabolism (Rampey et al., 2006). The authors suggested that BTS interacts with PYE binding partners such as ILR3 and bHLH115 to repress PYE or BTS inhibits downstream targets of PYE. However, these hypotheses require further confirmation.

### 1.5 Abiotic stress and nutrient homeostasis

Fe and other nutrients play crucial roles in growth and development of plants. However, both insufficient and excess nutrient quantity to plant's demand affect their growth and yield. If present in excess,  $\text{Fe}^{2+}$  can react with  $\text{H}_2\text{O}_2$  to form reactive oxygen species (ROS) via the Fenton reaction (Winterbourn, 1995; Fenton, 1894). The ROS can damage lipids, proteins and DNA within the plant cell, leading to cell death unless plant cells manage to trigger detoxification mechanisms. Both Fe deficiency and excess are abiotic stresses which plants may cope with.

#### 1.5.1 Abiotic stress

Plants growing in natural conditions, which have difficulties to achieve their full genetic potential for reproduction are considered "stressed" (Boyer, 1982). It was shown that only 22% of the genetic potential yield contributed the production of field-grown crops in the United States because of impacts of unfavorable adverse environments (Boyer, 1982). Therefore, to adapt with changing environment, plants have developed complex mechanisms to respond to stress. 2007 FAO statistics showed that only 3.5 % of the world land area is not impacted by some adverse environmental conditions. This problem is still growing, because of continued decrease of crop growing land, reduction of water resources and increased global warming effects and climate change (Lobell et al., 2011).



**Table 1.1. Estimates of the impacts of abiotic stresses on crop production.**

	<b>Stress Type</b>	<b>% of global land area affected*</b>
Water	Deficit or Drought	64
	Flooding or Anoxia	13
Temperature	Cold	57
Chemical/Soil	Salt or salinity	6
	Mineral deficiency or low fertility	9
	Acid soil	15

\*based on FAO World Soil resource Report 2000 <ftp://ftp.fao.org/agl/agII/docs/wrs.pdf>

Rarely is there only one single environmental factors affecting plants, usually there is a complex of factors. For example, high temperature and high light often go with low water supply, and the consequence is nutrient toxicity. Like iron-mediated oxidative stress, these abiotic stresses share a common response, which is the generation of ROS and therefore, result in oxidative stress (Dat et al., 2000). Although ROS was previously considered to harm cells, recent researches suggest that cells developed strategies to use ROS as signals in the regulation of various genetic stress-response mechanisms (Foyer et al., 1997; Dalton et al., 1999; Dat et al., 2000), or in the regulation processes during plant growth and development, like cell elongation (Foreman et al., 2003) and root differentiation (Tsukagoshi et al., 2010) Depending on the effects (acute vs chronic) and duration of stress, plants develop specific programs to respond to received signals at the different levels like tissue or cell specific transcriptional responses in roots (Dinneny et al., 2008).

### ***Nutrition and abiotic stress***

Along with Mn, Mo and Ni, micronutrients which are needed for plant growth are Fe, Cu and Zn. Both Fe and Cu have changing redox states, so they are common as cofactors of components in the electron transport chain of respiration. While Fe exists in the Fe-S clusters, the heme, Cu is mostly found in plastocyanin, a protein that functions as electrons transporter to photosystem I (PSI) (Marschner, 1995). Cu is involved in many processes such as in protection from ROS, cell wall lignification, development of pollen, carbohydrate metabolism, and phenolics production in response to pathogen attack (Marschner, 1995). Cu is present in the ethylene receptor for proper signaling (Rodríguez et al., 1999). Unlike Fe and

Cu, Zn is not redox active. It exists as a cofactor in many enzymes, including RNA polymerase, superoxide dismutase, alcohol dehydrogenase and carbonic anhydrase (Marschner, 1995) and is a component to construct transcription factors of Zn finger family, which are involved in responses to abiotic stress (Ciftci-Yilmaz & Mittler, 2008).

Despite being essential, three of them will restrict plant growth when they present in low or excessive concentrations because of deficiency or toxicity. Deficiency of these three micronutrients all showed chlorosis symptom in the leaves. While chlorosis only appears in young leaves in Fe and Cu limiting conditions, Zn starvation exhibits it on both the young and old leaves. Deprivation of both Zn and Cu cause the change of leaf form, even form necrosis (Marschner, 1995). So that three of them all affect the machinery of photosynthesis that causes to reduce plant yield. To deal with it, plants have evolved well-control networks in respect of uptake, utilization, homeostasis, and even detoxification due to present in excess of Fe and Cu result in generating ROS via Fenton reaction. ROS could greatly impact lipid, protein, DNA by causing lipid peroxidation, protein denaturation and DNA mutation. Excess Zn also results in the suppression of root growth. It disturbs basic cellular functions because zinc can easily replace Fe, Mg in the active sites of enzymes or transporters. For example, it suppressed the activities of Rubisco and PSII in *Phaseolus vulgaris* by substituting magnesium (Van Assche and Clijsters, 1986).

To control metal uptake, plants have developed different mechanisms. Non-graminaceous species release protons into the rhizosphere to increase the solubility of  $\text{Fe}^{3+}$  by acidification. Acidification would also cause to soluble Zn and Cu, by facilitating cation exchange and releasing the divalent metals from insoluble soil particle chelators. And the  $\text{Fe}^{3+}$  is then reduced to  $\text{Fe}^{2+}$  by FRO2. FRO2 is also likely to reduce Cu, but expression of FRO2 is not induced under Cu deficiency.  $\text{Fe}^{2+}$  and  $\text{Cu}^+$  are imported into the root cells by respective IRT1, COPT1 (Sancenón et al., 2004). COPT1 belongs to CTR-related copper transporter family in Arabidopsis, and members of CTR family were also identified in animals and yeast. COPT1 complemented the *ctr1-3* mutant defective in high affinity copper uptake in yeast *Saccharomyces cerevisiae* (Kampfenkel et al., 1995). Although it was expressed at the low level in the root tip, the decrease of Cu transport rates between seedlings of three different COPT1 antisense transgenic lines and the controls propose that COPT1 plays a role as a copper transporter in Arabidopsis and takes part in copper uptake from the surrounding medium (Sancenón et al., 2004). Besides COPT1, Cu is also taken up by member of the ZIP transporter family, transport divalent cations. It is found that expression of both ZIP2 and ZIP4 are induced by Cu deficiency and able to complement Cu uptake in *ctr1* yeast mutants

(Wintz et al., 2003). Gramineous plants use a strategy based on chelation, which involves the secretion of MA -phytosiderophores (PS).  $\text{Fe}^{3+}$  chelated MA complexes are imported into root cell. MAs are thought to be involved in mobilizing Zn in barley and the subsequent absorption of PS- $\text{Fe}^{3+}$  complexes (Suzuki et al., 2006). Expression of these genes involve in the synthesis and secretion of MAs were increased in both Zn-deficient and Fe-deficient barley roots. However, HvNAAT-B transcripts were found in Zn-deficient shoots, but not in Fe deficient shoots. Furthermore,  $\text{Zn}^{2+}$ -MA complexes were detected more than  $\text{Zn}^{2+}$  in the roots of Zn-deficient barley plants (Suzuki et al., 2006).

After importing into the root cells, metals will be transported or stored in tissues where they are required. Due to toxicity of these metals at the large amount, plants control very tight these processes. Two main strategies that plant utilizes to overcome this toxicity are sequestration and chelation. In addition to sequestration into the vacuole, Fe is kept in plastids via ferritin, 24 subunits of which builds a hollow sphere that may store up 4500 atoms  $\text{Fe}^{3+}$  in its core. Therefore, they were first considered to be involved in Fe homeostasis. Then they were shown to protect cells from oxidative stress damages. There are 4 ferritins identified in Arabidopsis. These transcripts were induced in response to high iron treatment in both roots and leaves and accumulated in seedlings under  $\text{H}_2\text{O}_2$  treatment (Petit et al., 2001). *FER2* is accumulated in the seed, but it is not major form of Fe storage in seeds. *fer2* knock-out mutant showed a high sensitivity to methylviologen (MV) compared with controls demonstrating that *FER2* role is to protect the seed from free-iron-mediated oxidative stress. *FER1*, *FER3* and *FER4* expressions are found in leaves. The absence of all three genes in *fer1-3-4* mutants results in increased activity of several ROS-detoxifying enzymes and enhanced ROS production in leaves and flowers under normal development conditions. This suggests that most plants use ferritin to sequester Fe that avoids Fe toxicity more than to store Fe (Ravet et al., 2009). It is known that  $\text{Fe}^{2+}$  can interact with  $\text{H}_2\text{O}_2$  to form ROS through Fenton reaction (Fenton, 1894).

To avoid toxicity, metals are also bound into chelated complexes with carriers. In plants, Fe is often chelated to NA, citrate or phytosiderophores (Curie et al., 2009).  $\text{Cu}^+$  is known bound with Arabidopsis copper chaperone (CCH) to recycle Cu from senescing tissue towards reproductive structures (Mira et al., 2001). The second chaperone of Cu is found is CCS, a functional ortholog of yeast copper chaperone for superoxide dismutase (SOD) Ccs1p/Lys7p. These complexes are thought to transfer Cu to SOD in the chloroplast (Abdel-Ghany et al., 2005). The third chaperone is cytochrome c oxidase 19 (COX19), induced by metals (including Cu) or ROS production and may transport Cu to cytochrome c oxidase in the

mitochondria (Attallah et al., 2007). Additionally, chelators for metals are also amino acids, organic acids, polypeptides such as phytochelatins (PCs) and metallothioneins (MTs) (Robinson et al., 1993; Briat & Lebrun, 1999; Rauser, 1999).

### **1.5.2 Plant responses to abiotic stress**

#### ***1.5.2.1 ROS as signaling molecule***

Most of abiotic stresses result in enhanced generation of ROS (Mittler, 2002). Initially, it is considered as toxic by-product of aerobic metabolism, but with its advantages, it was shown to act as a signaling molecule. There are several advantages making ROS as such good signaling molecules. The first is that the cell is capable to rapidly produce and scavenge different forms of ROS (for example: singlet oxygen ( $O_2^1$ ), superoxide radical ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) or hydroxyl radical ( $HO^\cdot$ )) in a simultaneous manner, so that changes in ROS levels are rapid and dynamic. The second is that the cell controls tightly the subcellular localization of ROS signals. ROS are produced in specific locations of the cell, such as a certain membrane patch, or an organelle (Monshausen et al., 2007; Takeda et al., 2008). The third is that they could be used as rapid long distance auto-propagating signals moved in the plant. Each individual cell along the path of the signal could activate its own ROS producing mechanism(s) in an autonomous manner carrying a ROS signal over long distances. It was recently reported for example that such a signal in *Arabidopsis* could travel at a rate of up to 8.4 cm/min (Miller et al., 2009). In addition, an advantage of ROS is that there are different forms of ROS having significantly different molecular characterizations. For example: while superoxide is a charged molecule and can not passively transfer across membrane, hydroxyl peroxide formed from superoxide can easily passively transport across membrane or through water channel (Miller et al., 2010). ROS can link with other signaling pathways such as calcium and protein phosphorylation networks (Ogasawara et al., 2008; Kobayashi et al., 2007).

In plant cells, ROS are produced in the compartments with a highly potential oxidizing metabolic activity or with a large rate of electron flow, including chloroplasts, mitochondria and peroxisome. In mitochondria, higher reduction level of components in the electron transport chain increases ROS production under specific stress conditions (Møller, 2001). In chloroplasts, besides the sources produce ROS like in mitochondria, the antenna pigments also contribute to generate ROS (Asada, 2006). As a part of this pathway, the activity of glycolate oxidase generates  $H_2O_2$  in peroxisomes (Kisaki & Tolbert, 1969).  $H_2O_2$  is also generated as a side-product of fatty acid oxidation in glyoxisomes (Cooper & Beevers, 1969).

Another source of ROS production is in the apoplast by way of ROS production enzymes such as amine oxidase, oxalate oxidase, NADPH oxidase (Mittler, 2002). Plasma membrane NADPH oxidase enzyme plays a crucial role in this network. In mammals, NADPH oxidase or respiratory burst oxidase (RBO) is a protein complex between a membrane-bound NADPH-binding flavocytochrome  $b_{558}$  and cytosolic regulatory proteins. In Arabidopsis, *Respiratory burst oxidase homolog (RBOH)* belongs to a multigenic family with ten members (Torres & Dangl, 2005). The structure of these RBOH proteins contain the same domains in mammalian RBO such as transmembrane domains, the functional oxidase domain at the C terminal, and regulatory regions at the N-terminal region like calcium-binding EF hands and phosphorylation domains important for the function of the plant oxidases (Suzuki et al., 2011). *RBOHs* are expressed in different parts of the plant, tissues and cell types. Most of them (except for *RBOH H and J*, whose expression pattern is yet to be identified) are expressed in the roots, only *RBOH D* is expressed not only in shoots but also in roots. They all were induced by pathogens and different abiotic stress such as oxidative, cold, wounding stresses etc. (Sagi et al., 2004; Davletova et al., 2005; Torres & Dangl, 2005). In recent years, several genetic studies revealed that plant *RBOHs* play a role in regulating signaling in response to pathogens and abiotic stresses such as heat, drought, cold, high-light (HL) intensity, salinity or wounding. The initial burst of ROS production could activate communication among cells and develop a ROS wave that propagates and transmits throughout the different tissues over long distances (Miller et al., 2009; Niethammer et al., 2009). This ROS wave can be blocked by the local treatment of catalase or an NADPH oxidase inhibitor, at distances that are up to 5–8 cm away from the signal initiation site. Moreover, the signal requires the presence of the NADPH oxidase *RBOH D* gene and spreads throughout the plant in both the upper and lower directions (Miller et al., 2009). In addition, plant NADPH oxidase regulates developmental programs such as polarized cell expansion in root hair formation and pollen tip growth (Foreman et al., 2003; Potocký et al., 2007) or seed ripening (Müller et al., 2009). For example, *AtRbohC* regulates cell expansion which needs  $Ca^{2+}$  influx from the extracellular store during root hair formation. Analysis of the Arabidopsis *rboh C* mutant (also named *root hair-defective 2-rhd2*) demonstrated that *AtRBOH C* produced  $H_2O_2$  to activate hyperpolarization of  $Ca^{2+}$  channels during the root hair development (Foreman et al., 2003).

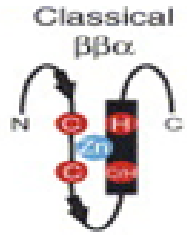
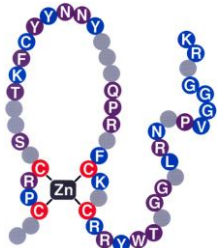
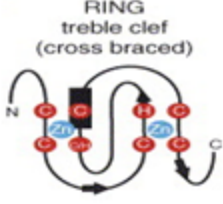
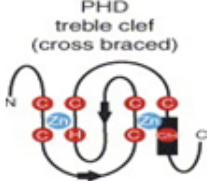

### ***1.5.2.2 H<sub>2</sub>O<sub>2</sub> signaling model***

ROS including H<sub>2</sub>O<sub>2</sub> is accumulated in the cytosol could be sensed by different redox response transcription factors such as HsfA4 and relayed to downstream effectors such as different transcription factors that include members of NAC, ZAT, WRKY, DREB, bZIP and MYB families (Miller et al., 2008; Petrov & Van Breusegem, 2012). Expression of *HsfA4a* and *HsfA8* was induced in the *Apx1* knockout mutant, whose H<sub>2</sub>O<sub>2</sub> levels highly increased and HsfA4 was demonstrated to be required for expression of *ZAT12*, *APX1* in response to light stress by analyzing a dominant-negative construct for HsfA4 (Dalevtova<sup>b</sup> et al., 2005; Miller & Mittler, 2006). HsfA4 belongs to class A with 21 members of Arabidopsis heat shock factors. They bind to the consensus sequence found in the promoters of many defense genes control the response of plants to different stresses (Miller & Mittler, 2006). By the plant CARE software, Rizhsky identified these sequences in the promoters of *ZAT12*, *ZAT7* and *WRKY25* (Rizhsky et al., 2004; Miller & Mittler, 2006). Both human and drosophila Hsfs are able to interact directly with H<sub>2</sub>O<sub>2</sub>, by forming DNA-binding-competent homotrimers in a reversible manner from inactive Hsf monomers (Zhong et al., 1998; Ahn & Thiele, 2003). Plants may have the same strategy for H<sub>2</sub>O<sub>2</sub> sensors. After that, by binding their promoters, Hsfs can activate the target transcription factors related to abiotic stress responses.

### **1.5.3 Zinc Finger (ZnF) Family**

The Zinc finger domain is a protein structural motif which stabilizes the protein by the coordination of a zinc ion. These proteins are classified according to their zinc binding topology into several different types: TFIIIA- type or classical C2H2, LIM, RING, PHD etc. (Table 1.2). These proteins play a pivotal role in many cellular functions, including transcriptional regulation (C2H2- type etc.), RNA binding, regulation of apoptosis, and protein-protein interactions (LIM- and RING-finer types etc.).

**Table 1.2. Classification of zinc finger proteins.** (Takatsuji, 1998; Gamsjaeger et al., 2007; Yanagisawa, 2004)

Type	Characterization	(Proposed) structure
<b>TFIIIA type</b> (classical C2H2)	This motif includes two cysteines and two histidines in a conserved sequence motif (Cys <sub>2</sub> -His <sub>2</sub> ) (Miller et al., 1985). It coordinates a zinc atom to form a compact structure that binds to the major groove of DNA (Pavletich and Pabo, 1991). For example: petunia EPF1, ZAT10/STZ (Takatsuji et al., 1994; Lippuner et al., 1996) etc.	 <p>Classical ββα</p>
<b>The Dof family</b> (DNA binding with one finger)	The proteins of Dof family contain a conserved domain (Dof domain) including a zinc-finger motif, Cys <sub>2</sub> -Cys <sub>2</sub> (Yanagisawa, 1995). This domain has been identified to bind to DNA. Such proteins function in response to light, phytohormones and defense, seed development and germination (Yanagisawa, 2002). For example: maize Dof1 (Yanagisawa & Izui, 1993), mDof2 (Yanagisawa & Sheen, 1998) etc.	
<b>RING-finger type</b> (Really Interesting New Gene)	The RING finger (Cys <sub>3</sub> -His-Cys <sub>4</sub> ) engages two zinc atoms (von Arnim & Deng, 1993). By searching database in Arabidopsis, 469 RING domain containing proteins were found to be potential E3 ligases which are involved in ubiquitination via the ubiquitin/26S proteasome pathway (Stone et al., 2005). For example: <i>Arabidopsis</i> COP1 (CONSTITUTIVE PHOTOMORPHOGENIC1), RING-H2 protein RHA2a in ABA signaling (Bu et al., 2009) etc.	 <p>RING treble clef (cross braced)</p>
<b>PHD-finger type</b> (plant homeodomain finger)	The PHD finger (Cys <sub>4</sub> -His-Cys <sub>3</sub> ) is similar to the RING finger (Cys <sub>3</sub> -His-Cys <sub>4</sub> ) in the arrangement of putative zinc binding amino acids. For example: <i>Arabidopsis</i> HAT3.1 (Schindler et al., 1993) and maize Zmhox1a (Bellmann and Werr, 1992) etc.	 <p>PHD treble clef (cross braced)</p>
<b>The LIM family</b> (LIN-11, Isl1 and MEC-3)	The LIM domain contains a cysteine-rich motif of Cys <sub>2</sub> -His-Cys-Cys <sub>4</sub> . Arabidopsis has six members which all exhibit actin-binding, -stabilizing, and-bundling activities. (Papuga et al., 2010). For example: WLIM2 and WLIM2a and b; PLIM2a-c etc.	



### 1.5.3.1 Classical (C2H2) ZnF motifs

One of the most common types of ZnFs is the C2H2-type in which two cysteines and two histidines coordinate a single zinc atom to form a finger construct consisting of a short beta hairpin and an alpha helix. Structure researches indicated  $\alpha$  helix bind the major groove of DNA (Choo & Klug, 1997; Pavletich and Pabo, 1991). The first identified protein is TFIIIA in the *Xenopus laevis* (Miller et al., 1985). It might bind to DNA and regulate the expression of the 5S rRNA gene. Although these type of proteins are thought to mainly bind DNA, they also contact RNA and proteins (Iuchi, 2001; Gamsjaeger et al., 2007) They are a crucial class of genomic regulators, in part regarding their large distribution (found in yeast, animals, plants), but also regarding their expansion within the genomes of eukaryotes.

In plants, ZPT2-1 (previous by named EPF1) is the first TFIIIA-type or C2H2-type zinc finger was found in a DNA-binding protein of petunia, that interacts with the promoter region of the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene. It comprises two canonical TFIIIA-type zinc-finger motifs that contain QALGGH conserved sequence that substitution of which any A,L,G,G or H results in completely loss of DNA binding activity (Kubo et al., 1998). Soon after, in wheat WZF1 was identified as a DNA-binding protein that specifically interacts with a *cis* element of histone genes (Sakamoto et al., 1993). Subsequently, many other C2H2-type proteins have been identified in plants.

Englbrecht et al., found that 176 zinc finger proteins in *Arabidopsis thaliana* form the largest family of putative transcriptional regulators. Based on protein sequences, a majority of them (81%) are plant specific, only 33 proteins are conserved in other eukaryotes (Englbrecht et al., 2004). The major plant ZnFs usually consist of invariant QAGGH motif in zinc finger helices, in contrast to animal and yeast (Takatsuji, 1998); and have long, variable spacers (in length and sequence) between zinc finger domains, while in yeast and animals these fingers are clustered and have short spacers (Schuh et al., 1986; Klug & Schwabe, 1995). According to these criteria ZnF proteins were grouped into three categories - A, B and C. The major difference between sets A, B and set C was that on proteins in the two former sets include tandem ZnF arrays whereas those in set C carried out single ZnF or several dispersed ZnFs. The majority of AT-ZnFPs (*Arabidopsis thaliana*-Zinc finger proteins) belong to category C. Each set is combined different subsets (e.g. C1, C2 and C3), whose ZnF types vary in their spacing between the two invariant zinc coordinating histidines by three (C1), four (C2) and five (C3) amino acid residues (Table 1.3) (Englbrecht et al., 2004).



**Table 1.3. Overview of Arabidopsis ZFPs in different sets. ZFPs in set C are further classified into families. ZAT12 is in boldface.**

Classified set of C2H2-type proteins	Characteristics of ZnF domain
Set A&B	include tandem ZnF arrays
<b>Set C</b>	carries out single ZnF or several dispersed ZnFs.

Classification of **Set C**

Families are classified in set C	Numbers amino acids between two invariant zinc coordinating histidines:	For example
<b>C1 family</b>	<b>3 amino acids</b>	<b>(-CKTCLKQFHSFQALGGHR <u>A</u> <u>SH</u>, ZnF of ZAT12, Ciftci-Yilmaz &amp; Mittler, 2008)</b>
C2 family	4 amino acids	(-CPFCSMLCGSFKGLQFHL <u>N</u> <u>S</u> <u>SH</u> , ZnF of VRN2, Gendall et al., 2001)
C3 family	5 amino acids	(-CKLCLTLHNNEGNYLAHT <u>Q</u> <u>G</u> <u>K</u> <u>RH</u> , ZnF of AT2g32600, Englbrecht et al., 2004, <a href="http://www.arabidopsis.org/">http://www.arabidopsis.org/</a> )

**ZFP: Zinc finger protein; ZnF: zinc finger; C2H2: two cysteines and two histidines**

Besides the zinc finger domain, C2H2 type zinc finger proteins contain three characteristic regions. One is a short basic region near the N-terminal region of protein with core sequence of KXKRSKRXXR (B-box) which might act as a potential nuclear localization signal (NLS) and participate in DNA binding. The second is a short, leu rich region located between B-box and the first zinc finger domain. The third is a Ethylene responsive element binding factor-associated Amphiphilic Repression (EAR) motif with a core sequence of [L/FDLNL/F(x)P] at C-terminus that is thought to cause repression activities in the mechanisms of defense and stress response (Sakamoto et al., 2000; Kagale et al., 2010).

### **C1 family**

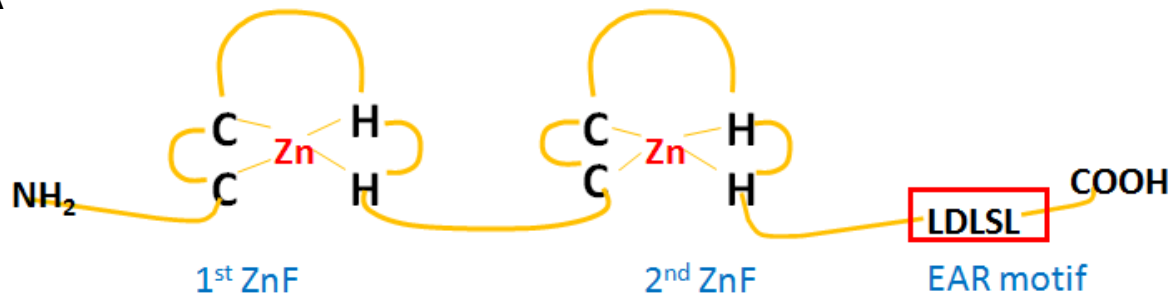
C1 family is found as one of the most abundant families of *Arabidopsis* specific ZnFPs contains 64 members. Based on the varying numbers of fingers, family C1 is divided into different subclasses (see below Table 1.4). Among these subclasses, with their function, members of C1-2i are some of the most researched plant C2H2 type zinc finger protein.

**Table 1.4. Classification of C1 family into different subclasses. Subclasses, number of zinc fingers and number of members in subclasses are given. ZAT12 belongs to the underlined subclass.**

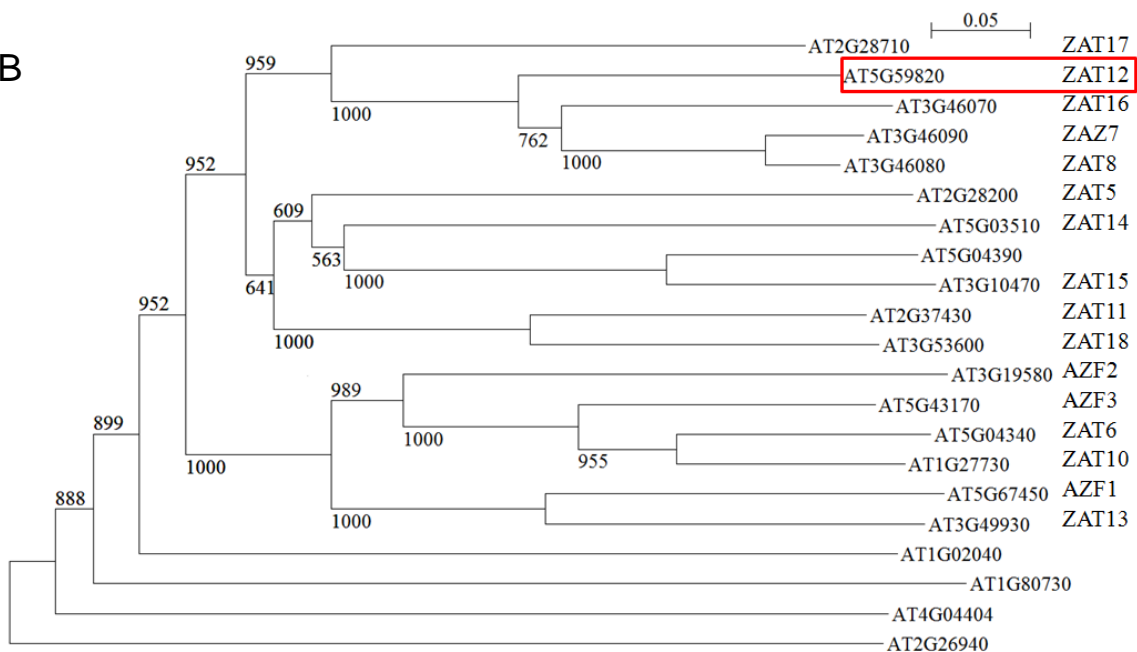
C1 family	Number of C2H2-type zinc fingers in protein structure	Number of members in subclass
C1-1i	<b>1</b> zinc finger	33
<u>C1-2i</u>	<u><b>2</b></u> zinc fingers	<u>20</u>
C1-3i	<b>3</b> zinc fingers	8
C1-4i	<b>4</b> zinc fingers	2
C1-5i	<b>5</b> zinc fingers	1

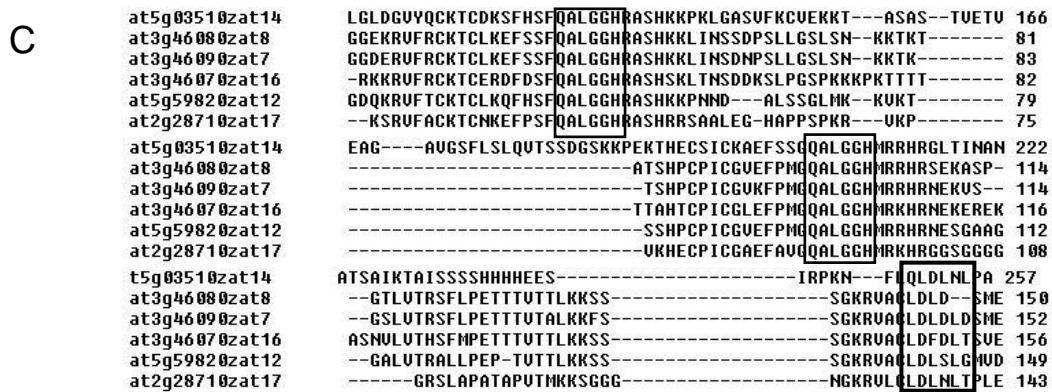
'i' indicates amount of C2H2-type zinc fingers is in bold

A



B





**Fig. 1.2. ZAT12 protein - a C1-2i subclass representative.**

Structure of ZAT12 zinc finger (ZnF) protein (A); A neighbor joining tree of C1-2i Arabidopsis proteins. ZAT12 is indicated as red rectangular (B). Alignments were performed with Clustal X; Alignment for conserved amino acids C1-2i subclass representative. The rectangulars show sequences of zinc finger. Bold rectangular represents sequences of EAR motif (C).

### 1.5.3.2 C1-2i subclass

It consists of 20 members including ZAT5, ZAT6, ZAT7, ZAT8, ZAT10, ZAT11, ZAT12, ZAT13, ZAT14, ZAT15, ZAT16, ZAT17, ZAT18, AZF1, AZF2, AZF3, At5g04390, At1g02040, At2g26940, and At4g04404 that show widely homology in two dispersed C2H2-type fingers (except for At2G26940 and AT4G04404). (Table 1.5) (Englbrecht et al., 2004)

These eighteen members have the invariant QALGGGH motif in the first and second zinc finger domain, a long spacer between two fingers and an EAR motif [L/FDLNL/F(x)P] at the C-terminal part of the protein. Some members such as ZAT10 and ZAT6, ZAT7 and ZAT8, ZAT11 and ZAT18 are the result of duplications (Meissner & Michael, 1997; Englbrecht et al., 2004). Some members of this subclass are thought to function in defense and stress response, because mRNA levels of these members are induced under stress conditions (Iida et al., 2000; Rizhsky et al., 2004; Davletova<sup>a</sup> et al., 2005).

**Table 1.5. Arabidopsis transcriptional regulator proteins of C1-2i subclass with conserved EAR motif sequence. (Ciftci-Yilmaz & Mittler, 2008)**

AGI code	Protein name	Protein size	Sequence of Core EAR motif site
AT1g27730	STZ/ZAT10	227	<u>DLNI</u> PP
AT1g02040		324	<u>LDLNL</u>
AT2g26940		286	<u>DLNQ</u> PP
AT2g28200	ZAT5	286	LPL <u>LDLNL</u> LPA
AT2g28710	ZAT17	156	LCL <u>LDLNL</u> TTP
AT2g37430	ZAT11	178	LSL <u>LDLNL</u> TTP
AT3g10470	ZAT15	398	LDL <u>LDLNL</u> LPA
AT3g19580	AZF2	273	<u>DLNL</u> LPA
AT3g46090	ZAT7	168	<u>LDL</u> <u>DL</u>
AT3g46080	ZAT8	164	<u>LDL</u> DS
AT3g49930	ZAT13	215	<u>DLNL</u> LPA
AT3g53600	ZAT18	175	<u>LDLNL</u> TTP
AT3g10470	ZAT15	398	<u>LDLNL</u>
AT3g46070	ZAT16	170	LDFDL
AT5g03510	ZAT14	292	LQL <u>LDLNL</u> LPA
AT5g04340	ZAT6	238	<u>DLNI</u> PP
AT5g04390		362	LDL <u>LDLNL</u> LPA
AT5g43170	AZF3	193	<u>DLNI</u> IP
<b>AT5g59820</b>	<b>ZAT12</b>	<b>162</b>	<b><u>LDL</u><u>SL</u></b>
AT5g67450	AZF1	245	<u>DLNL</u> LPA

### ZAT7

*ZAT7* is expressed in the roots, the stems, the leaves (Meissner & Michael, 1997). Along with *ZAT12* and *WRKY*, *ZAT7* is found to be highly expressed in *apx1* knockout mutants that maintain high H<sub>2</sub>O<sub>2</sub> level in the absence of stress (Pnueli et al., 2003). The expression level of *ZAT7* is also elevated under H<sub>2</sub>O<sub>2</sub>, heat shock, wounding, or paraquat (ROS generating chemical) treatment (Rizhsky et al., 2004). *ZAT7* overexpressing plants show suppressed growth and developmental phenotypes, but more tolerance to salinity stress (Rizhsky et al., 2004; Ciftci-Yilmaz et al., 2007). By deletion the EAR repressor motif Ciftci-Yilmaz demonstrated that *ZAT7* plays a key role in enhancing tolerance to salinity. Additionally, this motif is required for protein-protein interaction between *ZAT7* and *WRKY70* or *HASTY* (Ciftci-Yilmaz et al., 2007).

### **ZAT10/STZ (salt tolerance zinc finger)**

*ZAT10* was first recognized as cDNA that complements salt-sensitive phenotype of calcineurin (phosphoprotein phosphatase) defective mutants in yeast (Lippuner et al., 1996). It is expressed in roots, stems, leaves (Lippuner et al., 1996; Meissner & Michael, 1997; Sakamoto et al., 2000; Sakamoto et al., 2004). An increase in the *STZ* mRNA level was observed in response to salinity, low temperature, dehydration, whereas it responded weakly to ABA and was down-regulated by gibberellin (Lippuner et al., 1996; Sakamoto et al., 2000; Gong et al., 2001; Ogawa et al., 2003; Sakamoto et al., 2004). Moreover, plants expressing constitutively *ZAT10* had increased tolerance to osmotic, salinity, heat stresses, while *zat10* deficient mutants had enhanced their tolerance to osmotic and salinity stresses. This suggested that *ZAT10* regulates both positively and negatively mechanisms of plant defences (Mittler et al., 2006). *ZAT10* also contains an EAR repressor motif. By fusion with GAL4 (DNA-binding domain), STZ/*ZAT10* protein were shown to function as active repressors of transcription and the DLN box of EAR motif was identified as a repression domain (Ohta et al., 2001)

### **ZAT12**

By homology cloning, *ZAT12* was first found to express in roots, stems, flowers. Structural analysis of *ZAT12* protein showed that it is constituted of 162 amino acids divided into two C2H2-type zinc finger domains with a 22-amino acid inter-finger region, and a LDLSL core sequence of the EAR motif localized at the C terminus from amino acid 143 (Figure 1.2) (Meissner & Michael, 1997; Englbrecht et al., 2004; Kagale et al., 2010). Subsequently, *ZAT12* was identified among genes induced during the acclimation period to white light of 600  $\mu\text{Em}^{-2}\text{sec}^{-1}$ . Its expression is highly responsive to light stress (Iida et al., 2000). While tolerance of plants overexpressing *ZAT12* was enhanced in response to high irradiation, antisense transgenic plants displayed decreased tolerance to high irradiation (Iida et al., 2000). Therefore, *ZAT12* is proposed to play a key role in the acclimation response to changes in light intensity. Later studies suggested that *ZAT12* is also involved in the cold stress because overexpression of *ZAT12* in *Arabidopsis* caused a small, but reproducible, increase in freezing tolerance. Additionally, the expression of the *CBF* genes were repressed by *ZAT12* suggesting *ZAT12* functions in a negative regulation of the *CBF* cold response pathway (Vogel et al., 2005). The repression may be due to the presence of the EAR repressor motif in the *ZAT12* protein. At the same time, Rizhsky found an elevated expression of *ZAT12* under oxidative stress (*i.e.*  $\text{H}_2\text{O}_2$  or paraquat application), heat shock, or wounding, which did not occur in

response to light or osmotic stress. However, *APX1* was induced by all stresses tested. Seedlings expressing constitutively *ZAT12* showed more tolerance to oxidative stress. Moreover, *zat12* knock-out mutants were also more sensitive than wild type plants to H<sub>2</sub>O<sub>2</sub> application. Although both transgenic plants expressing *ZAT12* and *zat12*-deficient plants did not induce *APX1*, *ZAT7*, or *WRKY25* expression under controlled conditions or H<sub>2</sub>O<sub>2</sub> or paraquat application, a number of transcripts with a putative signaling function such as a monomeric G-protein, MAPK kinase 4, calcium binding protein etc. or related to ROS metabolism like NADPH oxidase, peroxidase 2a, and glutathione *S*-transferase suggesting that *ZAT12* plays an important role in this complicated network of the oxidative stress response in *Arabidopsis* (Rizhsky et al., 2004). Additionally, Davletova et al., showed the *ZAT12* transcript levels increased in response to cold, salinity, drought stresses. Since the transcriptome of plants subjected to hydrogen peroxide stress and the transcriptome of plants expressing *ZAT12* shared a wide overlap it was suggested that *ZAT12* expression might be involved in the response of plants to reactive oxygen species accumulation during abiotic stresses. Moreover, the role of *ZAT12* is necessary for plant tolerance to osmotic, oxidative, and salinity stresses, but it may have a negative effect on plant tolerance to heat stress (Davletova<sup>a</sup> et al., 2005). Another correlation of *ZAT12* and ROS is confirmed by finding out the lower induction *HsfA2* and *ZAT12* in the insertional *rbohD* mutant under anoxia indicating a role for this NADPH oxidase in inducing these genes under anoxia (Pucciariello et al., 2012).

### **1.5.3.3 EAR motif**

To adapt with various developmental and environmental changes, plants used for regulation of gene expression a network of regulators by orchestrating activators, repressors, co-activators and co-repressors. Repressors were grouped into 2 types: active and passive (Hanna-Rose & Hansen, 1996). The difference between them is the presence of an intrinsic repression domain. Active repressors generally constitute a distinct, small and portable repression domain(s) that disrupt transcription process either by inhibitory protein-protein interactions with components of the basal transcriptional machinery or transcriptional activators, and/or by recruiting histone deacetylases (HDACs) which modify chromatin structure and avoid other transcriptional activators attaching to their target cis-elements (Hanna-Rose & Hansen, 1996). In plants, the Ethylene responsive element binding factor-associated Amphiphilic Repression (EAR) motif is a transcriptional regulatory motif found as active repressor in members of the ERF, C2H2, and AUX/IAA families... By comparison the

core sequence within the EAR motif sites uncovered two conservation forms: LxLxL and DLNxxP. In Arabidopsis, an EAR motif appears in 219 proteins belonging to 21 different transcriptional regulator families. The majority of these proteins (approximately 72%) contain a LxLxL type of EAR motif including ZAT12, ZAT7, etc., whereas 22% contain a DLNxxP type of EAR motif such as EIN3, EIL2 etc., and the remaining 6% have a motif where LxLxL and DLNxxP are overlapping. These proteins functions diversely by negatively regulating genes involved in developmental, hormonal and stress-response signaling pathways. Published studies suggested that approximately 40% of these proteins regulate negatively gene expression (Kagale et al., 2010).

### 2. Aims of this work

To overcome Fe deficiency, Arabidopsis plants, belong to the strategy I plants, reduce Fe<sup>3+</sup> by FRO2 (ferric reductase) into the soluble Fe<sup>2+</sup> form (Yi and Geurinot, 1996; Robinson et al., 1999) that later allows the uptake of Fe<sup>2+</sup> to root cell through the divalent metal transporter IRT (iron-regulated transporter) (Eide et al., 1996; Vert et al., 2002). These processes are tightly controlled and regulated by FIT to supply Fe enough for metabolism and avoid toxicity (Colangelo & Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2005; Bauer et al., 2007). *ZAT12*, a zinc finger transcription factor, whose mRNA is up-regulated by abiotic stresses, was revealed as a candidate for a putative interaction partner of FIT in a yeast-two-hybrid screen (unpublished work, laboratory of Prof. Dr. Bauer). Initial studies conducted by a diploma student indicated that *ZAT12* was regulated in response to Fe and that Fe deficient gene expression was affected in *zat12* mutants (E.Weber, Diplomwork, 2008). Our starting hypothesis was that *ZAT12* be responsible for the coordination of Fe deficiency and stress responses. In detail, *ZAT12* may have a repression activity through its EAR motif which is thought as a repressor motif of gene expression (Kagale et al., 2010). To address this hypothesis, we established the below goals.

#### **Goal 1. Verification protein interaction between ZAT12 and FIT.**

In the previous experiment, *ZAT12* was identified in a total of 14 putative candidate cDNAs in the yeast two-hybrid screen (Lingam et al., 2011). The interaction was realized under growth conditions that would allow yeast colonies to grow if C-terminal FIT peptide fused to the GAL4 DNA binding domain (FIT-C-BD, excluding the N terminus and bHLH domain of FIT) interacts with an expressed peptide from a root –Fe cDNA library fused to the GAL4 activation domain. To confirm this, the yeast two hybrid assay was to be performed again. We generated vectors containing FIT-C-BD or *ZAT12*-AD. By cotransformation into yeast and growth of yeast colonies under selective growth condition interaction was to be confirmed.

#### **Goal 2. Investigation the function of the protein-protein interaction ZAT12-FIT.**

The expression level of *ZAT12* is known to be increased in response to several stresses such as cold, wounding, salt, heat, oxidative stresses (Rizhsky et al., 2004, Vogel et al., 2005; Davletova<sup>a</sup> et al., 2005). However, such an increased expression may have a negative effect on plant tolerance to heat stress (Davletova<sup>a</sup> et al., 2005). To clarify *ZAT12* function, the molecular and physiological consequences of a *ZAT12* loss of function in *zat12* mutant



transgenic plants was to be investigated. The expression of *ZAT12* was to be examined under physiological conditions related to Fe deficiency and stress by reverse transcription real-time PCR analysis. *ZAT12* mRNA levels would be increased in response to exogenous H<sub>2</sub>O<sub>2</sub> treatment. If *ZAT12* functions as a repressor of gene expression as the previous report, *zat12* loss-function mutant will show upregulation of its target, here could be *FIT*. The regulation of *ZAT12* at protein level in response to Fe supply and stresses was to be followed after the generation of tagged protein transgenic lines. With them, we could study *ZAT12* abundance and stability under Fe deficiency and H<sub>2</sub>O<sub>2</sub> stress conditions.

### **Goal 3. Study mechanistic explanations of the protein-protein interaction.**

The EAR motif is thought to provide the repressor function of *ZAT12* (Kagale et al., 2010). Therefore, we generated transgenic lines containing *zat12* mutant version with a deleted EAR motif (*ZAT12*Δ) for further study of the dynamics of interaction and protein regulation. The deletion of EAR motif was expected to abolish the suppressor role of *ZAT12*. This would lead to relieve *FIT* from repressor processes and Fe uptake would be induced and the plants expressing *ZAT12*Δ could be suffered from oxidative stress.

### 3. Materials and Methods

#### 3.1 Materials

##### 3.1.1 Plant materials

- *Arabidopsis thaliana* ecotype Columbia (Col-0) has been used as wild type
- other *Arabidopsis* lines:

Abbreviation/ Name	Gene/ AGI code	Characteristic genotype /phenotype	Reference
<i>rhd2</i>	<i>RHD2 (RBOH C)/</i> AT5G51060	identified in a Spm mutagenised population; phenotype: shorter and fewer root hairs	(Foreman et al., 2003)
<i>fit-3</i>	<i>FIT/</i> AT2G28160	T-DNA insertion line; Exon, 207 bp downstream of ATG; phenotype: chlorosis	(Jakoby et al., 2004)
HA-FIT 9/ FIT Ox	<i>FIT/</i> AT2G28160	Transgenic lines containing <i>p2xCaMV35S:HA3-FIT/Col-0</i>	(Meiser et al., 2011)
<i>zat12-3</i> (GABI- kat348H06)	<i>ZAT12/</i> AT5G59820	T-DNA insertion line, 426 bp downstream of ATG	This work E.Weber, Diplomwork
CH107	<i>ZAT12/</i> AT5G59820	Transgenic lines containing <i>pZAT12:ZAT12-GFP/Col-0</i>	This work
CHd107	<i>ZAT12/</i> AT5G59820	Transgenic lines containing <i>pZAT12:ZAT12ΔEAR-GFP/Col-0</i>	This work

- *Nicotiana benthamiana*

##### 3.1.2 Strains for molecular cloning

Strain	Genotype	Use	Manufacturer
<i>Bacterial strains for molecular cloning</i>			
<i>E. coli</i> , <i>ccdB</i> one shot survival T1- Phage resistant cells	F-mcrA (mrr-hsdRMS-mcrBC) 80lacZ M15 lacX74 recA1 ara 139 D (ara- leu) 7697 galU galK rpsL (StrR) endA1 nupG tonA::Ptrc -ccdA	Propagation	Invitrogen
<i>E. coli</i> INVαF'	F' endA1 recA1 hsdR17 (rk-, mk+) supE44 thi-1gyrA96 relA1 φ80lacZ_M15_(lacZYAargF) U169 λ-	Propagation and cloning	Invitrogen

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NovaBlue	<i>endA1 hsdR17</i> ( $r_{K12}^- m_{K12}^+$ ) <i>supE44 thi-1 recA1 gyrA96 relA1 lac</i> [F' <i>proA+B+lacI<sup>q</sup>ZDM15 ::Tn10(tet<sup>R</sup>)</i> ]	Generation recombinant colonies visualized by blue/white screening.	Novagen
Tuner(DE3)pLacI	F <sup>-</sup> <i>ompT hsdS<sub>B</sub></i> ( $r_B^- m_B^-$ ) <i>gal dcm lacY1</i> (DE3) pLacI (Cam <sup>R</sup> )	Protein expression	Novagen
<i>Agrobacterium tumefaciens</i>	C58C1; GV2260/pGV2260; Rif <sup>R</sup> , Cn <sup>R</sup> .	Plant transformation	

#### Yeast strain

<i>S. cerevisiae</i> strain AH109	<i>MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1<sub>UAS</sub> - GAL1<sub>TATA</sub> - HIS3, GAL2<sub>UAS</sub> - GAL2<sub>TATA</sub>-ADE2, observations URA3::MEL1<sub>UAS</sub>-MEL1<sub>TATA</sub>-lacZ, MEL1</i>	Yeast-Two-Hybrid	Clontech
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#### 3.1.3 Plasmids

Detailed information about pMDC Gateway vectors are available under:

[http://botserv1.uzh.ch/home/grossnik/curtisvector/index\\_2.html](http://botserv1.uzh.ch/home/grossnik/curtisvector/index_2.html)

#### Original plasmids used in this work

Name of plasmids	Manufacturer	Structure	Selection marker
pDONR207	Invitrogen	Entry vector containing Gateway cassette with P1 and P2 attachment sites	Selection marker: <i>ccdB</i> suicidal gene: insertion control, Gm <sup>R</sup> : transformation control in bacteria
pMDC107	Invitrogen	Binary destination vector for expression of native promoter-gene-of interest-GFP C-terminal fusions	Selection marker: <i>ccdB</i> suicidal gene: insertion control, Kan <sup>R</sup> : transformation control in bacteria, Hygromycin <sup>R</sup> : transformation control in plants
pGBKT7-GW	Clontech; Provided by Dr.Yves Jacob	derivative of yeast bait expression vector	Selection marker: Kan <sup>R</sup> : transformation in bacteria, <i>TRP1</i> : transformation control in yeast
pACT2-GW	Clontech; Provided by Dr.Yves Jacob	derivative of yeast prey expression vector	Selection marker: Can <sup>R</sup> : transformation in bacteria, <i>LEU</i> : transformation control in yeast

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pETBlue-2	Novagen	Protein expression: T7lac-driven, tightly controlled, high level expression in <i>E. coli</i>	Blue/white screening		
Name of plasmids	Original plasmid	Place in vectors	Inserted fragment	Bacterial/Yeast marker	Reference

#### Plasmids generated in this work for protein expression in plants

pDONR207- pZAT12: <i>ZAT12ns</i>	pDONR207 (Invitrogen)	Replaced <i>ccdB</i> gene between P1 and P2 attachment sites	pZAT12: <i>ZAT12ns</i> generated with 5'attB1-zat12 PROMOTER &3'attB2-zat12 NONSTOP primers	Gm <sup>R</sup> : transformation control in bacteria	This work
pMDC107- pZAT12: <i>ZAT12ns</i>	pMDC107 (Invitrogen)	Replaced <i>ccdB</i> gene between R1 and R2 attachment sites	pZAT12: <i>ZAT12ns</i> generated from pDONR207- pZAT12: <i>ZAT12ns</i>	Kan <sup>R</sup> : transformation control in bacteria, Hyg <sup>R</sup> : transformation control in plants	This work; see Fig. 3.1
pDONR207- pZAT12: <i>ZAT12ΔEARns</i>	pDONR207 (Invitrogen)	Replaced <i>ccdB</i> gene between P1 and P2 attachment sites	pZAT12: <i>ZAT12</i> <i>ΔEARns</i> generated with <i>ZAT12dEAR</i> - R1 & <i>ZAT12dEAR</i> -F2 primers	Gm <sup>R</sup> : transformation control in bacteria	This work
pMDC107- pZAT12: <i>ZAT12ΔEARns</i>	pMDC107 (Invitrogen)	Replaced <i>ccdB</i> gene between R1 and R2 attachment sites	pZAT12: <i>ZAT12</i> <i>ΔEARns</i> generated from pDONR207- pZAT12: <i>ZAT12</i> <i>ΔEARns</i>	Kan <sup>R</sup> : transformation control in bacteria, Hyg <sup>R</sup> : transformation control in plants	This work; see Fig.3.1

#### Plasmids generated in this work for protein expression in *E.coli*

pETBlue-2- <i>ZAT12:His</i>	pETBlue-2 (Novagen)	inserted into EcoRV (278) at multiple cloning region of vector	<i>ZAT12:His</i> generated with 5'ZAT12_PET & ZAT12_CT His 3' primers	Blue/white screening	This work
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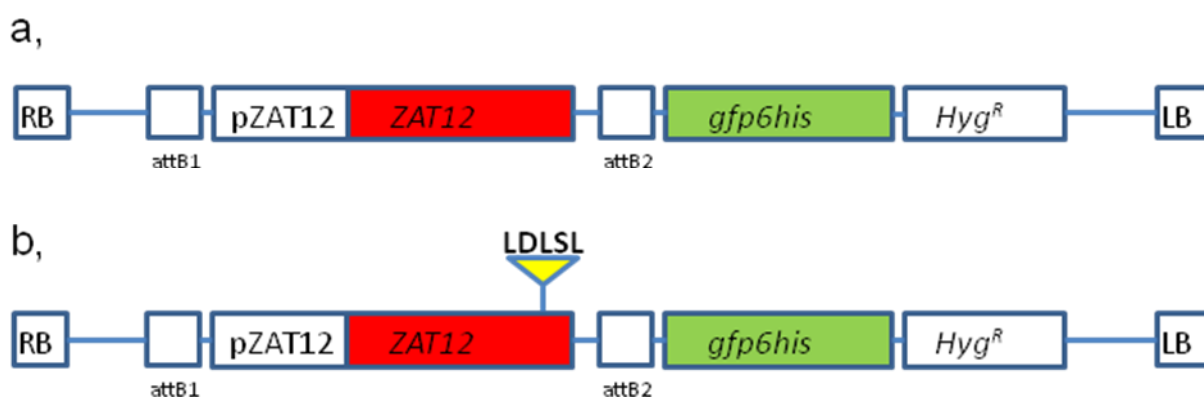
#### Plasmids generated for protein expression in Yeast-Two-Hybrid (Y2H) assay

pDONR207- ZAT12	pDONR207 (Invitrogen)	Replaced <i>ccdB</i> gene between P1 and P2 attachment sites	<i>ZAT12</i> generated with 5'attB1_Zat12 and 3'-attB2_Zat12 primers	Gm <sup>R</sup> : transformation control in bacteria	Provided by Dr.F. Mauer
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pGBKT7-FIT-C	pGBKT7-(Clontech)	Replaced <i>ccdB</i> gene between R1 and R2 attachment sites	<i>FIT-C</i>		Kan <sup>R</sup> : Provided by Dr.T. Brumbarova
					<i>TRP1</i> : transformation control in yeast
pACT2-GW-ZAT12	pACT2-GW (Clontech; Provided by Dr.Yves Jacob)	Replaced <i>ccdB</i> gene between R1 and R2 attachment sites	<i>ZAT12</i> generated from pDONR207-ZAT12		Car <sup>R</sup> : This work
					transformation in bacteria, <i>LEU</i> : transformation control in yeast

ns: nonstop; R: resistance



**Fig. 3.1. T-DNA containing *ZAT12* and *ZAT12* $\Delta$ EAR gene constructs.**

*ZAT12* (a) and *ZAT12* $\Delta$ EAR (b) were cloned in front of *GFP* (*gfp6his*) in the binary destination vector pMDC107. Selection marker in transgenic plants is Hygromycin (*Hyg*<sup>R</sup>). Attachment sites (attB), right (R) and left (L) border are indicated.

#### 3.1.4 Antibodies

- rat IgG monoclonal anti-HA antibody (clone 3F10, Roche) for detection of HA tagged proteins (dilution used in this work 1:1000)
- mouse IgG monoclonal anti-GFP antibody (clone 7.1, Roche) for detection of GFP tagged proteins (dilution used in this work 1:2000)
- polyclonal goat anti-rat horseradish peroxidase secondary antibody (Sigma Aldrich) for detection of anti-HA antibodies (dilution used in this work 1:10000)
- polyclonal goat anti-mouse horseradish peroxidase secondary antibody (Pierce) for detection of GFP antibodies (dilution used in this work 1:5000)
- *ZAT12* Antiserum generated by Prof. U. Müller (Zoology Department, Saarland University) (undiluted)

## 3.1.5 Oligonucleotides

All primers were ordered in a concentration of 100  $\mu$ M.

**Table 3.1. List of primer sequences for PCR reactions.**

Gene/ T-DNA /PROMOTER	Primer name	Sequence
<b>Primer for standard-PCR reactions</b>		
<i>ZAT12</i>	ZAT12 genotyping 5'	5`TCGCATCCTTGTCCCATATGT 3`
	ZAT12 genotyping 3'	5`TTCAAATTGTCCACCATCCCTAG 3`
	ZAT12 Standard 5'	5`ACACCAAACCTCAAAAACACAAACC 3`
	ZAT12 Standard 3'	5`CGTCGGCAAATAGGCTAATAGATT 3`
35S promoter	35S FORWARD	5`ATCCCACTATCGTTCGCAAGACCC 3`
GABI-kat T-DNA (G)	GABI T-DNA check	5`CCCATTGGACGTGAATGTAGAC 3`
<i>GFP</i>	GFP rev	5`AAAGGGCAGATTGTGTGGAC 3`
<i>HA</i>	attB1 HA3	5`GGGGACAAGTTTGTACAAAAAAGCA GGCTCCATGGCATAACCCATACGACGT-3`

**Primer for RT-PCR**

<i>EF1B-alpha-g</i> (STD)	AtEF-gen-3'(2726)	5`CCGGGACATATGGAGGTAAG 3`
	AtEF-gen-5'(2522)	5`TCCGAACAATACCAGAACTACG 3`
<i>EF1B-alpha-g</i> (Q, gDNA)	AtEF-gen-3'(2726)	5`CCGGGACATATGGAGGTAAG 3`
	AtEF-gen-5'(2522)	5`TCCGAACAATACCAGAACTACG 3`
<i>EF1B-alpha</i> (STD)	AtEF-c-5'(2125)	5`ACTTGTACCAGTTGGTTATGGG 3`
	AtEF-c-3'(2251)	5`CTGGATGTACTCGTTGTTAGGC 3`
<i>EF1B-alpha</i> (Q, cDNA)	AtEF-c-5'(2125)	5`ACTTGTACCAGTTGGTTATGGG 3`
	AtEF-c-3'(2251)	5`CTGGATGTACTCGTTGTTAGGC 3`
<i>ZAT12(STD)</i>	ZAT12 Standard 5'	5`ACACCAAACCTCAAAAACACAAACC 3`
	ZAT12 Standard 3'	5`CGTCGGCAAATAGGCTAATAGATT3`
<i>ZAT12(Q)</i>	ZAT12 Real-Time 5'	5`GAGTCACAAGAAGCCTAACAACGA 3`
	ZAT12 Real-Time 3'	5`AAGCCACTCTCTTCCCACTGCTA 3`
<i>IRT1</i> (STD)	AtIRT1-c-3'(1622)	5`TTAGGTCCCATGAACTCCG3`
	AtIRT1-c-5'(1523)	5`AAGCTTTGATCACGGTTGG3`
<i>IRT1</i> (Q)	AtIRT1-c-3'(1622)	5`TTAGGTCCCATGAACTCCG3`
	AtIRT1-c-5'(1523)	5`AAGCTTTGATCACGGTTGG3`
<i>FIT</i> (STD)	AtFRU-c-5'(1392)	5`GGAGAAGGTGTTGCTCCATC3`
	AtFRU-c-3'(1483)	5`TCCGGAGAAGGAGAGCTTAG3`
<i>FIT</i> (Q)	AtFRU-c-5'(1392)	5`GGAGAAGGTGTTGCTCCATC3`
	AtFRU-c-3'(1483)	5`TCCGGAGAAGGAGAGCTTAG3`
<i>BHLH39</i> (Q)	RT5`bHLH39	5`GACGGTTTCTCGAAGCTTG3`
	RT3`bHLH39	5`GGTGGCTGCTTAACGTAACAT3`

**Primer for molecular cloning**

<i>ZAT12</i>	5`attB1-zat12 PROMOTER	5`GGGGACAAGTTTGTACAAAAAAGCAGG CTCAAATGGACCCGATTTGAGA 3`
	3` attB2-zat12 NONSTOP	5`GGGGACCACTTTGTACAAGAAAGCTGGGGTGATA AACTGTTCTTCCAAGCTCCA 3`

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5`-attB1_Zat12	5`GGGGACAAGTTTGTACAAAAAAGCAGGCTCC ATGGTTGCGATATCGGAGATCAA3`
3`-attB2_Zat12	5`GGGGACCACTTTGTACAAGAAAGCTGGGT TCAATAAACTGTTCTTCCAAGCTCCA3`

#### Primer for ZAT12 antibody preparation

ZAT12	5` ZAT12_PET	5` – ATGGTTGCGATATCGGAGATCAA3`
	ZAT12_CT His 3`	5`TCAAGAGGCCATACCGTGATGATGATGATGATG AGAACCACGATAAACTGTTCTTCCAAGCTCCA3`

#### Primer for sequencing

ZAT12 inserted	pDSeq 1	5` GCAGTTCCTACTCTCGG 3`
in pDONR207	pDSeq 2	5`CATCAGAGATTTTGAGACAC3`

#### Primer for mutation

ZAT12	ZAT12dEAR-R1	5`TTCAAATTGTCCACCATCCCACAAGCCACTCTCTT-3`
	ZAT12dEAR-F2	5`GTGGGAAGAGAGTGGCTTGTGGGATGGTGGACAAT 3`

Standard amplification (STD); Quantification (Q)

#### 3.1.6 Peptides for antibody

<u>Gene</u>	<u>Sequences</u>
ZAT12	C-RVGQENV DGGDQKRVFT

#### 3.1.7 Enzymes and Kits

<u>Application</u>	<u>Description</u>	<u>Manufacturer</u>
cDNA synthesis	RevertAid First Strand cDNA Synthesis	Fermentas
DNase I treatment	DNase I, RNase-free	Fermentas
RNA Isolation (RT-qPCR)	Spectrum Plant Total RNA Kit	Sigma-Aldrich
Standard PCR	JumpStartREDTag Ready Mix	Sigma-Aldrich
Real time RT-qPCR	Premix ExTaq	TaKaRa
Amplification DNA for cloning	Phusion Polymerase	New England Biolabs
Cloning (Insert Amplification)	Phusion DNA Polymerase	Finnzymes
BP reaction (Gateway cloning)	Gateway BP clonase II Enzyme Mix	Invitrogen
LR reaction (Gateway cloning)	Gateway LR clonase II Enzyme Mix	Invitrogen
Plasmid DNA Isolation	InnuPREP Plasmid Mini Kit	AnalytikJena
Isolation and concentration of DNA fragments from agarose gels	InnuPREP Gel Extraction Kit	AnalytikJena

**3.1.8 Antibiotics**

	<b>Dissolve in</b>	<b>Stock concentration</b>	<b>Dilution for use</b>
Carbenicillin	H <sub>2</sub> O	60 mg/ml	1:1000
Gentamycin	H <sub>2</sub> O	50 mg/ml	1:2000
Hygromycin B	H <sub>2</sub> O	15 mg/ml	1:1000
Kanamycin	H <sub>2</sub> O	50 mg/ml	1:1000
Rifampicin	DMSO	50 mg/ml	1:1000

**3.1.9 Markers**

<b>Application</b>	<b>Description</b>	<b>Manufacturer</b>
Mi 1kb DNA Marker Go	Agarosegel-Electrophorese	Metabion
Page ruler 1kb DNA ladder	Agarosegel-Electrophorese	Fermentas
PageRuler Prestained Protein ladder	SDS-Gelelectrophorese	Fermentas

**3.1.10 Media and buffers****3.1.10.1 Buffer for ImmunoBlot****a, SDS-PAGE**

	<b><u>Running gel for 2 Gels</u></b>	<b><u>Stacking gel for 2 Gels</u></b>
	<b>15%</b>	<b>5%</b>
H <sub>2</sub> O	2.5 ml	3 ml
1xBuffer *	2.5 ml	1.25 ml
30% Acrylamide/ Bisacrylamide, (37.5:1)	5 ml	0.67 ml
10 % APS	50 µl	50 µl
TEMED	5 µl	5 µl

**\* 4x Running gel buffer (pH8,8)**

1.5 M Tris-HCl

0.4 % SDS

**\* 4x Stacking gel buffer (pH 6,8)**

0.5 M Tris-HCl

0.4% SDS



#### **b, Other Solutions**

##### **2x Laemmli loading buffer**

125 mM Tris-HCl (pH 6,8)  
10% Glycerol  
2% DTT  
2.5% SDS  
0.02% Bromphenolblue  
fill dest. H<sub>2</sub>O up to 10ml

##### **1x Transfer buffer (pH 8,3)**

10% Running buffer  
20 % Ethanol  
fill dest. H<sub>2</sub>O up to 1l

##### **1x TBS-T (pH 7,4)**

150 mM NaCl  
2.7 mM KCl  
0.25 M Tris  
0.5 % Tween

##### **Antibody solution**

(depend on working concentration of antibody)  
Blocking solution  
Antibody  
1x TBS-T

##### **10x Running buffer**

250 mM Tris  
2.5 M Glycine  
1% SDS

##### **1x Ponceau S**

0.2% Ponceau S (Sigma)  
3% TCA  
3% Sulfosalicylic

##### **Blocking solution**

2.5 % Milk powder  
fill 1x TBS-T up to 50ml

##### **ECL-Solution (Amersham)**

50% Luminol (Solution A)  
50% Peroxid (Solution B)

#### **3.1.10.2 Solutions for tobacco infiltration and Arabidopsis transformation**

##### **1x Infiltration solution (pH 5,6)**

2mM NaH<sub>2</sub>PO<sub>4</sub>  
50 mM MES  
5% Glucose  
100µM Acetosyringon

##### **1x Arabidopsis Transformation solution**

5% Sucrose  
10 mM MgCl<sub>2</sub>  
0.02% Silwet Gold

#### **3.1.10.3 Media**

##### **a, Bacteria medium**

##### **LB-Medium (pH 7)**

0.17 M NaCl  
0.5% Yeast Extract  
1% Bacto Tryptone

##### **LB-Agar (pH 7)**

1l LB-Medium  
1.6% Micro Agar

**b, Plant medium**

**Fe deficient Hoagland Medium (pH 6)**

0.375 mM Ca(NO<sub>3</sub>)<sub>2</sub>  
 0.1875 mM MgSO<sub>4</sub> x7 H<sub>2</sub>O  
  
 0.3125 mM KNO<sub>3</sub>  
 0.125 mM KH<sub>2</sub>PO<sub>4</sub>  
 1 ml Micronutrient  
 50µM Ferrozine (1ml 50mM Ferrozine)  
 10g Sucrose  
 0.8% Plant Agar  
 fill dest. water up to 1l

**Standard Hoagland Medium for 1 l (pH 6)**

50 µM FeNaEDTA (0.5 ml 100 mM)  
 Other components are the same as +Fe  
 Hoagland medium

**Micronutrients**

12.5 µM KCl  
 12.5 µM H<sub>3</sub>BO<sub>3</sub>  
 2.5 µM MnSO<sub>4</sub> x H<sub>2</sub>O  
 0.5 µM ZnSO<sub>4</sub>x 7 H<sub>2</sub>O  
 0.375 µM CuSO<sub>4</sub> x 5 H<sub>2</sub>O  
 0.01875 µM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> x 4 H<sub>2</sub>O

**MS-Medium (Duchefa)**

<b>Micro Elements</b>	<b>Macro Elements</b>	<b>Vitamins</b>
0.11µM CoCl <sub>2</sub> .6H <sub>2</sub> O	2.99mM CaCl <sub>2</sub>	26.64µM Glycine
0.10µM CuSO <sub>4</sub> .5H <sub>2</sub> O	1.25 mM KH <sub>2</sub> PO <sub>4</sub>	0.56µM myo-Inositol
0.10µM FeNaEDTA	18.79 mM KNO <sub>3</sub>	4.06µM Nicotinic Acid
0.10µM H <sub>3</sub> BO <sub>3</sub>	1.50mM MgSO <sub>4</sub>	2.43µM Pyridoxine HCl
5.00µM KI	20.61 mM NH <sub>4</sub> NO <sub>3</sub>	0.30µM Thiamine HCl
0.10µM MnSO <sub>4</sub> .H <sub>2</sub> O		
1.03µM Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O		
29.91µM ZnSO <sub>4</sub> .7H <sub>2</sub> O		

**3.1.10.4 Others**

- Petri dish (Round/Square) (Greiner Bio One)
- Sterilisation solution (6 % NaClO, 0,1 % Triton-X)
- 0,1 % Plant Agar solution
- 0,1 M NaOH for adjust pH
- 10 mM dNTP Mix
- 25 mM MgCl<sub>2</sub>
- 6xDNA Loading Dye (Thermo Scientific)
- GelRed (Biotinum)
- 50xTAE (10M EDTA; 0.2M acetic acid; 0.4M Tris)
- Ethanol
- 0,2 M citric acid in H<sub>2</sub>O
- High performance chemiluminescence film (Amersham Hyperfilm ECL)
- Turf substrate and vermiculte
- Jiffy (yiffypot.com)

### 3.1.11 Devices

RealTime qRT-PCR-Cycler	MyIQ Bio-Rad
Confocal Microscopy	Zeiss LSM310 Laser Scanning Microscope
Fluorescent Microscopy	Keyence, BZ-9000
Primus 96	Peqlab
GelDoc 2000	BioRad
Mini Trans-Blot Cell	BioRad
Plant Climatics	CLF
Safire <sup>2</sup>	Tecan

### 3.1.12 Databank and softwares

ApE- A plasmid Editor	<a href="http://biologylabs.utah.edu/jorgensen/wayned/ape">http://biologylabs.utah.edu/jorgensen/wayned/ape</a>
TAIR	<a href="http://www.arabidopsis.org/">http://www.arabidopsis.org/</a>
LSM software	<a href="http://www.zeiss.de/micro">www.zeiss.de/micro</a>
Bio-Rad iQ5	<a href="http://www.bio-rad-iq5.software.informer.com/">www.bio-rad-iq5.software.informer.com/</a>

## 3.2 Methods

### 3.2.1 Physiological methods

#### 3.2.1.1 Plant Growth

After being surface-sterilized with bleach for 9 minutes and washed later five times with distilled water, Arabidopsis seeds were stratified for 2-3 days in 0.1% plant agar in the dark at 4°C. Arabidopsis plants were grown on soil or agar plates later.

- In the seedling growth system, depend on the age of harvesting time, 30-45 plants per plate were directly grown on 50µM FeNa-EDTA (+Fe, standard condition) or 0µM FeNa-EDTA (-Fe) for six to ten days on square plates placed at 21°C/19°C and 16 h light, 8 h dark cycles (long-day condition) in plant chambers. The agar plate was prepared by using 1x Hoagland solution with a concentration of 0.8% plant agar supplied with 50 µM FeNa-EDTA (+Fe) or 50µM ferrozine (-Fe) and 1% sucrose. When seedlings were harvested for molecular analysis, one sample consists of 30-45 plants.
- The growth on soil was performed utilizing a mixture a turf substrate and vermiculte (3:1).

### ***Oxidative stress assay***

For the analysis of stress tolerance, seedlings were subjected to 20mM H<sub>2</sub>O<sub>2</sub> for 1h. Subsequently, the whole seedlings were harvested for analysis.

#### ***3.2.1.2 Iron measurement***

To determine Fe content in plant material, shoots of the plants grown for 10 days were harvested and dried over night at room temperature (RT) and again dried for 1 day at 120°C in the oven. After drying, harvested shoots was powdered with an Ahart mortar. Iron contents were finally determined in material with direct solid sampling graphite furnace absorption spectrometry (GF AAS 6; Analytik Jena) at the Leibniz Institute für Neue Materialien (INM, Saarbrücken). Experiment replications were four.

#### ***3.2.1.3 H<sub>2</sub>O<sub>2</sub> measurement and localization***

10-day old seedlings were used to measure H<sub>2</sub>O<sub>2</sub> production by using an Amplex red hydrogen peroxide-peroxidase assay kit (Molecular Probes). The AmplexR Red reagent (10-acetyl-3, 7-dihydroxyphenoxazine), along with horseradish peroxidase (HRP), has been used to detect H<sub>2</sub>O<sub>2</sub> because reaction between each other with H<sub>2</sub>O<sub>2</sub> causes production of red-fluorescent oxidation product, resorufin. Resorufin was quantified using Safire<sup>2</sup> (absorbance at ~560 nm, Tecan). Root tissue was harvested and ground in liquid nitrogen. After 30mg of ground frozen tissue were diluted in 200 µl of phosphate buffer (20 mM K<sub>2</sub>HPO<sub>4</sub>, pH 6.5) and centrifuged, 50 µl of the supernatant was added with 100 µM Amplex Red reagent (10-acetyl-3,7-dihydrophenoxazine) and 0.2 U/ml horseradish peroxidase and incubated at room temperature for 30 min under dark conditions. Experiments were performed three times.

The generation of ROS was also localized in the root. For these experiments plant roots were incubated with CM-H2DCFDA (5-(and-6)-chloromethyl-2, 7-dichlorodihydrofluorescein diacetate, acetyl ester; (Molecular Probes)) for 30 minutes and washed with medium three times. CM-H2DCFDA exists nonfluorescent stage until enter into the cell and its acetate groups are removed by intracellular esterases and oxidation within the cell. Therefore, the oxidation of samples can be detected by appearance and increase of fluorescence. All fluorescence images were obtained with a fluorescence microscope (Keyence, BZ-9000).

#### ***3.2.1.4 Confocal imaging***

To image confocal microscopy of ZAT12-GFP fusion proteins, 10-day old roots of plants expressing ZAT12-GFP were observed under a LSM510 confocal microscope (located at the

department of Pharmacy, Saarland University) with excitation wavelength: 488nm and detection filter: 500-530 nm.

The authenticity of the GFP was confirmed by a lambda scan and GFP signals were specifically detected at the expected wavelength of 510 nm.

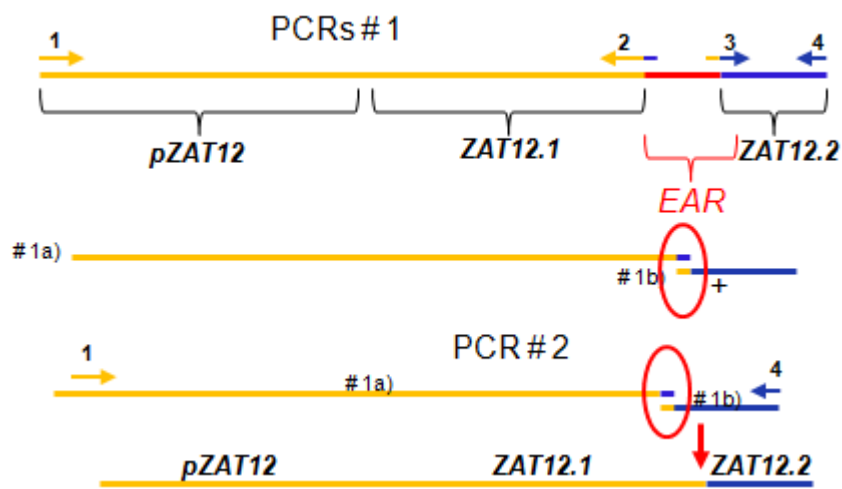
### 3.2.2 Molecular methods

#### 3.2.2.1 Molecular cloning

##### *a, Molecular cloning for expression in plants*

Two protein fusion constructs were generated using the Gateway cloning system (Invitrogen). First, *ZAT12* genomic DNA sequences and *ZAT12* deleted EAR motif including the native promoter regions (approximately 1600 bp upstream of ATG) and nonstop codon were introduced by Gateway cloning (based on homologous recombination) into the pDONR207 vector. These sequences were amplified by PCR using primer mixture 5'attB1-zat12 PROMOTER and 3' attB2-zat12 NONSTOP (see Material) in order to be flanked by two attachment sites B1 and B2. For deletion of EAR motif we based on overlap extension PCR. In the first round of PCR, two partially overlapping pieces of *ZAT12* were amplified using 5'attB1-zat12 PROMOTER and ZAT12dEAR-R1 for one piece and ZAT12dEAR-F2 and 3' attB2-zat12 NONSTOP for the other piece. The second round of PCR was performed with mixture of these two PCR products and the outer primers (5'attB1-zat12 PROMOTER and 3' attB2-zat12 NONSTOP) (see Fig. 3.2). These PCR products were later purified from agarose gel (InnuPREP Gel Extraction Kit, Analytikjena, according to manufacturer' manual) and inserted into pDONR207 by BP recombination. Finally, the products of reaction were transformed into *E. coli* INVαF' competent cell. Empty Gateway vectors contain a *ccdB* gene (suicidal gene, whose protein interferes with *E. coli* DNA gyrase, impairing growth of most *E. coli* strains) between the attachment sites that will be replaced by the gene of interest. Hence, only clones that contain a vector with gene of interest are able to grow. Small cultures were prepared from these colonies in order to harvest plasmid contain DNA fragment of interest by miniprep procedure (InnuPREP Plasmid Mini Kit, Analytikjena, according to manufacturer' instructions). After being verified by colony PCR and/or restriction enzyme digestion and sequencing, they were transferred by Gateway cloning into the binary vectors pMDC107 by LR recombination to obtain protein-GFP fusion constructs of the respective *ZAT12* genes (Curtis & Grossniklaus, 2003) . The same procedures of verification were performed after this cloning step. The pZAT12:*ZAT12-GFP* and pZAT12:*ZAT12ΔEAR-GFP* fusion constructs were obtained with the pMDC107 vector in *E.coli* cells as Fig. 3.1. Subsequently, both

destination vectors were transferred into *Agrobacterium tumefaciens* strain GV2260. Only clones containing these vectors could grow under LB medium supplemented Rif, Car, and Kan antibiotics and were verified transient transformation by infiltration of tobacco leaves. After confirmation of vector constructs and gene expression, these clones were transformed into *Arabidopsis thaliana* plants (ecotype Col-0) by the “floral dip” method (Clough & Bent, 1998). Transformants are selected based on hygromycin resistance of transgenic plants constitute vector of interest (Harrison et al., 2006). The positive transgenic *Arabidopsis* lines were multiplied and plants containing homozygous single insertions were selected.



**Fig. 3.2. *ZAT12*Δ*EAR* amplification scheme.**

**Reaction 1a:** 5′attB1-zat12 PROMOTER (labeled #1) and ZAT12dEAR-R1 (labeled #2) primers. Expected product size (pZAT12-ZAT12.1): 2100bp.

**Reaction 1b:** ZAT12dEAR-F2 (labeled #3) and 3′attB2-zat12 NONSTOP (labeled #4) primers. Expected product size ZAT12.2: 103bp.

**Reaction 2:** 5′attB1-zat12 PROMOTER (labeled #1) and 3′attB2-zat12 NONSTOP (labeled #4) primers. Expected product size (pZAT12-ZAT12.1-ZAT12.2, deleted EAR motif): 2203bp.

#### *b, Molecular cloning for expression in yeast.*

Following Gateway cloning, pACT2-GW - *ZAT12* and pACT2-GW - *ZAT12*Δ*EAR* were made for Yeast-Two-Hybrid assay. *ZAT12* gene and *ZAT12*Δ*EAR* with stop codon were amplified by primer combination 5′-attB1\_Zat12 and 3′-attB2\_Zat12 (Material) to attach two attB1 and attB2 sites, were also introduced into the pDONR207 vector and pACT2-GW vectors later. We obtained vector pACT2-GW harboring *ZAT12* and mutant version *ZAT12*Δ*EAR*. Vector pGBKT7 harboring FIT-C was obtained from previous experiment (Lingam et al., 2011).

#### *c, Molecular cloning for expression in E.coli*

For ZAT12 antibody preparation, we used another cloning system – pETBlue system (Novagen). *ZAT12* was attached His-Tag at the 3' end (for purification of ZAT12 protein later) by amplification PCR using 5' ZAT12\_PET and ZAT12\_CT His 3' primers (see Material). After checking size of these fragments by agarose electrophoresis, *ZAT12-His* fragments were purified and cloned into the EcoRV cloning site of pETBlue-2 vector and transformed into NovaBlue competent cells. Identification of successful cloning events was performed by blue/white screening. When *ZAT12-His* inserted into pETBlue-2 vector leads to disrupt expression of the *lacZ*  $\alpha$ -peptide and thereby produces white colony on plate's supplemented X-gal and IPTG. Inversely, colonies containing vector without insertion turn blue. After verification of sequence and PCR mediated orientation, selected positive colonies were multiplied in small culture and vector was isolated, and used to transform into Tuner (DE3)pLacI competent cells for protein expression by IPTG induction.

#### **3.2.2.2 Real-time reverse transcription quantitative PCR (RT-qPCR)**

To study gene expression levels, we used Reverse transcription real time quantitative PCR (RT-qPCR). Due to its high sensitivity, this method is considering as a precise choice to quantify mRNA levels. While semi-quantitative RT-PCR based on end-point detection of PCR product, RT-qPCR quantify the amount PCR products based on the detection of fluorescent marker molecules emitting light proportional to them during the PCR reaction. Therefore, the fluorescence can be monitored and precisely quantified in real time during each cycle. By this, its detection limit can be up to one single molecule per reaction tube. All set-up steps of the RT-qPCR were conducted following recommendations for accurate real-time reverse transcription quantitative PCR of Klatté and Bauer 2008 (Klatté and Bauer, 2008).

#### *RNA isolation and cDNA synthesis*

Total RNA was extracted from about 100 mg deep frozen seedlings. Plant material was powdered and homogenized under liquid nitrogen and finally applied with the Spectrum Total RNA Kit from Sigma-Alrich to isolate total RNA according to the manufacturer's protocol. Then RNA concentration was measured and 1 $\mu$ g of total RNA was used for cDNA synthesis following the Fermentas cDNA synthesis kit. To minimize genomic DNA contaminations in the samples, total RNA samples were digested DNAaseI. And cDNA final product was diluted 1:10 for next step.

#### *Experimental setup*

Master mix for real time RT-qPCR:

10 µl Takara Premix

0.2 µl Primer 5`

0.2 µl Primer 3`

0.1 µl SYBR green

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10.5 µl Total volume

For RT-qPCR quantification, cDNA was diluted a 1:10 second time. 10µl cDNA or standard respectively along with 10µl master mix were tranfered to 96- well plate. Plate then was sealed with ICycleriQTM Optical Tape.

**Table 3.2: Thermopprofile of RT-qPCR**

Initial denaturation	95 °C	3 min	
Denaturation	95 °C	10 s	} 40x
Annealing	58 °C	18 s	
Elongation	72 °C	18 s	
Final elongation	72 °C	7 min	
Melt curve analysis	55 °C - 95 °C		
Cooling	12 °C	forever	

#### *Data evaluation*

For each gene, the absolute quantity of initial transcript was determined by mass standard curve analysis. Before analyzing data, the quality and integrity of each individual PCR reaction was verified such as Ct (threshold cycle) value to test the reproducibility of technical repetitions, standard curve to determine the PCR efficiency, and melt curve to validate the PCR specificity. Expression data were obtained by exporting from the Bio-rad operating software into Microsoft Excel. Absolute expression values (SQ mean values) were subtracted by expression value of water control and expression values obtained from genomic DNA.

To determine the normalization factors, the absolute expression values of a control sample (e.g. wild type, root, standard condition) were divided by the absolute expression of each individual sample of the housekeeping gene (*EFc/UBP6*). These obtained normalization factors were multiplied with the respective expression values of all measured genes. Mean values and standard deviation were finally calculated from biological replica and absolute normalized expression values were presented in bar diagrams.



### **3.2.2.3 ZAT12 antibody preparation**

Based on predicted antigenic propensity scores, a peptide corresponding to the N-terminal of ZAT12 was chemically synthesized and conjugated with KLH (Bio Trend) and later injected into rat to obtain a polyclonal antiserum (This work was conducted by Prof. U. Müller, Zoology Department, Saarland University). The antiserum was tested positive for its specificity by detecting bacterially expressed ZAT12 peptide. For use in Immunoblots with plant protein extracts anti-ZAT12 antiserum need to be purified. This work will be continued later.

### **3.2.2.4 Immunoblot analysis**

After harvesting and grinding in liquid nitrogen, total plant protein from ten 10-day old seedlings were extracted in 2x Laemmli loading buffer and then centrifuged for 5 min at 10000 x g. The amount of 2x Laemmli loading buffer added was proportional to the weight of ground material (20µl of 2 x Laemmli loading buffer/10 mg materials). The supernatants containing the total protein extracts were denatured at 95°C for 5 min and loaded onto a 15 % SDS-polyacrylamide gel for separation. Subsequently, samples were transferred to Protran nitrocellulose membrane (Schleier & Schuell), stained with Ponceau S (Sigma-Aldrich) for loading control. Later on, the membranes with ZAT12-GFP and ZAT12Δ-GFP fusion protein were probed with anti-GFP (Roche, 1:2000) followed by goat anti-mouse horseradish peroxidase secondary antibody (Pierce Chemical, 1:5000), whereas the membranes with FIT-HA protein were probed by anti-HA (Roche, 1:1000) followed by goat anti-rat horseradish peroxidase secondary antibody (Sigma Aldrich, 1:10000). Immunoblots were developed using ECL chemiluminescence detection reagents (GE Health Care) according to the manufacturer's instructions.

### **3.2.2.5 Yeast Two-Hybrid assay**

The yeast two-hybrid screen was performed according to the protocol of the Matchmaker library construction and screening kit as described by the manufacturer (Clontech Laboratories, Inc.).

Yeast-two-hybrid method is used to screen novel interaction between proteins before the further verification of the interaction. A bait gene is expressed as a fusion to GAL4 DNA binding domain, whereas suspected (prey) gene is expressed as a fusion to the GAL4 activation domain. When bait and prey fusion proteins interact leads to activate transcription of reporter genes (such as *HIS3*, *ADE2*, *lacZ* and *MEL1*).

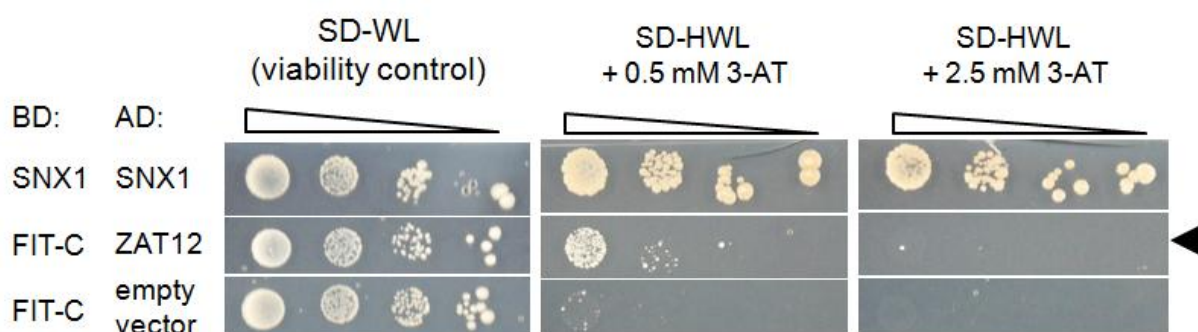
The sequences encoding *ZAT12* and *ZAT12 $\Delta$ EAR* mutants were cloned into the activation domain (AD) vector (pACT2-GW) and *FIT-C* was inserted into the DNA-binding domain (BD) vector (pGBKT7-GW). Construct combinations were transformed into the yeast AH109 strain and selected on SD agar medium lacking Leu and Trp (SD-WL). AH109 contains four reporters: *ADE2*, *HIS3*, *MEL1*, and *lacZ* regulated by three distinct GAL4 upstream activating sequences (UASs) and TATA boxes. To reduce the incidence of false positives, *HIS3* were selected for screening of interaction (James et al., 1996). *HIS3* expression may slightly elevate in transformants using AH109 strain because of intrinsic DNA-binding properties of the bait protein. Using 3-amino-1, 2, 4-triazole (3-AT) which is a competitive inhibitor of the yeast *HIS3* protein (His3p) inhibits leaky expression of His3p and thus to prevent background growth on SD medium lacking histidine (Fields, 1993; Durfee et al., 1993). Therefore, 10-fold serial dilutions ( $A_{600} = 1$  to  $10^{-4}$ ) of the transformants were spotted on agar plates of SD medium lacking His, Trp, Leu (SD-HWL) and supplemented with 2.5 mM or 0.5 mM 3-AT and were incubated at 30 °C for 14 days.

## 4. Results

### 4.1 Functional study of the protein-protein interaction ZAT12-FIT

#### 4.1.1 Verification of protein interaction between ZAT12 and FIT

In FIT over-expression Arabidopsis lines, the iron deficiency genes *IRT1* and *FRO2* showed enhanced expression levels only in response to Fe deficiency, but not in Fe sufficiency, similar to the situation in wild-type. This suggested that there exists an additional level of regulation acting on either the abundance or the activity of the FIT protein (Jakoby et al., 2004). Later on, FIT abundance was shown to be very high under both conditions but its rate of degradation was enhanced under iron deficiency (Lingam et al., 2011; Meiser et al., 2011; Sivitz et al., 2011). In order to identify factors responsible for the post-transcriptional regulation of FIT, a yeast two-hybrid screen was performed, resulting in the identification of a total of 14 putative candidates (Lingam et al., 2011). One of the candidates was the Zn finger transcription factor ZAT12 (J. Mohrbacher and P. Bauer, unpublished). To test the interaction, we performed the yeast two hybrid assay again, this time using the full ZAT12 sequence, instead of the C-terminus identified in the screen, and the C-terminal FIT peptide (FIT-C), excluding the N-terminus and the bHLH domain. A triple selection was applied to select for the complementation of the yeast auxotrophy to Trp (W, selection for the presence of the FIT-C-containing vector), Leu (L, selection for the presence of the ZAT12-containing vector) and His (H, marker for the interaction between FIT-C and ZAT12).



**Fig.4.1. Protein Interaction of FIT with ZAT12 in Yeast.**

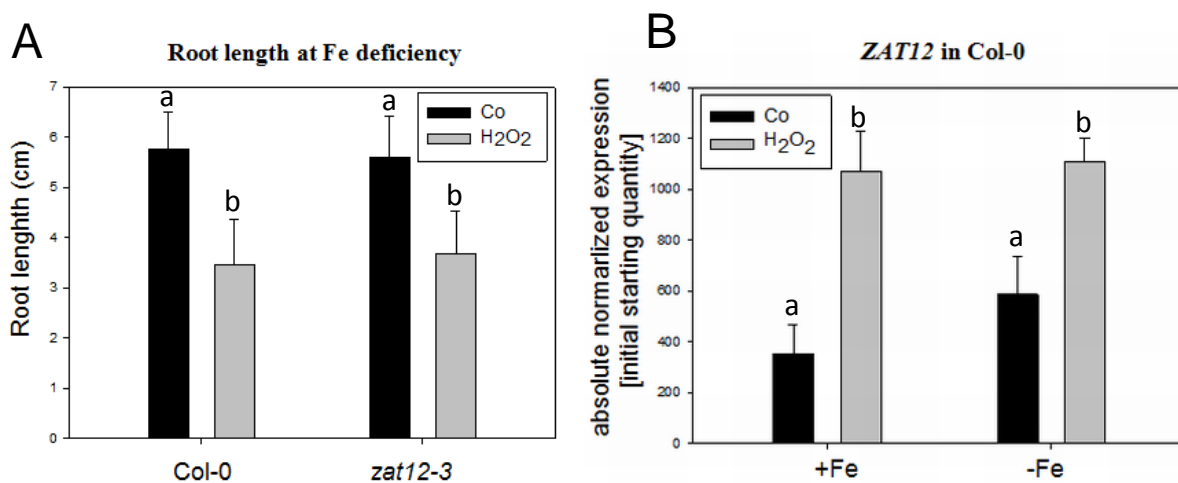
10-fold dilutions ( $A_{600}$  of  $1-10^{-4}$ ) of the yeast AH109 strain harboring either an activation domain (AD)-ZAT12 or an empty DNA- AD-vector and binding domain (BD)-FIT-C constructs were spotted on SD selection media without Leu and Trp (-WL; selection for positive transformants) or without Leu, Trp, and His (-HWL and 2.5 or 0.5 mM 3-amino-1,2,4-triazole; selection for interaction) and incubated for 14 days at 30 °C. Yeast two-hybrid assays indicate strong interaction of FIT with ZAT12 as indicated by *arrowheads*. Positive control is interaction between SNXs (Pourcher et al., 2010). This work was performed with Dr. Tzvetina Brumbarova.

Due to intrinsic activation properties of the FIT-C protein, leaky *HIS3* expression may occur leading to partial growth of the host yeast strain on medium lacking His. Therefore, small amounts of 3-amino-1,2,4-triazole (3-AT) was used to limit this unspecific His biosynthesis. Yeast containing both FIT-C and ZAT12, showed significant growth on His selection plates (Fig.4.1). This demonstrates the interaction between the two proteins. The yeast growth was limited at higher dilutions, suggesting that the interaction might be only transient, which may be expected, considering that FIT may serve as a scaffold for numerous interactions serving to fine tune the iron deficiency responses (Yuan et al., 2008; Lingam et al., 2011; Wang et al., 2012). In *planta*, we tried to confirm this interaction by bimolecular fluorescence complementation (BiFC) and Co-Immunoprecipitation (Co-IP). For Co-IP, we crossed Arabidopsis lines containing ZAT12-GFP with lines containing FIT-HA for stable expression and also transiently expressed by infiltration vectors harboring ZAT12-GFP and FIT-HA constructs into tobacco. By targeting FIT with anti-HA antibody it may help pull the entire protein interaction complex out of solution and thereby identify ZAT12 of the complex by anti-GFP antibody. To conduct BiFC, FIT-C and ZAT12 sequences were first attached with the N-terminal (YN) or C-terminal (YC) part of the yellow fluorescent protein (YFP), and later infiltrated in leaves mediated *Agrobacterium tumefaciens*. The interaction is confirmed by fluorescent observation of reconstituted YFP fluorescent protein under confocal microscopy. However, all above methods still not yet confirm the interaction between ZAT12 and FIT in *planta*. Reason explains for them will be shown later.

#### **4.1.2 Functional characterization investigation of *zat12-3* knock-out mutant Arabidopsis lines in response to Fe supply and H<sub>2</sub>O<sub>2</sub> stress**

*ZAT12* gene encoding a zinc finger protein containing an EAR motif function as a negative regulator of gene expression and its mRNA abundance is elevated in response to oxidative stress (the application of H<sub>2</sub>O<sub>2</sub> or methyl viologen), cold, wounding stresses etc. (Rizhsky et al., 2004; Davletova<sup>a</sup> et al., 2005; Vogel et al., 2005; Iida et al., 2000; (Kagale et al., 2010). Along with enhanced quantity, by *ZAT12* gain-/loss of function studies *ZAT12* was also suggested to involve in the tolerance to oxidative stress (under the application of superoxide-generating agent, methyl viologen) (Davletova et al., 2005). And in the cell when Fe<sup>2+</sup> presents in excess often leads to oxidative stress because it can react with the hydrogen peroxide, a normal by-product of metabolism and abiotic stress, to form Fe<sup>3+</sup>, a hydroxyl radical, and a hydroxide anion. Moreover, at Fe deficiency the level of H<sub>2</sub>O<sub>2</sub> in sunflower and maize was shown to be enhanced (Ranieri et al., 2001; Sun et al., 2007). This can also cause oxidative stress. Therefore, there is a direct connection between response to iron deficiency

and oxidative stress responses. We asked if *ZAT12* may represent a molecular link between these two processes because of its interaction with FIT. At first, we aimed to investigate the response of plants to strong oxidative stress. We treated wild type Col-0 and a *zat12* knockout mutant, *zat12-3*, previously characterized in our lab.  $H_2O_2$  was applied to 5 day-old seedlings, which were then transferred to new medium for another 3 days. A control condition without hydrogen peroxide treatment was also included. Each condition was applied to at least 30 single plants. Those seedlings were harvested for phenotype and gene expression level analysis.  $H_2O_2$  treated plants exhibited short root phenotypes and red shoots in contrast to control plants. However, no phenotypic difference was observed between Col-0 and *zat12-3* mutant regardless Fe supply. Root length of those plants was measured at Fe deficient condition (Fig.4.2A). This result of root length contradicts the previous results showing that *ZAT12* is required for tolerance of plant to oxidative stress (Davletova et al., 2005). A possible explanation could be the difference in the growth system. However, in term of gene expression, its level was increased after  $H_2O_2$  treatment regardless of Fe supply (Fig.4.2B). This is consistent with the previous report of Rizhsky et al., (2004).



**Fig.4.2. Response of *ZAT12* to oxidative stress (n=3).**

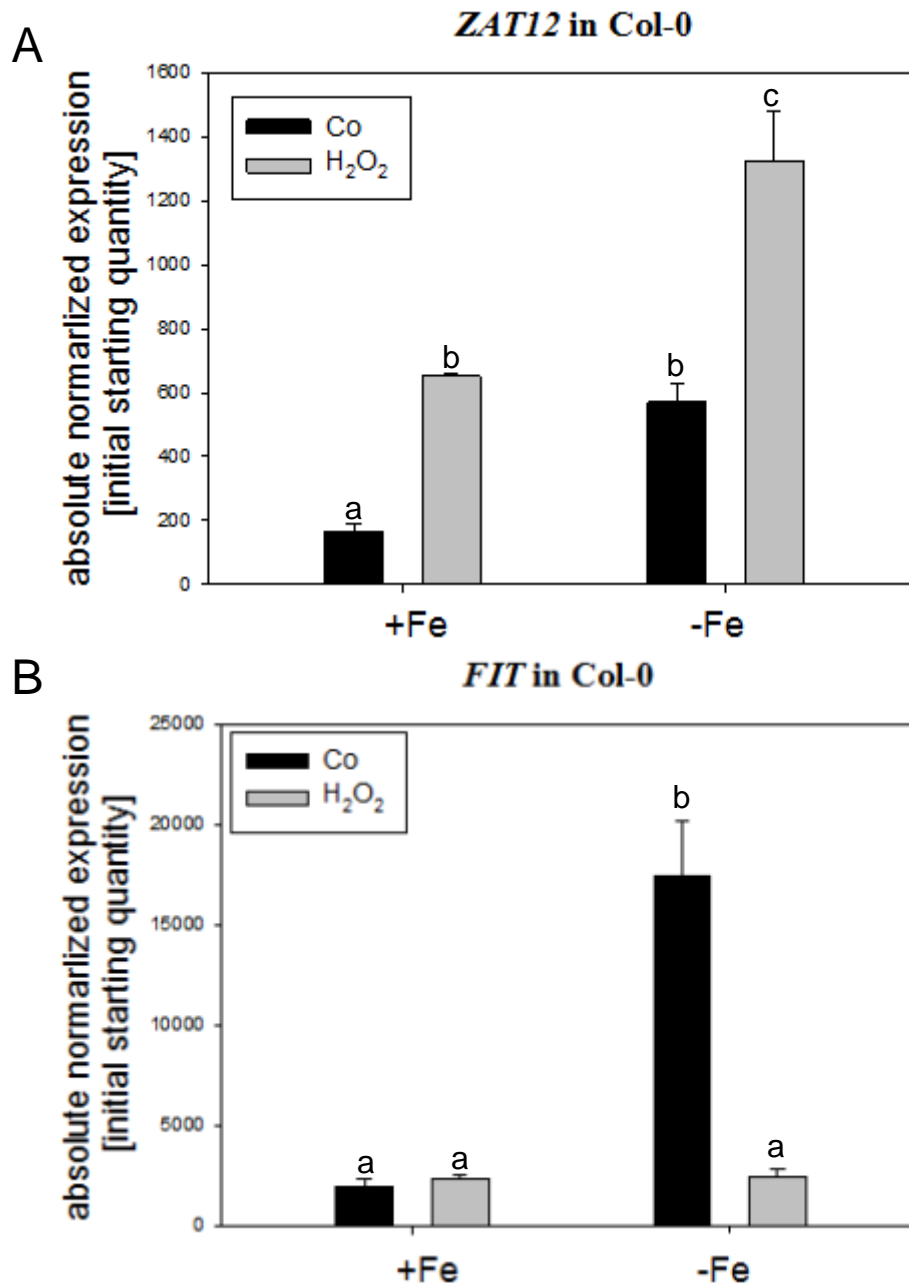
(A) Tolerance of *zat12-3* knock-out (KO) mutant seedlings to oxidative stress (n=30). (B) *ZAT12* gene expression in Col-0 (n=3) (Co means control without  $H_2O_2$ ). 5-day-old wild type (Col-0) and *zat12-3* KO plants were germinated on standard agar plates and transferred to fresh medium in the presence or absence of Fe for 3 days then treated with  $H_2O_2$ . Those plants were put back to correspondent plates for root length measurement (A) or harvested for gene expression analysis (B). The data were analyzed by analysis of variance (ANOVA). Error bars represent the standard deviation. In each figure, means with the same letter are not significantly different from each other at  $P < 0.05$ .

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#### 4.1.3 Analysis of *ZAT12* and *FIT* gene expression under physiological conditions related to Fe deficiency and oxidative stress by Reverse Transcription Real-time PCR

Although *zat12-3* mutant did not exhibit higher sensitivity to H<sub>2</sub>O<sub>2</sub> stress than Col-0, mRNA levels of *ZAT12* were elevated after H<sub>2</sub>O<sub>2</sub> treatment (Fig.4.2B). To understand how *ZAT12* functions in Fe deficiency and H<sub>2</sub>O<sub>2</sub> stress, we first investigated the expression of *ZAT12* and *FIT*. By using quantitative reverse transcription real-time PCR, we addressed questions such as: how is *ZAT12* expressed in response to oxidative stress and changes in the external Fe concentration, and how the absence of a functional *ZAT12* gene affects the regulation of *FIT* and iron uptake.

For this analysis, 8-day old Col-0 and *zat12-3* seedlings grown in Hoagland medium were subjected to +/- Fe and later +/- H<sub>2</sub>O<sub>2</sub> treatment. The experiment was performed in three biological replicates. The results showed that *ZAT12* responded to changes in Fe supply. *ZAT12* level was enhanced after 8-day iron starvation that did not see it in the early 5- day Fe deficiency system (Fig. 4.2B and Fig. 4.3A). Similar results were recently reported by Ravet et al., (2012). As expected, *ZAT12* transcripts were elevated in response to H<sub>2</sub>O<sub>2</sub> (Fig.4.3A). At the same time, *FIT* expression was repressed under Fe deficiency after H<sub>2</sub>O<sub>2</sub> treatment (Fig.4.3B). This result suggests that this seedling growth system is good for induction of iron deficiency and repression.



**Fig. 4.3. Expression of *ZAT12* and *FIT* in response to Fe deficiency and H<sub>2</sub>O<sub>2</sub> stress. (n=3)**

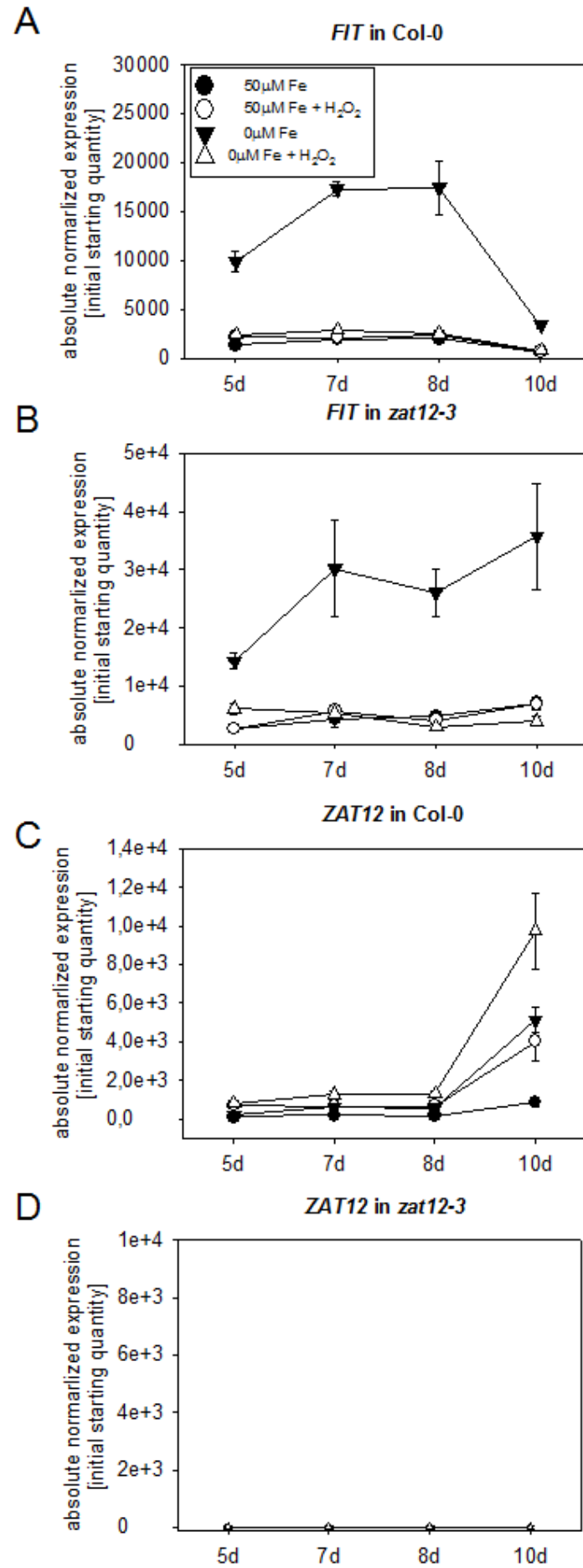
Gene expression analysis of *FIT* (A) and *ZAT12* (B) in Col-0 by reverse transcription real time quantitative PCR (RT-qPCR) (Co means control without H<sub>2</sub>O<sub>2</sub>). Seedlings were germinated and grown under Fe deficient and sufficient conditions in the 8 day agar seedling growth system. Seedlings were later harvested and processed for RT-qPCR (n=3). The data were analyzed by analysis of variance (ANOVA). Error bars represent the standard deviation. In each figure, means with the same letter are not significantly different from each other at P < 0.05.

Taken together, *ZAT12* is induced in response to Fe deficiency at 8-day-old seedling system, but not in 5 -day-old seedling system. With the different responses observed depending on the age of the seedlings, we wanted to understand the dynamics of Fe deficiency responses and

their cross-talk with oxidative stress signaling in a larger window of early seedling development. We therefore analyzed *ZAT12* and *FIT* expression at different growth stages of wild-type plants. 5, 7, 8, and 10-day old seedlings were grown in Hoagland medium (+/- Fe) and treated with +/- H<sub>2</sub>O<sub>2</sub>. The results shown in Fig. 4.4 revealed a correlation between *ZAT12* and *FIT* expression. From time point day 5 to day 8, at the iron deficiency condition *FIT* was induced regularly, but repressed dramatically after H<sub>2</sub>O<sub>2</sub> application, while *ZAT12* expressed nearly constantly or changed slightly under H<sub>2</sub>O<sub>2</sub> treated condition. Interestingly, on day 10, *ZAT12* was strongly induced, even more so in response to H<sub>2</sub>O<sub>2</sub>. At the same time, *FIT* expression under -Fe was significantly repressed. Under sufficient iron supply, *FIT* gene expressed a lower extent as usual. Therefore, H<sub>2</sub>O<sub>2</sub> and presence of *ZAT12* have negative effect on *FIT* gene expression, especially at the 10 days old stage (Fig. 4.4A, C). One possible explanation is that *ZAT12* acts as a repressor of *FIT* in the presence of H<sub>2</sub>O<sub>2</sub>.

To confirm this result, an expression analysis was performed on the *zat12-3* knock-out mutants. *FIT* expression was stronger in the *zat12-3* mutants under iron deficiency compared to Col-0 and the decline of expression at the 10<sup>th</sup> day could not be observed in the absence of *ZAT12* (Fig. 4.4B).



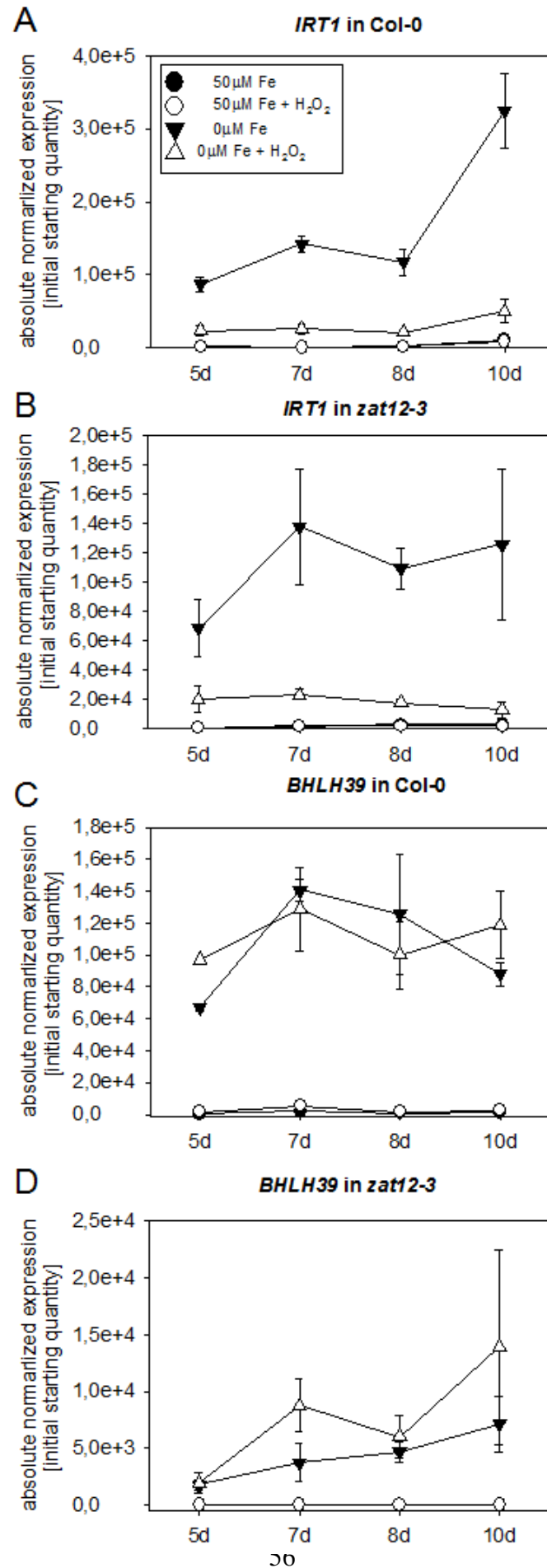


**Fig.4.4. H<sub>2</sub>O<sub>2</sub> affects transcriptional level of ZAT12 and FIT. (n=3)**

(A, B) Effect of H<sub>2</sub>O<sub>2</sub> on the *FIT* mRNA abundance in Col-0 and *zat12-3*. (C, D) Effect of H<sub>2</sub>O<sub>2</sub> on the *ZAT12* mRNA abundance in Col-0 and *zat12-3*. Arabidopsis seedlings were germinated and grown in agar Hoagland medium adding Fe (50μM Fe) or not (0μM Fe) for 5, 7, 8 and 10 day before treated with 20 mM H<sub>2</sub>O<sub>2</sub> for 1 h or not for control. Seedlings were collected and determined mRNA accumulation by RT-qPCR. Values and standard deviations were obtained from three independent experiments (nd. means not detected).

**4.1.4 Analysis of Fe-acquisition gene expression in response to Fe deficiency and oxidative stress by Reverse Transcription Real-time PCR**

Evidence from recent years shows that in order to regulate its target genes, such as *IRT1* encoding the principal iron importer, FIT may require at least one of the four Group Ib bHLH proteins bHLH038, bHLH039, bHLH100 and bHLH101 (Wang et al., 2007; Yuan et al., 2008; Wang et al., 2012; Sivitz et al., 2012). A recent study using multiple mutant combinations showed that among these four partially redundant proteins, bHLH39 plays the most significant role in regulation of iron uptake (Wang et al., 2012). Therefore, the expression of *BHLH039* gene in response to iron and oxidative stress was tested in wild-type and *zat12-3* mutant plants by quantitative RT-PCR. In addition, we tested the expression of *IRT1* as a target of FIT and bHLH039. The results of this experiment are shown in Fig.4.5.



**Fig.4.5. Effect of *FIT* repression on genes related to Fe uptake. (n=3)**

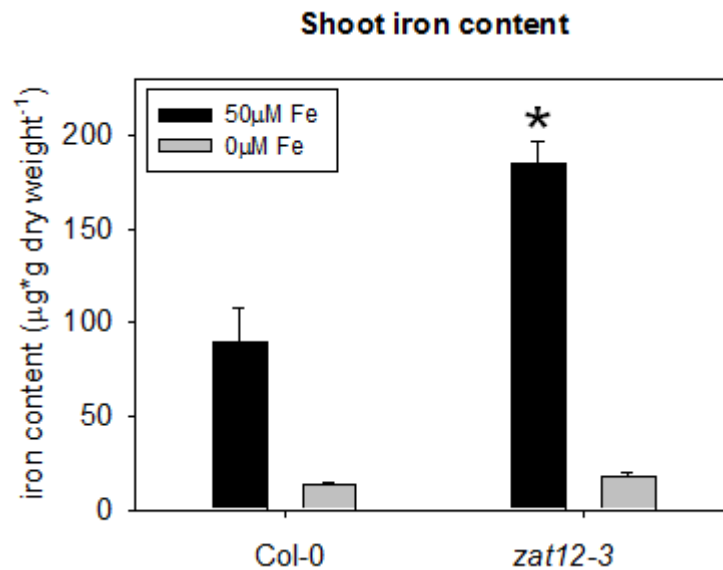
(A, B) Effect on the *IRT1* transcription in Col-0 and *zat12-3*. (C, D) Effect on *BHLH39* transcription in Col-0 and *zat12-3*. Arabidopsis seedlings were germinated and grown in agar Hoagland medium adding Fe (50 $\mu$ M Fe) or not (0 $\mu$ M Fe) for 5, 7, 8 and 10 day before treated with 20 mM H<sub>2</sub>O<sub>2</sub> for 1 h or not for control. Seedlings were collected and determined mRNA accumulation by RT-qPCR. Error bar represents the standard deviation.

Both genes were induced upon Fe deficiency but unlike *FIT* expression levels of *BHLH39* in *zat12-3* mutant were lower than those in wild-type. This shows that the negative regulation of *ZAT12* is specific to *FIT*, but not other iron-related *BHLH* genes. *IRT1* levels were comparable between the two genotypes, with the exception of day 10. There, the *zat12-3* mutant failed to further increase *IRT1* expression to the levels seen in the wild-type.

It seems that *bHLH039* might play a role in balancing iron deficiency responses. Thus, in *zat12-3* the upregulation of *FIT* is counteracted by the downregulation of *BHLH039* and the net result is a stable expression of the target *IRT1* until day 10.

**4.1.5 Determination of Fe contents in *zat12* knock-out mutants**

Because at the 10<sup>th</sup> day *IRT1* expression under iron deficiency became lower in the *zat12-3* mutant, we wanted to understand how this reflects the ability of the mutant to take up iron. Direct Fe measurement was performed on the shoots of 10-day old Col-0 and *zat12-3* seedlings. The result shown on Fig. 4.6 shows that the iron content in Col-0 was high under normal iron supply and decreased dramatically in plants, grown under iron deficiency. The tendency was the same in shoots of *zat12-3* mutant plants, however, under sufficient iron supply they contained approximate 2 fold more Fe than Col-0. The iron content under iron deficiency was comparable in both genotypes. The result shows that the main consequence of *ZAT12* absence occurs already at normal iron supply, where the activity of the iron uptake machinery is normally suppressed to avoid metal overaccumulation. Therefore, *ZAT12* can be considered a negative regulator of iron uptake. A similar conclusion can be drawn for the iron deficiency situation, however in this case *ZAT12* seems to be required at a much later stage – after 10 days of deficiency. As the metal determination reflects the whole period of seedling growth, it can be expected that iron content under iron deficiency will be equal for both genotypes. A difference might be observed only at a later stage; however we have chosen the 10<sup>th</sup> day as the final time point to avoid the accumulation of secondary effects and phenotypes due to prolonged treatment.



**Fig.4.6. Determination of Fe content in shoots of *zat12-3* and wild-type seedlings. (n=4)**

Col-0 and *zat12-3* were germinated and grown following 10-day seedlings growth system. Shoots of those plants were harvested and determined iron contents by direct solid sampling graphite furnace absorption spectrometry (GF AAS 6; Analytik Jena) at the Leibniz Institute für Neue Materialien (INM, Saarbrücken). Error bars represent standard deviation of four independent experiments. T-test was used to show significant differences ( $p < 0.05$ ). \* indicates significant change between Col-0 and *zat12-3* in Fe sufficient condition ( $p < 0.05$ ).

## 4.2 ROS signaling

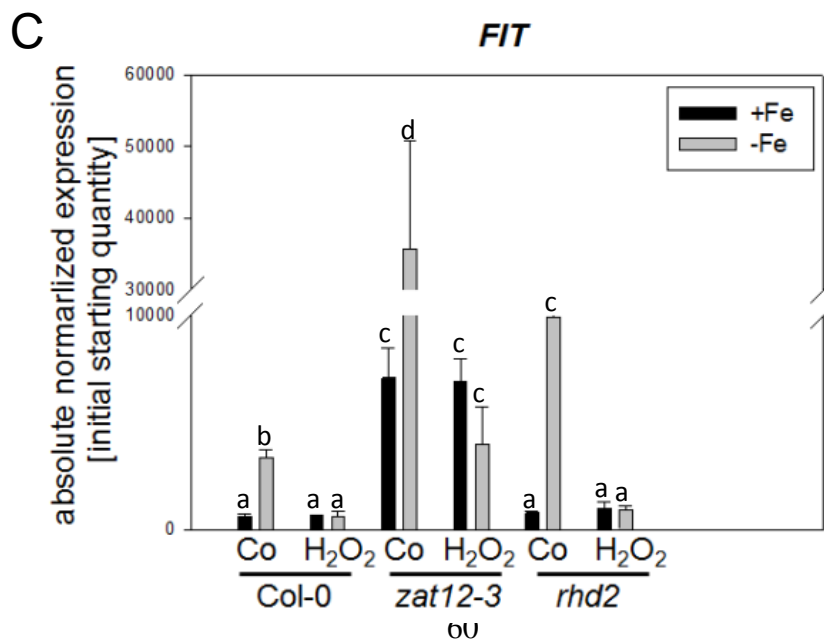
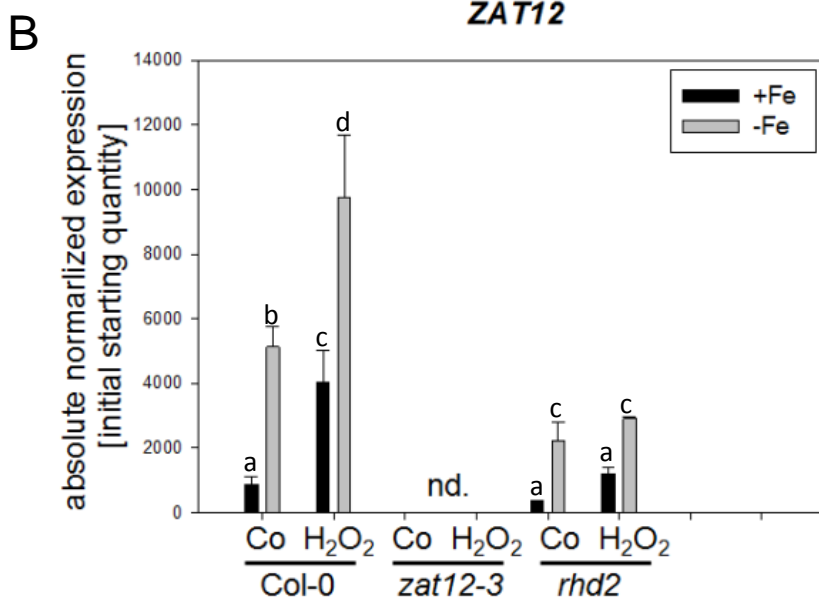
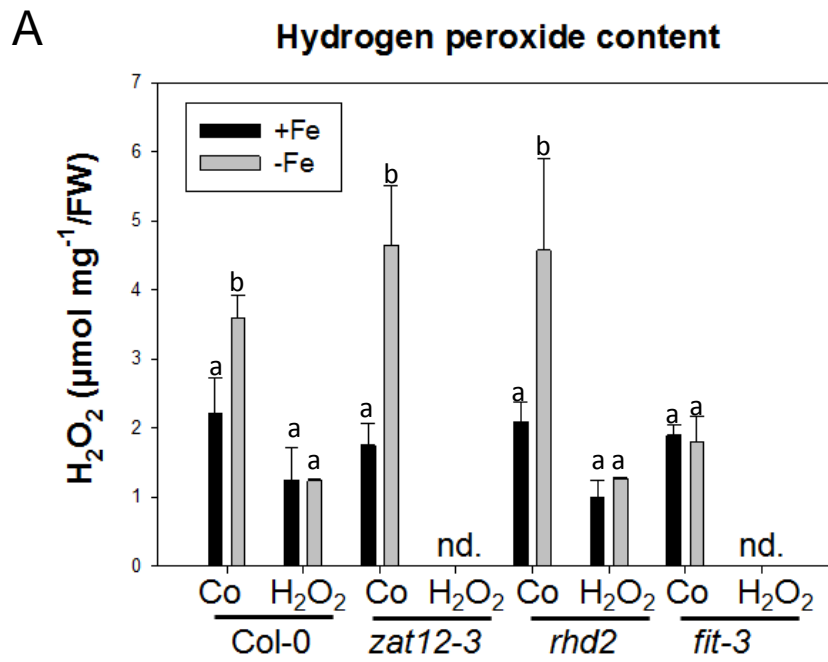
### 4.2.1 H<sub>2</sub>O<sub>2</sub> modulates gene expression under Fe deficiency and H<sub>2</sub>O<sub>2</sub> stress

Enhanced H<sub>2</sub>O<sub>2</sub> production was under nutrient deprivation such as potassium, nitrogen and phosphorus has been previously reported (Shin et al., 2005). This led to the interpretation that H<sub>2</sub>O<sub>2</sub> may be involved in cellular signaling in response to nutrient starvations.

To determine whether H<sub>2</sub>O<sub>2</sub> plays a signaling role under iron deficiency, we measured the level of H<sub>2</sub>O<sub>2</sub> production under iron deficiency in Col-0, *zat12-3* and *fit-3* seedlings. In addition, we used *rhd2* mutant seedlings, which lack *RHOB C*, an iron-dependent root hair-specific NADPH oxidase. *RHOB C* functions in transferring electrons from NADPH to an electron acceptor, thus leading to the formation of reactive oxygen species (ROS). ROS levels were shown to be markedly decreased in the *rhd2* mutant (Foreman et al., 2003). H<sub>2</sub>O<sub>2</sub> concentrations in roots of Col-0, *zat12-3* and *rhd2* increased after depriving plants of iron for 10 days (Fig.4.7A). So H<sub>2</sub>O<sub>2</sub> may potentially be used as an intermediate Fe-response signal. Interestingly, H<sub>2</sub>O<sub>2</sub> production was lower in response to Fe deficiency in *fit-3* mutant than in Col-0, suggesting that *FIT* is involved in iron deficiency induced H<sub>2</sub>O<sub>2</sub> production (Fig. 4.7A). We did not observe the reported decrease of H<sub>2</sub>O<sub>2</sub> levels in *rhd2* mutant. A probable

explanation is the different age of the seedlings, which we use. We also treated roots in each condition with external  $H_2O_2$ . The measured  $H_2O_2$  concentrations in these samples were surprisingly comparable or lower compared to the corresponding untreated condition (Fig. 4.7A). It seems that exogenous application of  $H_2O_2$  resulted in unbalancing the redox state of the plant cell and triggered a set of  $H_2O_2$  scavenging enzymes. In term of gene expression, although  $H_2O_2$  content decreased after external  $H_2O_2$  application, *ZAT12* was still induced by  $H_2O_2$  in Col-0 under Fe starvation, but not in *rhd2* mutant (Fig. 4.7B). This suggests that *RBOHC* plays a role in signaling transduction and  $H_2O_2$  produced by it may control *ZAT12* expression. We also observed that *FIT* was repressed by  $H_2O_2$  in the absence of *ZAT12*. This means that there exists a *ZAT12*-independent mechanism for *FIT* down-regulation by  $H_2O_2$  (Fig.4.7C).

Taken together,  $H_2O_2$  is a signaling molecule of iron deficiency; in turn this signal is amplified and relayed to regulate *ZAT12* expression by *RBOHC*.



**Fig.4.7. H<sub>2</sub>O<sub>2</sub> content (A) and changed expression level of ZAT12 (B) and FIT (C) in response Fe deficiency in *fit-3*, *rhd2*, *zat12-3* knock-out mutant and Col-0 plants. (n=3)**

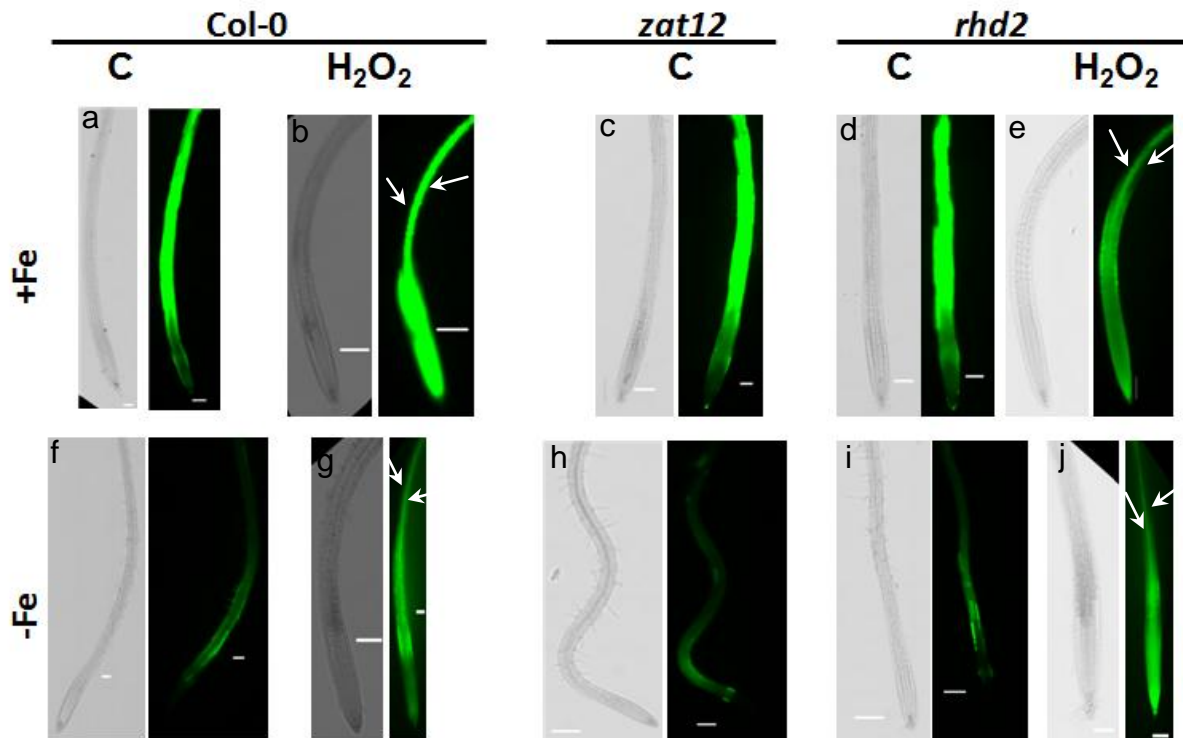
Col-0, *zat12-3*, *rhd2* and *fit-3* were subjected 20mM H<sub>2</sub>O<sub>2</sub> after germinating and growing following 10-day seedlings growth system (Co means control without H<sub>2</sub>O<sub>2</sub>). Seedlings of those plants were harvested for gene expression and hydrogen peroxide analysis. Error bars represent the SD of 3 separated experiments. (nd. in (A)= no determination; nd. in (B)= not detected). In each figure, means with the same letter are not significantly different from each other at P < 0.05

**4.2.2 ROS localization in iron-deficient roots**

H<sub>2</sub>O<sub>2</sub> is only a kind of ROS. In the presence of Fe, the different other ROS are produced from H<sub>2</sub>O<sub>2</sub> as a result of the Fenton reaction. Therefore, we also conducted ROS localization studies using a membrane permeable fluorescent probe CM-H<sub>2</sub>DCFDA that mainly detects the ROS species including HO<sup>-</sup>, ROO<sup>-</sup>, ONOO<sup>-</sup> and H<sub>2</sub>O<sub>2</sub>. CM-H<sub>2</sub>DCFDA was loaded into root cells and then the ROS localization was observed under fluorescence microscope.

To determine where ROS were produced in response to iron deficiency and/or H<sub>2</sub>O<sub>2</sub> application, seedlings of Col-0, *zat12-3* and *rhd2* mutant grown on Fe deficient condition for 10 days would be subjected with 20 mM H<sub>2</sub>O<sub>2</sub> for 1h and finally incubated with 50 μM CM-H<sub>2</sub>DCFDA. Experiment was performed three replicates per each condition. Results shown in Fig. 4.8 indicate that only small amounts of ROS could be visualized in roots of Col-0, *zat12-3* and *rhd2* mutant seedlings under Fe deficiency (Fig.4.8 f, h, i). However, under sufficient Fe conditions, there was an increase in ROS dye reaction product as compared with plants grown under iron deficiency (Fig. 4.8 a, c, d). And after treating H<sub>2</sub>O<sub>2</sub>, ROS dye reaction product was observed in root tip and stele regardless of genotypes and Fe supply (Fig. 4.8 b, e, g, j). This result is accordance with cell type-specific microarray analysis of Dinneny et al. (2008) in Fe-deficient *Arabidopsis* roots. Dinneny et al., 2008 showed genes involved in signaling and stress were enriched among the stele-activated genes (Dinneny et al., 2008). Therefore, ROS may also relate to Fe uptake and oxidative stress signaling.





**Fig.4.8. Localization of ROS in Arabidopsis roots during Fe deficient condition.**

Col-0, *zat12-3* and *rhd2* were germinated and grown Fe sufficiency (+Fe) and Fe deficiency (-Fe) following 10-day seedlings growth system, then subjected 20mM  $H_2O_2$  ( $H_2O_2$ ) or not for control (C). Fluorescence indicated the presence of ROS under fluorescent microscope after seedlings were loaded with 50  $\mu$ M CM-H2DCFDA (white arrows indicated ROS signal in the stele of root). Six roots for each treatment showed similar results (Scale bar =100 $\mu$ m).

### 4.3 Investigation ZAT12 protein regulation in response to Fe supply and $H_2O_2$ stress by generating tagged protein transgenic lines

#### 4.3.1 Generation and characterization of GFP tagged ZAT12 lines regulated by its own promoter

We observed that *ZAT12* gene was induced by  $H_2O_2$  produced RBOHC and along with ROS repressed *FIT* expression. This causes increased expression of *FIT* in *zat12-3* mutant. But the *zat12-3* mutant failed to further increase *IRT1* expression to the levels seen in the wild type. As we known *FIT* is required to induce *IRT1* and *FRO2* expression in response to Fe deficiency. Therefore, it is interesting to study protein regulation of *ZAT12* here. To monitor *ZAT12* protein abundance in respect to Fe supply, we generated Green Fluorescence Protein (GFP)-tagged *ZAT12* transgenic lines regulated by its native promoter. Such transgenic lines have been useful to reveal protein stability regulation in many studies since the protein under investigation can be easily monitored through the immunogenic GFP tag using specific commercial monoclonal antibodies (Youvan, 1995; Prasher et al., 1992; Chalfie et al., 1995).

The fusion constructs were generated using the Invitrogen Gateway cloning technology. The cloning scheme is showed in Methods. The final destination vectors contain a pZAT12:*ZAT12-GFP* fusion in the pMDC107 backbone (Curtis & Grossniklaus, 2003).

#### **4.3.2 Verification of generated ZAT12 reporter constructs by transient transformation of tobacco leaves**

The expression of pZAT12:*ZAT12-GFP* fusion protein was verified with by transient transformation of *Agrobacterium tumefaciens*-mediated infiltration of tobacco leaves. Green fluorescent protein (GFP) signals of tobacco leaves were observed two days after infiltration under the fluorescence microscope. As shown Fig.4.9A, the GFP signals were observed in nuclei of tobacco leaf cells partly indicating that successful expression of construct and ZAT12 activities take place in the nucleus of plant cell. To confirm further the expression of this fusion protein in correct size, we also verified it by Immunoblot with anti-GFP antibodies. After observing under microscope, tobacco leaves infiltrating pZAT12:*ZAT12-GFP* constructs were harvested to conduct Immunoblot with anti-GFP antibodies. Result in Fig.4.9B showed ZAT12-GFP fusion protein possessed electrophoretic mobility corresponding well to the predicted molecular weight of 48 kDa.

#### **4.3.3 Analysis of ZAT12 protein abundance and localization in roots of stably transformed transgenic Arabidopsis plant lines**

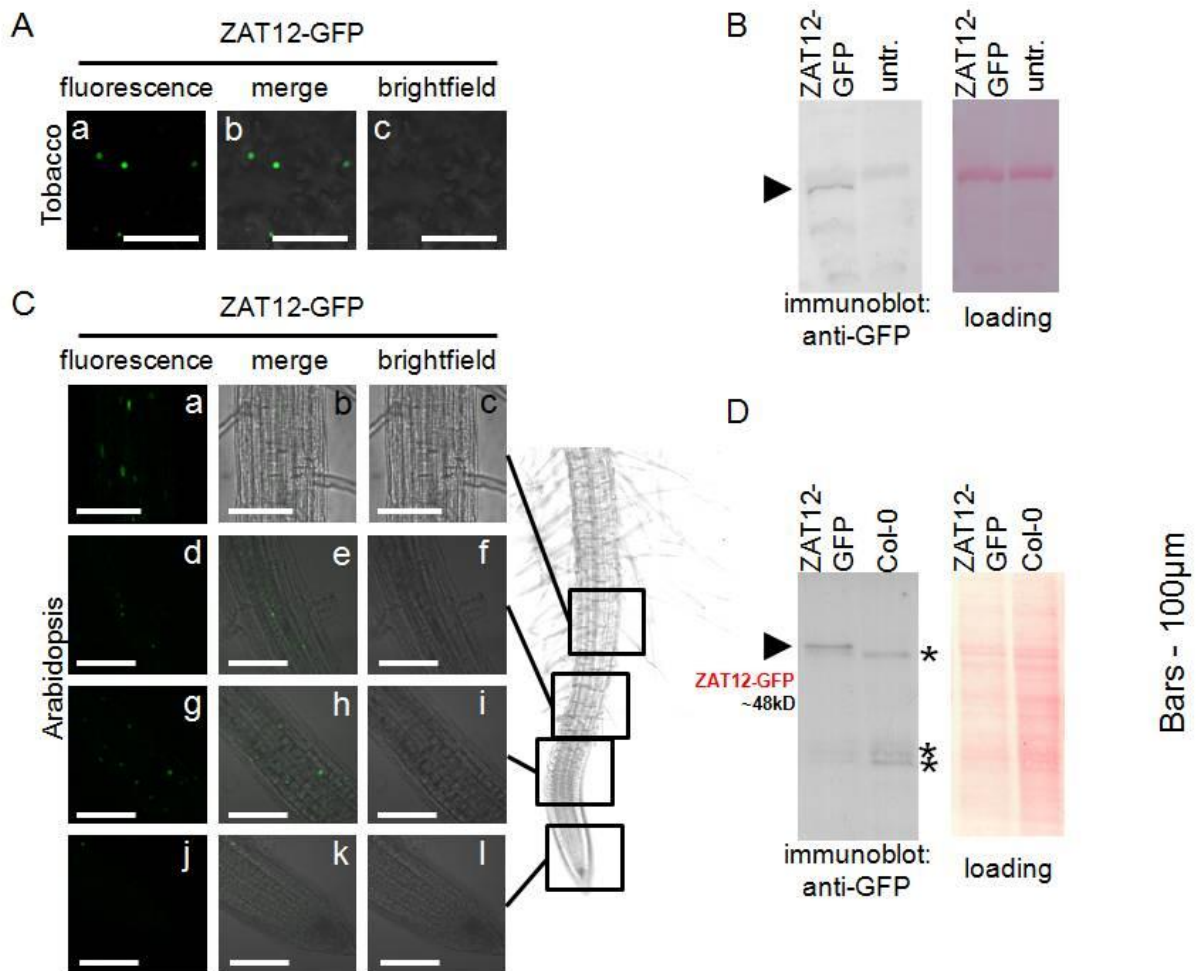
To investigate functionality of GFP tagged ZAT12 protein downstream Fe deficiency and H<sub>2</sub>O<sub>2</sub> stress response, we transformed pZAT12:*ZAT12-GFP* construct into wild-type Arabidopsis plants.

Transformants were screened by Hygromycin selection and genotyping to obtain homozygous lines. Four transformants were obtained with pZAT12:*ZAT12-GFP* construct.

Firstly, transformants were confirmed under confocal microscope. This could also show ZAT12 fusion protein expressed in the nuclei of Arabidopsis root cells starting from the elongation zone and going upwards through the root hair zone (Fig.4.9C). Secondly, we detected ZAT12-GFP fusion protein in transformed Arabidopsis seedlings by Immunoblot with anti-GFP (Fig.4.9D). This demonstrated that ZAT12-GFP expressed successfully in Arabidopsis under regulation of its native promoter. This line is a useful tool for further studies of ZAT12 function and stability related to H<sub>2</sub>O<sub>2</sub> stress and Fe deficiency.

In order to determine how ZAT12-GFP fusion proteins respond to Fe supply and H<sub>2</sub>O<sub>2</sub> stress, we applied seedling growth system at the time point of 10 days when *ZAT12* was upregulated

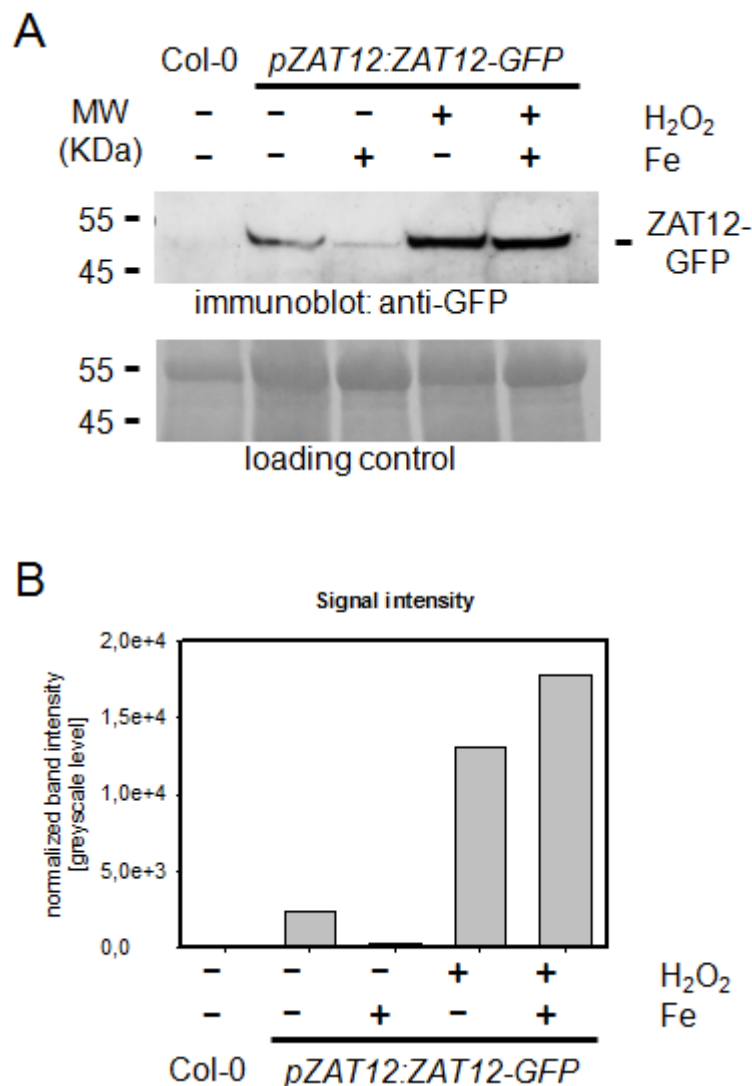
at transcriptional level. The results shown in Fig.4.10 indicate that GFP tagged ZAT12 proteins were induced by Fe deficiency and their abundance increases in response to H<sub>2</sub>O<sub>2</sub> stress after 3h treatment; regardless of iron supply. This is consistent with result of *ZAT12* gene expression. So that ZAT12 protein also induced by Fe deficiency and oxidative stress.



**Fig.4.9. Verification of ZAT12-GFP fusion protein in transiently transformed tobacco leaves and stably transformed Arabidopsis roots.**

Tobacco leaves were infiltrated with a solution of Agrobacteria containing the reporter construct pZAT12:*ZAT12-GFP*. After two days expression of GFP fusion proteins has been observed under confocal microscope (A) and detected by Immunoblot with anti-GFP (B). In transgenic Arabidopsis were verified by GFP signal occurring in nuclei of root cells (C) and Immunoblot with anti-GFP (D). GFP signal were detected on the left column (a, tobacco) and (a, d, g, g, Arabiopsis) ; right column [(c, tobacco) and (c, f, I, j, l, Arabidopsis), differential interference contrast (DIC) microscopy; middle column [(b, tobacco); and (b, e, h, k, Arabidopsis)], merge of fluorescent signal and DIC Arrowhead indicates ZAT12-GFP fusion protein with the predicted molecular weight of 48 kDa. \* shows unspecific protein bands.

GFP fluorescence was observed by confocal microscopy at 500-530 nm and identity of true signal was confirmed with a lambda scan.



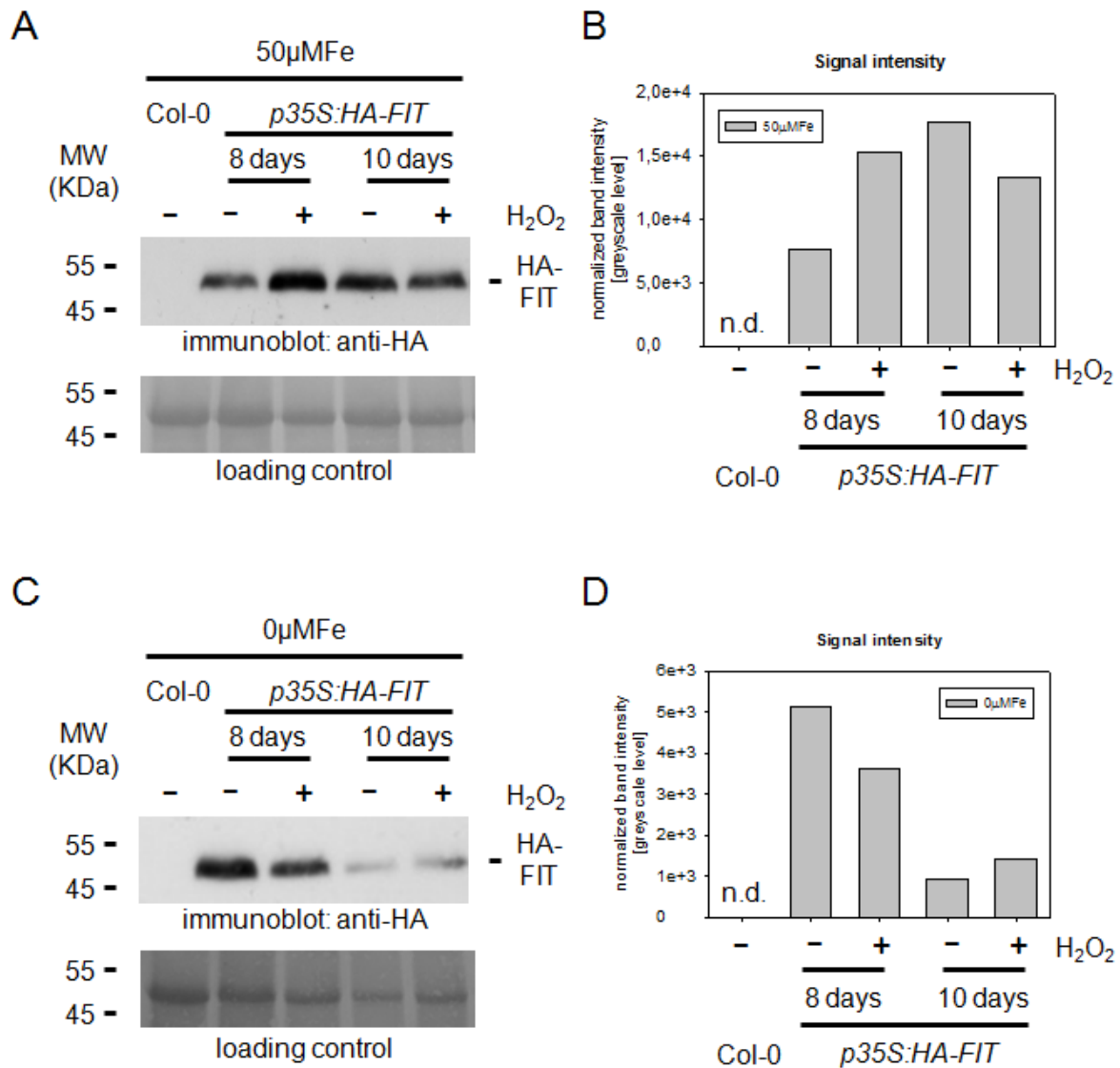
**Fig.4.10. ZAT12-GFP protein quantity in Col-0 background in response to Fe deficiency and H<sub>2</sub>O<sub>2</sub> stress.**

A, Immunoblot analysis using anti-GFP antibody. Arabidopsis homozygous lines containing pZAT12:ZAT12-GFP construct grown following 10-day seedling growth system were treated with 20mM H<sub>2</sub>O<sub>2</sub> for 1h, and harvested for immunoblot analysis using anti-GFP antibody in 3h later. The Ponceus staining control is presented below. B, Quantification of band intensities by measuring grey scale pixel quantity using Image J.

#### 4.3.4 Analysis of FIT protein abundance under Fe deficiency and H<sub>2</sub>O<sub>2</sub> stress in roots of stable transgenic Arabidopsis plant lines

We found that H<sub>2</sub>O<sub>2</sub> treatment causes strong repression of *FIT* gene expression and therefore we wanted to know if this effect is mirrored on protein level. To address this question, we performed timecourse experiments of Western Blot with 8- and 10-day old HA tagged FIT

expressing seedlings (HA-FIT9 lines provided by Dr. J. Meiser, Meiser et al., 2011) using anti-HA antibody. Results presented in Fig.4.11 show that FIT stability and responses change with the time of iron deficiency treatment. After 8 days of sufficient iron supply HA-FIT abundance was higher in the H<sub>2</sub>O<sub>2</sub>-treated sample but in comparison at the same time point in plants grown under iron-deficient conditions, the response was the opposite. Such opposite regulation is not surprising in the light of the finding that the general stability of HA-FIT is decreased under iron deficiency (Meiser et al., 2011). After 10 days, the levels of HA-FIT were lower in comparison to 8 days and the effect of H<sub>2</sub>O<sub>2</sub> was much weaker. Nevertheless, one could observe the dramatic decrease in the HA signal under iron deficiency at 10 day compared to 8 day, which corresponds well to the transcriptional regulation of FIT. As the HA-FIT expression is controlled by a constitutive promoter, this effect should only be due to post-transcriptional regulation.



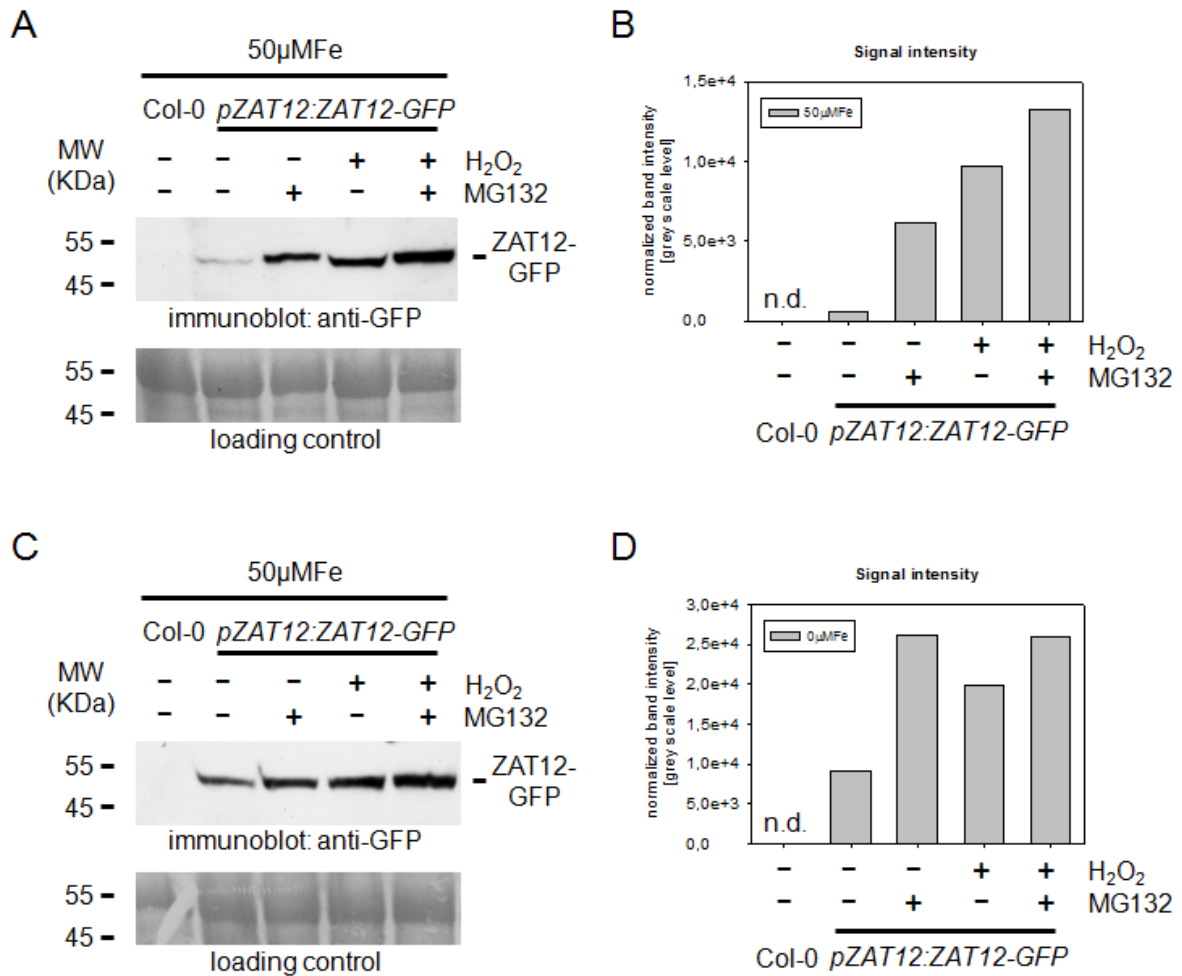
**Fig. 4.11. FIT protein abundance. (n=2)**

Immunoblot analysis using anti-HA antibody and quantification of band intensities under +Fe (A, B, respectively) and -Fe (C, D, respectively). FIT overexpress lines containing *p35S:FIT-HA* construct were grown on Fe sufficient (+Fe) and deprivation (-Fe) according to 8-and 10-day seedling growth system, and harvested for Immunoblot with anti-HA antibody after H<sub>2</sub>O<sub>2</sub> treatment. The Ponceau S staining control is presented below.

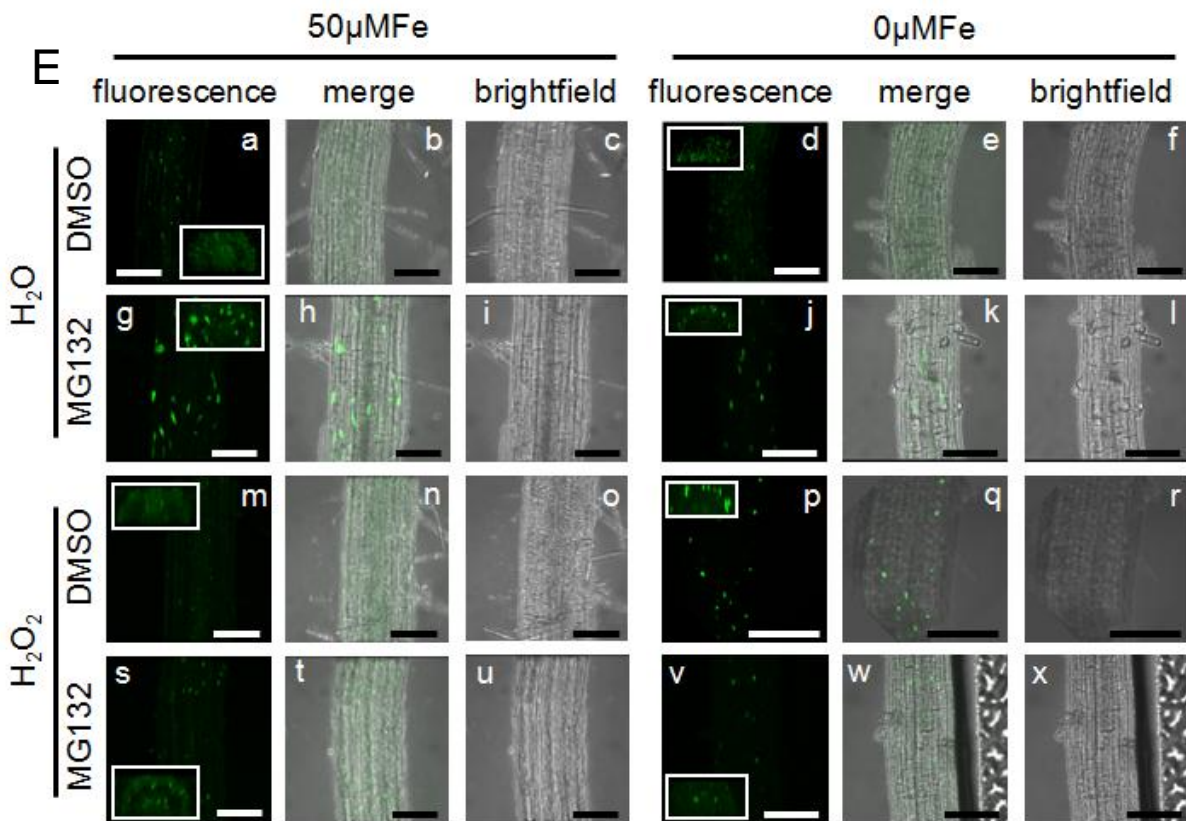
#### 4.3.5 Localization and stability of ZAT12-GFP in roots of stable transgenic Arabidopsis plant lines

Recent research demonstrated changes in the endogenous concentration of stress hormones might result in specific degradation of EAR-repressors through the proteasome pathway. For instance, by degrading the auxin/indole-3-acetic acid (AUX/IAA) proteins containing EAR motif were inactive results in the transcriptional activation of auxin-responsive genes (Tao et al., 2005).

Meiser et al., 2011 showed FIT protein abundance was controlled and is subject of a turnover control. The above results showed that ZAT12 interacts with FIT and in turn now repress FIT activity. In order to investigate stability of ZAT12, we treated 10-day old *pZAT12:ZAT12-GFP* transgenic plants with the proteasome inhibitor MG132 after subjecting these plants with 20mM H<sub>2</sub>O<sub>2</sub> for 1h. Seedlings were then observed under confocal microscope and collected for Immunoblot with anti-GFP antibodies.







**Fig.4.12. ZAT12 protein abundance after Fe deficiency and H<sub>2</sub>O<sub>2</sub> stress with application of proteasome inhibitor-MG132.**

Immunoblot analysis using anti-GFP antibody and quantification of band intensities of Arabidopsis lines containing pZAT12:ZAT12-GFP grown in the Fe sufficiency (A, B, respectively) and Fe deficiency (C, D, respectively). Those 10-day old seedlings grown at +Fe/-Fe were treated 20mM H<sub>2</sub>O<sub>2</sub> and 42µM MG132 (proteasome inhibitor) for 1h and 3h respectively. The whole seedlings were harvested for Immunoblot with anti-GFP antibody. The PonceuS staining controls are presented below.

**E**, Images of GFP signal were detected under confocal microscope in the roots after MG132 application. Left part and right part indicate GFP fluorescent signal detection in roots of seedling grown in standard and Fe deficient condition, respectively for 10days; Two upper rows and two lower rows represent GFP signal of H<sub>2</sub>O<sub>2</sub> untreated and treated root, respectively. The first and the third row show GFP signal observed in root for control. The second and the fourth rows show GFP signal detected in root after MG132 treatment. In each part, left column [(a, g, m, and s) or (d, j, p, and v)], GFP fluorescent signal; right column [(c, i, o, and u) or (f, l, r, and x)], differential interference contrast (DIC) microscopy; middle column [(b, h, n, and t) or (e, k, q, and w)], merge of fluorescent signal and DIC. Small rectangular showed the cross-section of root.

The results shown in Fig.4.12A indicate that ZAT12 abundance increased after MG132 treatment, compared to untreated seedlings in both Fe supply conditions and even after H<sub>2</sub>O<sub>2</sub> stress. This result could also be observed on tissue level, following the GFP fluorescence. The increase of GFP signal after H<sub>2</sub>O<sub>2</sub> and MG132 treatment was most prominent in the epidermal



layer of the root. As this is the place, where iron uptake occurs, it can be speculated that in the epidermis ZAT12 turnover rate is very high and therefore, there is a requirement of large amounts of freshly synthesized protein (Fig.4.12B).

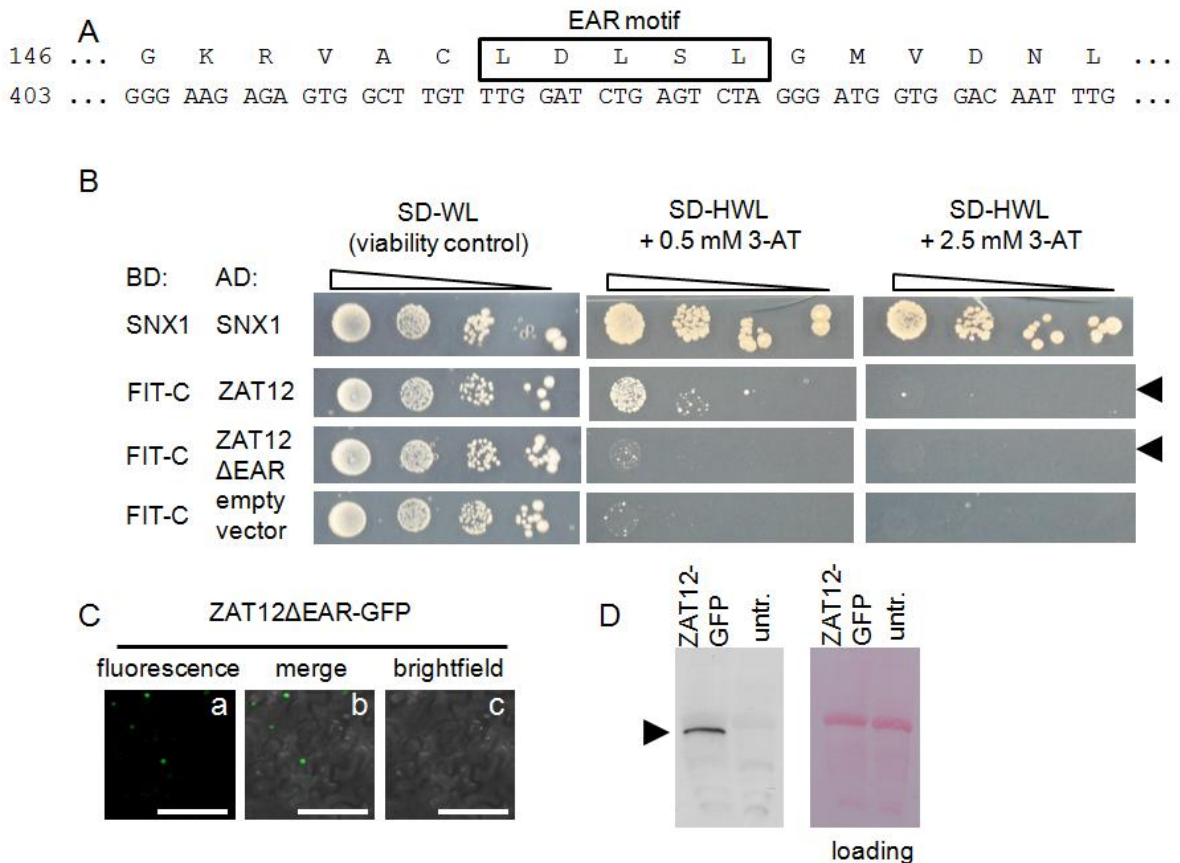
#### 4.4 Identification of functional core site in ZAT12

##### 4.4.1 Identification of core site interacted with FIT in ZAT12

The Ethylene responsive element binding factor-associated Amphiphilic Repression (EAR) motif is a transcriptional regulatory motif which acts as repressor of genes involved in developmental, hormonal and stress signaling pathways (Kagale et al., 2010). It is found in many transcription factor families and transcriptional regulators such as: ERFs (8 proteins; (Ohta et al., 2001; Mcgrath et al., 2005; Yang et al., 2005), ZFPs (4 proteins including ZAT12; (Ohta et al., 2001; Sakamoto et al., 2004; Mittler et al., 2006), etc.. Comparison of protein sequence in the core site of these proteins revealed two different conserved sequences: LxLxL and DLNxxP. Deletion of EAR motif in ZAT10 and ZAT11 abolished their transcriptional repression capacity (Ohta et al., 2001). Therefore, we asked whether ZAT12 repression function would be changed with the absence of this motif. To address this question, we first generated transgenic lines expressing an EAR-motif deleted ZAT12 variant (ZAT12 $\Delta$ ) regulated by its ZAT12 native promoter. The core amino acid sequence of the EAR motif in ZAT12 is LDLSL. Using PCR, we truncated that sequence (422-437) from ZAT12 full sequence (Figure 4.13 A). These PCR products were integrated as GFP fusion into pMDC107 vector (Curtis & Grossniklaus, 2003).

Construction and expression was first verified by observation of GFP signal in tobacco leaves under confocal microscope and by conduction of Immunoblot with anti-GFP antibody (Fig.4.13C and D).

As shown in Fig.4.13C, ZAT12 $\Delta$ -GFP fusion proteins also expressed in the nuclei of tobacco leaf cells. The size of these fusion proteins was correct (Fig.4.13D).



**Fig.4.13. ZAT12ΔEAR-GFP construct.**

A. DNA fragment used to generate the EAR motif deleted ZAT12 lines (ZAT12Δ). B. Yeast Protein Interaction of FIT with truncated ZAT12. 10-fold dilutions ( $A_{600}$  of  $1-10^{-4}$ ) of the yeast AH109 strain containing either an activation domain (AD)-ZAT12Δ or an empty DNA-AD-vector and binding domain (BD)-FIT-C constructs were spotted on SD selection media lacking Leu and Trp (-WL; selection for positive transformants) or Leu, Trp, and His (-HWL and 2.5 or 0.5 mM 3-AT; selection for interaction) and incubated for 14 days at 30 °C. Positive control is interaction between SNXs (Pourcher et al., 2010). This work was performed with Dr. T. Brumbarova; C, D Verification of successfully transformation and expression by tobacco infiltration (C) and Immunoblot using anti-GFP antibody (D).

After obtaining a construct containing pZAT12:ZAT12Δ-GFP, we conducted a targeted yeast two-hybrid analysis using FIT-C as bait and empty vector or vector containing EAR-motif deleted ZAT12 as preys.

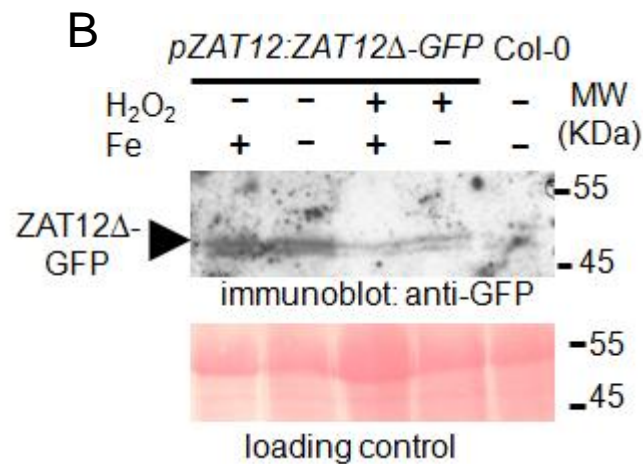
Result in Fig.4.13B shows that there is no interaction between FIT-C and the EAR domain lacking ZAT12. As positive control, we also implemented to confirm interaction between FIT and ZAT12 in parallel. This suggests that the EAR motif of ZAT12 is the site where interaction with FIT occurs.

#### 4.4.2 Functional characterization of ZAT12Δ

The EAR motif is found to play a role as a repressor in these proteins containing it. We asked if the role of this motif in ZAT12 function in response to Fe supply and H<sub>2</sub>O<sub>2</sub> stress. To do this aim, we transformed pZAT12:ZAT12Δ-GFP constructs in Arabidopsis wild-type plants.

Transformants were selected by Hygromycin until obtaining homozygous lines to do further experiment. As a result, we obtained 8 lines containing pZAT12:ZAT12Δ-GFP. These plants were first analyzed at morphological level. Plants grown under regular conditions in soil exhibit reddish brown, upward-pointing curly leaves, late flowering compared to Col-0. However, there was different from line to line (Fig. 4.14 A). Subsequently, pZAT12:ZAT12Δ-GFP containing, homozygous lines were grown follow to seedlings growth system for 10 days, treated with H<sub>2</sub>O<sub>2</sub> and harvested for Immunoblot experiment with anti-GFP antibody. The result is shown in Fig.4.14 B. After H<sub>2</sub>O<sub>2</sub> application, quantity of fusion protein decreased in comparison with those in untreated plants. Under Fe deprivation, abundance of ZAT12Δ-GFP was lower than that under Fe sufficiency. It is possible that the EAR motif plays a stabilizing role for ZAT12 under Fe deficiency and H<sub>2</sub>O<sub>2</sub> stress. Therefore, deletion of EAR motif impaired functionality of ZAT12. This led to degradation of ZAT12 protein. However, the result needs to be confirmed.



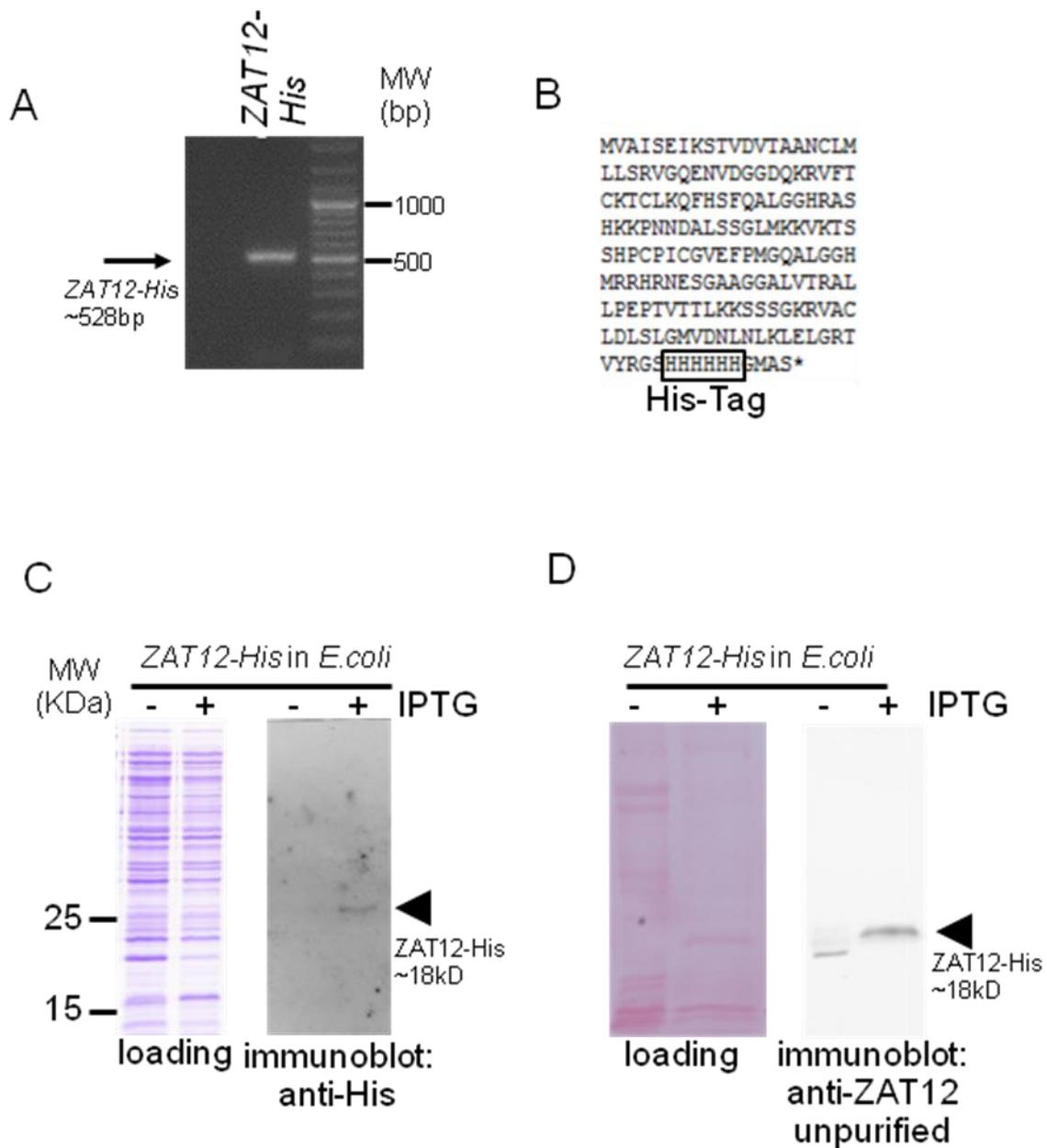


**Fig.4.14. Function of EAR motif in response to Fe deficiency and  $H_2O_2$  stress. (n=1)**

A, Effect of deletion the EAR motif in ZAT12 on plant morphology. (Scale bar = 1cm). B, Immunoblot analysis using anti-GFP monoclonal antibody; the PonceuS-stained image serves as loading control is presented below. ZAT12Δ-GFP lines in wild-type background were grown in the 10-day agar seedling growth system, and then treated 20mM  $H_2O_2$ , and harvested for Immunoblot

#### 4.5 ZAT12 antibody preparation

Although fusion proteins with tags were reported to be good tools to evaluate protein regulation in *planta*, it is more convincing when studies of protein regulation using immunoblot are conducted in parallel with antibodies against the native proteins. We generated a specific polyclonal affinity-purified antiserum directed against the N-terminal peptide of ZAT12. By using PCR, ZAT12 was attached with His Tag at 3' end for purification later, and cloned into pETBlue-2 for protein expression in *E.coli* (Fig.4.15A,B). After verification of sequences and orientation (Fig.4.15B), small culture of positive clone was prepared for induction to express protein using IPTG. To confirm successful expression of ZAT12-His in *E.coli*, we performed SDS-PAGE and Immunoblot using the anti-His antibody. The results shown in Fig.4.15C indicated the corrected ZAT12-His fusion protein band (expected molecular weight = 18kDa). At the same time, peptide was injected into rat to obtain a polyclonal antiserum. The specificity of the unpurified antiserum anti-ZAT12 was tested with ZAT12-His protein expressed in *E.coli*. The result showed the highly specific ZAT12 antiserum (Fig.4.15D). This partly demonstrated success in generation ZAT12 antibody. However, for further use, the antiserum needed to be purified. Because of time, this result will be continued later.



**Fig. 4.15. ZAT12 antibody preparation.**

Amplification ZAT12-His using Phusion Taq (A). Verification of successful cloning of ZAT12-His construct by sequencing (B). Confirmation of successful expression of ZAT12-His protein in *E. coli* by SDS-PAGE (on the left side) or Immunoblot with anti-His anti body (on the right side) (C). Immunological detection using antisera anti-ZAT12 (conducted by Prof. U. Müller, Zoology Department, Saarland University) (D).

## 5. Discussion

In this work, we demonstrated a direct molecular link between Fe deficiency response and oxidative stress regulators. We showed that FIT, the central transcription factor of the Fe uptake pathway, interacts with ZAT12, a zinc finger protein involved in the ROS signaling through the EAR motif.

Our results indicate that *ZAT12* level is increased in response to Fe deficiency and oxidative stress mediated by *RBOH C*. The increased ROS production upon Fe supply negatively regulates *FIT* at transcriptional level through a ZAT12-dependent mechanism. In the *zat12-3* mutant, the *BHLH39* expression level is decreased, while *FIT* and the Fe acquisition gene *IRT1* are upregulated. This repression by ZAT12 is confined to *FIT*, but not other iron-related *BHLH* genes. We propose that by controlling *FIT* expression, ZAT12 is one of the downstream factors of the ROS signaling that regulate Fe uptake responses to avoid excessive Fe. We suggest that this ZAT12-mediated influence may contribute to a crosstalk between Fe acquisition and environmental responses.

### 5.1 FIT may interact with ZAT12 through EAR motif which brings repressor activities and stability of ZAT12

ZAT12 may interact with FIT in root cells. The strongest evidence for this is provided by the fact that ZAT12 is one of 14 candidates identified to interact with FIT in the yeast-two-hybrid screening (Y2H) (Lingam et al., 2011). Later on, also using Y2H, we confirmed this interaction in yeast (Fig.4.1). ZAT12-GFP was detectable in the nuclei of root cells where FIT could also be observed, indicating that ZAT12 is in principle available for an interaction with FIT in the root cells (Fig.4.9). FIT belongs to bHLH transcription factor family containing a characteristic bHLH domain is known to build homo or heterodimers with other bHLH proteins (Murre et al., 1989; Ferre-D'Amare et al., 1994; Robinson et al., 2000; Heim et al., 2003). Recent studies demonstrated that FIT interacted with AtbHLH38 or AtbHLH39 and two homologs AtbHLH100, AtbHLH101, belong to the subgroup Ib bHLH genes to induce expression of Fe acquisition genes like *IRT1* and *FRO2* (Yuan et al., 2008; Wang et al, 2012). Besides other bHLH protein, FIT was shown to physically interact with EIN3/EIL1, the central transcription factors in the ethylene pathway, that do not contain any helix-loop-helix domain, however, EIN3 contains an EAR motif (DLNIPN, Kagale et al., 2010) at the C-terminus (Lingam et al., 2011). Therefore, we suggest that it is highly possible that FIT interacts with ZAT12 via the EAR motif. Indeed, it was found here that the EAR motif is required for the interaction in yeast. Perhaps the EAR motif is also needed for the interaction



of FIT-EIN3 in yeast. However, to date, efforts to verify the ZAT12-FIT interaction have not been successful in *planta*. We tried to conduct BiFC and Co-IP as well, but the attempts failed. Reasons could be that the interaction of FIT-ZAT12 is dependent on the presence of ROS or other Fe status and oxidative stress may cause difficulty to find situations to have enough amount of the protein complex for detecting the interaction. BiFC experiments using tobacco plants grown under regular conditions in soil or attempts with different conditions in the Co-IP experiments all did not showed any results. Perhaps, another factor is needed for interaction. For example, JAZ8 (JASMONATE ZIM), an EAR motif-containing repressor, interacts with the corepressor TOPLESS (TPL) in order to repress transcriptional activity (Shyu et al., 2012).

The EAR motif seems to involve in FIT-ZAT12 interactions. Interestingly, ZAT7, a close member in the same family of ZAT12, also failed to interact with WRKY 70 (determine the balance between SA-dependent and JA-dependent defense pathways) and HASTY (involved in miRNA transport) after deletion of the EAR motif. By interacting with EIN3/EIL1, FIT avoids being degraded through 26S proteasome (Lingam et al., 2011). The EAR-motif of ZAT7 was shown to suppress the activity of a suppressor that is a negative regulator of defense response activation during salinity stress (Ciftci-Yilmaz et al., 2007). ZAT12, whose mRNA was upregulated in response to different abiotic stresses such as wounding, cold, heat, oxidative stresses (Iida et al., 2000; Rizhsky et al., 2004; Davletova<sup>a</sup> et al., 2005), was suggested to play a major role in the response of plants to all of these stresses. However, ZAT12 gain- and loss-of-function studies suggest that ZAT12 is only involved in tolerance to a few of these stresses such as osmotic, oxidative, and salinity stresses, and even had a negative impact on tolerance of plants to heat stress (Davletova<sup>a</sup> et al., 2005). Moreover, under cold stress, ZAT12 represses the expression of *CBF1*, *CBF2* and *CBF3*, which are the key cold-stress-response transcription factors (Vogel et al., 2005). The EAR motif might make ZAT12 function as a repressor. Therefore, we proposed ZAT12 may have a repression activity on the transcription factor FIT through its EAR motif.

## **5.2 To avoid excessive Fe uptake, FIT is negatively regulated by ZAT12 in the presence of ROS**

After H<sub>2</sub>O<sub>2</sub> application, we found that ZAT12 expression is enhanced (Fig.4.2). Previously, ZAT12 expression level was demonstrated to increase in many different stress conditions including oxidative stress (Iida et al., 2000; Rizhsky et al., 2004; Davletova<sup>a</sup> et al., 2005; Vogel et al., 2005). In addition, it controls the expression of defense enzyme cytosolic

ascorbate peroxidase 1 (APX1) during oxidative stress (Rizhsky et al., 2004). In addition, *ZAT12* overexpressing plants are more tolerant to oxidative stress compared to wild-type (Davletova et al., 2005). All this suggests that *ZAT12* plays a role in response to oxidative stress. Beside oxidative stress, we found that *ZAT12* is involved in response to Fe deficiency. Here we observed that *ZAT12* is induced after 8 day-old starvation of Fe. By transcriptome analysis, Ravet et al., 2012 also found that *ZAT12* was modulated by Fe. Therefore, we strongly suggest that *ZAT12* could be a molecular link between in response to Fe responses and oxidative stress.

Here we found that both *ZAT12* and ROS are involved in repressing the expression of *FIT*. As shown Fig. 4.8, ROS production level at +Fe is higher than at -Fe. This can be explained by the interaction between H<sub>2</sub>O<sub>2</sub> and Fe via the Fenton reaction resulting in the formation of ROS (Fenton, 1894). *FIT* transcription is increased under -Fe, but not in +Fe (Fig.4.3 B; Fig. 4.4 A; Jakoby et al., 2004). Therefore, it is possible that ROS is involved in *FIT* repression in +Fe. Although ROS level is low at - Fe, H<sub>2</sub>O<sub>2</sub> production has the opposite result (Fig 4.7 A). And H<sub>2</sub>O<sub>2</sub> induces *ZAT12* transcription (Fig.4.3A; Fig.4.4C). Results also indicate that *ZAT12* and ROS repress *FIT* expression (Fig.4.4A). As a result, when plants were treated with H<sub>2</sub>O<sub>2</sub>, *FIT* expression was not up-regulated at both +Fe and -Fe (Fig. 4.4 A, B). Moreover, *FIT* is upregulated in *zat12-3* mutant. Taken together, we suggest that both ROS and *ZAT12* repress *FIT* expression. Meiser et al., 2011 showed that *FIT* is controlled at multiple steps from transcription to active FIT protein (Meiser et al., 2011). The primary control process in FIT activation happens at transcriptional level. The induction of *FIT* gene expression is observed at - Fe compared to +Fe (showed in Fig. 4.3B, Colangelo & Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2005). This *FIT* induction requires transcription factors acting upstream of *FIT*. Perhaps, a negative regulator might repress *FIT* transcription at + Fe. By removing the repressor at - Fe an induction of *FIT* appeared. Interestingly, *FIT* was up-regulated at +Fe after cycloheximide (CHX- protein translation inhibitor) application. This was explained that CHX may destroy the *FIT* repression at + Fe (Meiser et al., 2011). It is possible that *de novo* protein synthesis is necessary to increase repressor quantity. Perhaps, *ZAT12* may function in repressing *FIT* transcription because *ZAT12* is affected by proteasomal degradation (Fig. 4.12) so that CHX might lead to removal of *ZAT12*.

The second control of FIT occurs at translation level. After 8 days of sufficient iron supply HA-FIT abundance is higher in the H<sub>2</sub>O<sub>2</sub>-treated sample, while the response in plants grown under iron-deficient conditions contradicts (Fig. 4.11). Meiser et al., 2011 showed that the general stability of HA-FIT is decreased under iron deficiency. Therefore FIT was regulated



by posttranslational turnover. In addition, at 10-day time point, FIT abundance is lower than at 8-day stage (Fig.4.11). This is consistent with the transcriptional regulation of *FIT* (Fig.4.4A), so that this effect should only be due to post-transcriptional regulation.

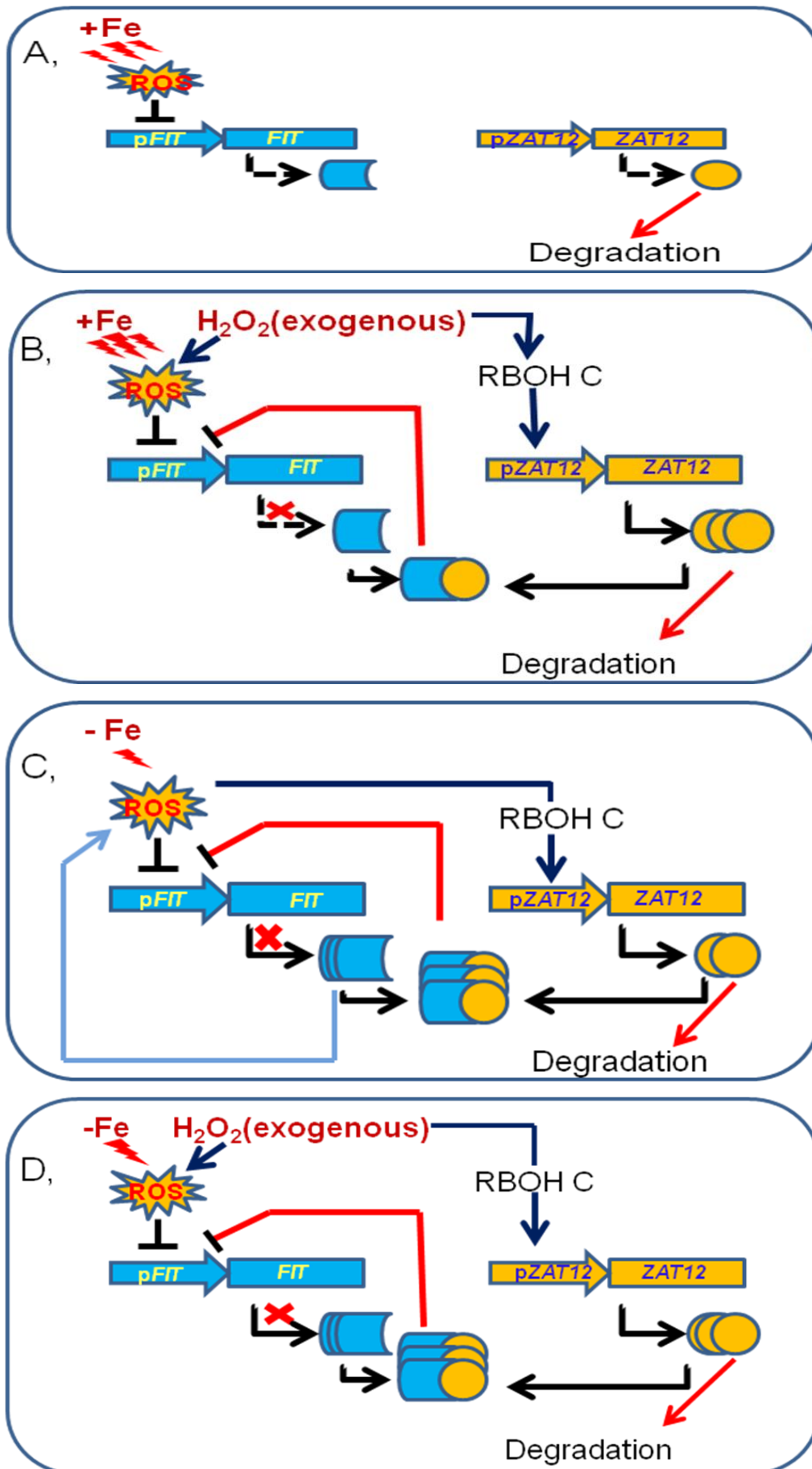
The following FIT control takes place at the level of protein degradation. Here, we observed that ZAT12 was elevated regardless of Fe supply and H<sub>2</sub>O<sub>2</sub> application after MG132 (26S proteasome inhibitor) treatment (Fig. 4.12). Lingam et al., (2011), Sivitz et al., (2011), Meiser et al. (2011) showed that FIT abundance increases after MG132 treatment. It was concluded that FIT was controlled posttranslationally by proteasome-mediated degradation, perhaps by 26S proteasome. Recent research indicated that auxin-dependent inactivation of the auxin/indole-3-acetic acid (AUX/IAA) proteins, EAR-repressors, through protein degradation results in the activation of auxin-responsive genes (Tao et al., 2005). In addition, we observed that after H<sub>2</sub>O<sub>2</sub> treatment, ZAT12Δ abundance is lower than control with no H<sub>2</sub>O<sub>2</sub> (Fig. 4.14A). This demonstrated that both H<sub>2</sub>O<sub>2</sub> and the EAR motif are involved in ZAT12 stability. Therefore, we suggest that after finishing repression activities, ZAT12 along with FIT and perhaps in the presence of H<sub>2</sub>O<sub>2</sub> are degraded via the 26S proteasome to relieve the effect of repression (Fig.5.1).

The last level of FIT regulation happens at the level of protein activity (Fig. 4.5). The downstream responses of FIT activity were measured as the induction of *IRT1* and *FRO2*. Here *IRT1* levels were comparable between Col-0 and *zat12-3*, except for day 10 where the *zat12-3* mutant failed to upregulate *IRT1* expression to the levels seen in the wild-type. Lingam et al. (2011), Meiser et al.(2011) and Sivitz et al. (2011) indicated the quantity of FIT protein was not found proportional to the level of the activity of FIT protein. By using plants overexpressing FIT, it could be shown that an elevated level of FIT/HA-FIT protein does not imply a general increase of FIT activity. Moreover, *BHLH39* mRNA accumulation in *zat12-3* mutant was also lower than those in wild-type. Recent studies shows evidence that in order to regulate its target genes, such as *IRT1* and *FRO2*, FIT may act in concert with at least one of the four Group Ib (bHLH proteins bHLH038, bHLH039, bHLH100 and bHLH101 (Wang et al., 2007; Yuan et al., 2008; Wang et al., 2012; Sivitz et al., 2012). By using multiple mutant combinations, Wang et al., 2012 showed that among these four partially redundant proteins, bHLH39 plays the most significant role in the regulation of iron uptake (Wang et al., 2012). Thus we conclude that bHLH039 might help balance iron deficiency responses. In *zat12-3* the decreased level of *BHLH039* perhaps reduces effect of the upregulation of *FIT* and maintains a stable expression of the target *IRT1* until day 10.

### 5.3 Explanation about ROS signalling of Fe responses

Abiotic stress leads to the accumulation of ROS and the interruption of the redox balance in the cell. Initially, ROS are thought to be toxic, but now there are more evidences demonstrating their other role such as messenger in the signaling pathways of cell. Here we also found that Fe deficiency resulted in the accumulation of H<sub>2</sub>O<sub>2</sub> (Fig.4.7C). Shin & Schachtman, 2005 have shown that H<sub>2</sub>O<sub>2</sub> production was increased in plants starving for potassium, nitrogen, and phosphorus, so that H<sub>2</sub>O<sub>2</sub> involved in cellular signaling of these nutrient starvations. Therefore, H<sub>2</sub>O<sub>2</sub> might also function as a signal of Fe deficiency. Signals can be detected by at least three types of ROS receptors such as heat shock transcription factors (HSF) or redox sensitive transcription factors or phosphatase (Mittler et al., 2004). Rizhsky found heat shock element (HSE), a putative DNA binding site, in the promoters of *ZAT12*, *ZAT7*, *WRKY25*, and *APX1*. *ZAT12* and *APX1* expressions were inhibited in plants expressing the dominant-negative *HSF21* construct suggest that *HSF21* functions in *ZAT12* and *APX1* expressions during light stress. It is possible that they are involved in regulating the expression of these genes in response to abiotic stress or oxidative stress (Rizhsky et al., 2004; Dalevtova<sup>b</sup> et al., 2005). Therefore, *ZAT12* might play a role in Fe responses through H<sub>2</sub>O<sub>2</sub> signal. After being detected, ROS can be transferred via transduction pathways and amplified by NADPH oxidase to trigger defense responses (Mittler et al., 2004; Mittler et al., 2011). Here for further experiment, we chose to work with mutant for *RHD2*, an NADPH oxidase - *RBOHC*, involved in ROS formation to activate Ca<sup>2+</sup> channels for cell elongation. My result did not show lower production level of H<sub>2</sub>O<sub>2</sub> in *rhd2* mutant as shown in Foreman et al., 2003 (Fig.4.7C). This can be explained by different growth systems. Interestingly, after H<sub>2</sub>O<sub>2</sub> application *ZAT12* transcriptional level was not induced in NADPH oxidase-deficient mutants (*rhd2*) like in Col-0 (Fig. 4.7 B). We proposed that RBOH C plays a role in ROS signaling to the level of the transcription factor *ZAT12*. Moreover, we detected a ROS signal along the stele of both Fe sufficient and deficient roots after H<sub>2</sub>O<sub>2</sub> treatment (Fig. 4.8). This is consistent with cell type-specific microarray analysis of Dinneny et al. (2008) in Fe-deficient *Arabidopsis* roots. There, the authors showed genes involved in signaling and stress were enriched among the stele-activated genes (Dinneny et al., 2008). Localized at the plasma membrane and by transferring electrons from NADPH to an electron acceptor, NADPH oxidase can form a ROS wave throughout the different tissues and bring the signal far away from the first burst of ROS (Miller et al., 2009; Niethammer et al., 2009). Therefore, we strongly suggest that ROS is considered as the second messenger relayed and amplified by NADPH oxidase in the stele to downstream components. We found that the absence of a

functional ZAT12 in plants expressing ZAT12 $\Delta$  led to the delayed growth and the late flowering. These plants have upward-pointing curly, reddish leaves which are typical symptoms of stress (Fig.4.14B). This indicates ZAT12 $\Delta$  lacks a functional EAR-motif, and acts as dominant-negative repressor of the endogenous ZAT12 protein. It competes with the endogenous ZAT12 protein, but it lacks the capability to suppress repressors of defenses mechanism, thus causing more susceptibility to stress. Rizhsky showed that ZAT12 is required for ascorbate peroxidase 1-APX1 expression, a cytosolic H<sub>2</sub>O<sub>2</sub>-scavenging enzyme, during oxidative stress (Rizhsky et al., 2004). Thus, we propose that ZAT12 might be a necessary component of the pathway to detoxify ROS. To sum up, it is reasonable to conclude a function of ZAT12 in response to oxidative stress caused by Fe responses.



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**Fig. 5.1. Model summarizing the FIT repression regulation of ZAT12 in the presence of ROS in wild type plants.**

A, In + Fe-treated wild type roots, *FIT* and *ZAT12* induction do not take place (shown by dashed, black arrows), but ROS production is increased (Fig.4.8) (indicated by a red lightning). ROS repress *FIT* (*FIT* gene and *FIT* promoter are represented as blue square and arrow, respectively).

B, In + Fe treated wild-type roots, *FIT* is also not upregulated under exogenous H<sub>2</sub>O<sub>2</sub> treatment (dashed black arrow). However, *ZAT12* expression induced by H<sub>2</sub>O<sub>2</sub> through RBOH C (black arrow) (Fig 4.3A; 4.4C). Both ROS (red lightning) and *ZAT12* (yellow) might repress *FIT* (shown as red **X** symbol). In addition, *ZAT12* interacts with *FIT* and therefore we propose that *ZAT12* inhibits *FIT* via interaction with *FIT* (in blue, interaction is shown by red line). Later on, *ZAT12* is degraded through 26S proteasome (red arrow) (Fig.4.12).

C, In – Fe wild-type roots, *FIT* transcription is induced regularly that makes increase H<sub>2</sub>O<sub>2</sub> production (lighter blue arrow, Fig. 4.7 A), but it is repressed after 10 day of Fe deficiency where *ZAT12* expression and H<sub>2</sub>O<sub>2</sub> level are increased. This indicates again that *ZAT12* represses *FIT*. Subsequently, *ZAT12* is degraded through 26S proteasome.

D, Under H<sub>2</sub>O<sub>2</sub> stress, *FIT* is not normally upregulated in – Fe wild-type roots where the *ZAT12* expression is induced dramatically by both –Fe and H<sub>2</sub>O<sub>2</sub> (Fig.4.4). *ZAT12* represses *FIT* and is degraded through 26S proteasome later to relieve repressor activities (red arrow).

## 6. Perspectives

With the here presented results we identified that the Zn-finger transcription factor ZAT12 is a molecular link between Fe deficiency responses and oxidative stress. However, in order to gain deeper and more mechanistic understanding of signal integration, more in depth molecular analysis is required. The future perspectives for this project can be categorized in the following main goals.

The first is to confirm the physical interaction between ZAT12 and FIT. To address this question, there are some analyses conducting such as BiFC (Bimolecular fluorescence complementation) or in vivo immunoprecipitation.

The second perspective is to research the role of the EAR motif in ZAT12- mediated FIT inhibition. This can be done by transgenic lines that expressing a mutant version (ZAT12 $\Delta$ EAR) of ZAT12 in which the LDLSL sequence was deleted. Recent alternative method was reported by using a heterologous carrot (*Daucus carota*) protoplast transfection system and thereby test directly whether ZAT12 functions as a transcriptional repressor (Shyu et al., 2012)

The third perspective will be the investigation about the connection between ROS and NO. Nitric oxide (NO) was proposed to improve the availability of iron within the plant. NO application rescued the iron-deficiency symptoms in maize plants growing with a sub-optimal iron concentration and also in *ys1* and *ys3* maize mutants (Graziano et al., 2002). Sun et al., 2006 showed that NO protected maize (*Zea mays*) plants against iron deficiency-induced oxidative stress by reacting with ROS directly or by changing activities of ROS-scavenging enzyme. In addition, in tomato roots grown in Fe deficient condition, the NO production was enhanced to regulate iron uptake-related gene transcriptions and physiological and morphological adaptive changes of roots (Graziano & Lamattina, 2007). Moreover, NO increases FIT stability (Meiser et al., 2011). Therefore, a correlation might exist between iron uptake and oxidative stress and NO.

The fourth perspective is the verification of protein regulation by using an antiserum against ZAT12. Although the generation of antibody of transcription factor is not easy because of the expected low expression of the latter, we confirmed the specificity of our generated ZAT12 antiserum. To use it, it is first necessary to be purified against a crude bacterial extract containing recombinant ZAT12 fusion protein.

Next, along with hormonal signaling networks, ROS signaling can regulate many developmental processes and environmental responses as well. Ethylene is found as

component in response and tolerance to potassium deprivation that activates ROS production in *Arabidopsis* (Jung et al., 2009). Moreover, ethylene is produced more in  $-Fe$  than  $+Fe$  (Romera et al., 1999). Loss-function mutations of *EIN3* and *EIL1*, two central components of ethylene signaling pathway, showed the increased expression of Fe acquired genes such as *IRT1* and *FRO2* under Fe deficient condition. In addition, FIT interacts with EIN3 and EIL1 to prevent its degradation via 26S proteasome (Lingam et al., 2011). It could be promising for further studies to identify overlapping points between ROS and ethylene signaling pathways. Finally, many players in ROS signaling are still not known. It would be interesting to answer questions such as: How ROS is perceived? How specific ROS signal is? How ROS is propagated, sensed through cells or tissues? How possible in capacity of cells scavenge ROS?

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