

**Iron dependent post-translational regulation of the bHLH transcription factor FIT in  
*Arabidopsis thaliana***

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## Summary

Iron (Fe) is an essential micronutrient for most organisms, but too high Fe contents can cause the formation of free radicals. Hence, Fe uptake must be tightly regulated. Root iron acquisition in non-graminaceous plants, like the model plant *Arabidopsis thaliana*, is achieved by reduction of soil Fe by the reductase *FRO2* and its subsequent uptake by the metal transporter *IRT1*. The bHLH transcription factor *FIT* is required for high-level expression of *FRO2* and *IRT1* upon Fe deficiency.

In this work we investigated post-transcriptional regulation of *FIT*. We found Fe dependent post-transcriptional regulation of *FIT* in way of constant turnover. Small amounts of active *FIT* were found to be sufficient to trigger the expression of *FRO2* and *IRT1*. *FIT* protein stability relies on the presence of the signalling compound nitric oxide (NO). NO mediated stabilisation of *FIT* is independent of transcriptional regulation and is most probably achieved by counteracting proteasome dependent degradation of *FIT*. We summarise our results in an integrative model and based on this we made further efforts to identify post-translational modifications that could regulate *FIT* activity. Based on *in silico* prediction we identified four amino acids in the c-terminal part of *FIT* as putative phosphorylation sites. With newly generated *FIT* forms containing phosphomimicking or non-phosphorylatable mutations we can draw further conclusions and suggest that phosphorylation may regulate *FIT* activity.

# Zusammenfassung

Eisen (Fe) ist ein essentieller Mikronährstoff für Lebewesen. Jedoch können zu hohe Konzentrationen an Eisen die Bildung freier Radikale zur Folge haben. Die Eisenaufnahme aus dem Boden erfolgt bei der Modellpflanze *Arabidopsis thaliana*, sowie bei allen Nicht-Gräsern, mittels Reduzierung des im Boden enthaltenen Eisens, durch die Reduktase FRO2 und durch die Aufnahme des Metalltransporters IRT1. Bei Eisenmangel wird der bHLH Transkriptionsfaktor *FIT* für die verstärkte Transkription von *FRO2* und *IRT1* benötigt.

In dieser Arbeit wurde die post-transkriptionelle Regulation von FIT untersucht und wir konnten eine eisenabhängige Regulation von FIT nachweisen. Geringe Mengen an FIT waren ausreichend um die Expression seiner Zielgene zu induzieren. Die Stabilität von FIT ist abhängig von dem Signalmolekül Stickstoffmonoxid (NO). Die transkriptionsunabhängige Stabilisierung von FIT durch NO erfolgt wahrscheinlich durch eine Hemmung der Proteasom-abhängigen Degradation von FIT. Wir haben unsere Ergebnisse in einem Modell zusammengefasst und weisen, darauf basierend post-translationalen Mechanismen auf, welche die Aktivität von FIT regulieren könnten. Vier Aminosäuren innerhalb des c-terminalen Teils von FIT wurden anhand von *in silico* Analysen als mögliche Phosphorylierungsstellen identifiziert. Neu hergestellte FIT Formen, welche eine Phosphorylierung oder eine Dephosphorylierung imitieren, zeigten dass Phosphorylierung ein potentieller Faktor ist, um die FIT-Aktivität zu regulieren.

## Abbreviations

bHLH	basic Helix Loop Helix
BiFC	Bimolecular Fluorescence Complementation
CHX	Cycloheximide
Col-0	Columbia-0
cPTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
Fe	Iron
FIT	FER LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR
FRO2	FERRIC REDUCTASE OXIDASE 2
GFP	GREEN FLUORESCENT PROTEIN
GSNO	S-nitrosoglutathione
HA	Hemagglutinine
IRT1	IRON REGULATED TRANSPORTER 1
MAPK	MITOGEN ACTIVATED PROTEIN KINASE
MG132	N-(benzyloxycarbonyl)leucinylleucinylleucinal Z-Leu-Leu-Leu-al
NO	Nitric Oxide
PAGE	Polyacrylamid Gelelectrophoresis
PCR	Polymerase Chain Reaction
RT qPCR	Reverse transcription real time quantitative PCR
VIT1	VACUOLAR IRON TRANSPORTER 1

# 1. Introduction

## 1.1. The importance of Fe for the physiology of living organisms

Iron (Fe) is an essential micronutrient for most organisms. Bio-available Fe is present only in limited amounts, for example in human diets or in calcareous field conditions for crop plants.

Fe plays an essential role in redox reactions that occur in many biological processes. It serves as a prosthetic group in proteins to which it is associated either directly or through a haem or an [Fe–S] cluster. It exists under two redox stages, the reduced ferrous Fe (II) and the oxidised ferric Fe(III) form and is able to gain or lose an electron, respectively, within metalloproteins (e.g. Ferredoxin and superoxide dismutase (SOD)). It functions as a component of many important enzymes and proteins involved in fundamental biochemical processes like the electron transfer chains of respiration and photosynthesis, the biosynthesis of DNA, lipids and hormones, the detoxification of reactive oxygen species (ROS) and the nitrogen assimilation (Marschner, 1995, Balk et al., 2005). Fe is usually bound to chelators which range from simple molecules like amino acids to complex proteins. In this way, the binding of free Fe, reduces the formation of free radicals through the Fenton reaction.

Plants deficient on Fe, show decreased photosynthetic activity and develop leaf chlorosis, caused by a decreasing number of chloroplasts. Such leaves turn yellow to white depending on the stage of chlorosis. Subsequently, Fe deficiency results in reduced biomass and fruit production.

Fe frequently appears in lower than required amounts in human diets, since bio-available Fe is present only in limited amounts, especially in third world countries where soils tend to be washed out and diets are mostly based on vegetables. Also in highly developed countries Fe deficiency caused anaemia is a problem, particular for women in pregnancy or with menstruation. Consequently, Fe deficiency related anaemia affects one third of the world population (Mc Lean et al., 2009).

To prevent anaemia, bio-fortification programs are underway to increase Fe in staple crops. Such approaches include the generation of plant varieties that are genetically optimized to contain higher Fe contents in the edible plant parts (Vasconcelos et al., 2003). Understanding the molecular basis of Fe homeostasis in plants will help to breed higher nutritious quality food crops by enabling the targeting of the major key genes of traits related to Fe content.

## 1.2. Plant Fe uptake and homeostasis

### 1.2.1. Root Fe uptake in non-graminaceous plants

Although Fe is the fourth most abundant element in the earth's crust, it is found mainly as stable, insoluble oxyhydroxide polymers that effectively limit free Fe (III) to an equilibrium concentration of 10<sup>-17</sup> M at neutral pH, a value far below that is required for the optimal growth of plants (Guerinot and Yi, 1994). To overcome this problem, plants react to Fe starvation by morphological alterations such as swollen root tip and increased root hair formation (Schmidt, 1999), as well as with physiological changes to enable them to dissolve and take up Fe into the rhizosphere. To solubilise Fe, all dicotyledonous and non-graminaceous monocotyledonous plants, including the model plant *Arabidopsis thaliana*, reduce ferric Fe<sup>3+</sup> and import the more soluble ferrous Fe<sup>2+</sup> into the root cells. This strategy is also known as the Strategy I (Figure 1.2). On the other hand, grasses extrude chelators into the soil to form stable complexes with Fe (III) to subsequently take up these complexes by specific transporters belonging to the Yellow Stripe family (YS) (Curie et al., 2009; Marschner, 1995; Römheld and Marschner 1986).

In the strategy I plant *Arabidopsis thaliana*, solubilisation of Fe is facilitated by the proton extruder *AHA2* (ARABIDOPSIS H<sup>+</sup>-ATPASE) in way of soil acidification (Palmgren, 2001; Santi and Schmidt 2009; Ivanov et al., 2011 (in press)). Reduction of Fe<sup>3+</sup> is carried out by one of the members of the ferric reductase oxidase (FRO) family. The *FRO* family consists of eight members and is differentially expressed throughout the whole *Arabidopsis* plant (Mukherjee et al., 2006). *FRO2* is located at the plasma membrane of root epidermis cells to reduce ferric Fe in the soil (Robinson et al., 1999). This reduction is thought to be the rate-limiting step in Fe uptake (Grusak et al., 1990; Eide et al., 1996). Once reduced, Fe can be taken up by the divalent metal transporter *IRT1* (Eide et al., 1996; Vert et al., 2002). *IRT1* belongs to the *ZIP* (ZRT, IRT-like Protein) family of metal transporters and consists of eight transmembrane domains and one histidine-rich intracellular domain. This loop is supposed to be the metal binding domain. Besides Fe, *IRT1* can also import other divalent metals such as Mn and Zn (Eide et al., 1996). Such metals are coincidentally taken up under Fe starvation.

*AHA2*, *FRO2* and *IRT1* are regulated by the bHLH transcription factor FIT (FER-LIKE FE DEFICIENCY INDUCED TRANSCRIPTION FACTOR) in an Fe dependent manner (Jakoby et al., 2004; Colangelo and Guerinot, 2004, Ivanov et al., 2011 (in press)) (Figure

1.2). FIT is supposed to be the key regulator in Fe uptake (Jakoby et al., 2004; Colangelo and Guerinot, 2004; Bauer et al., 2007).

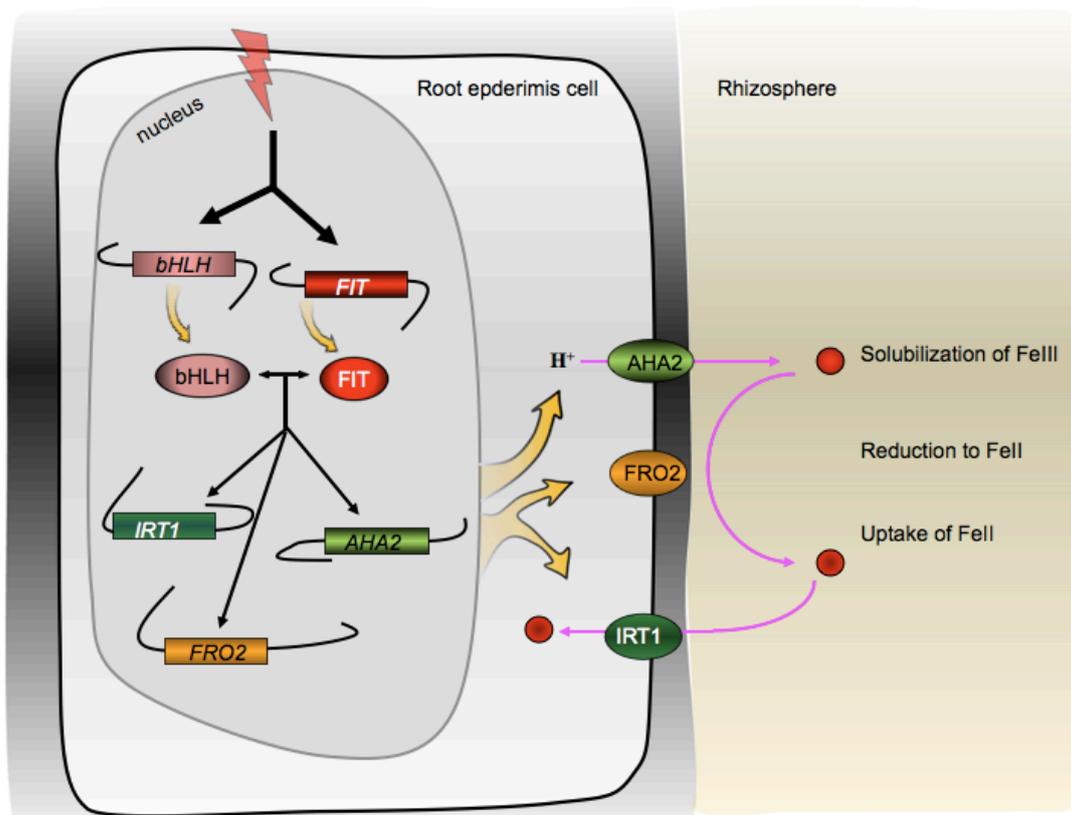


**Figure 1.1: Phenotype of Columbia-0 wild type (left) and *fit-3* (right) mutant plant**

Plants were grown for six weeks on soil in long day conditions. *fit-3* mutant plants are small and display severe leaf chlorosis and are unable to produce viable seeds unless they are supplied with external Fe.

The bHLH transcription factor *LeFER* (*Lycopersicon esculentum*) was identified first and later *FIT* was found to be the ortholog (Ling et al., 2002 ;Bauer et al., 2004, Jakoby et al., 2004; Colangelo and Guerinot, 2004; Bauer et al., 2007). The relation between *FIT* and *FER* was confirmed by Yuan et al. (2005) who complemented the *fer* mutant with the *AtFIT* gene. The loss of function mutant *fer*, fails to activate the Fe uptake machinery and develops less root mass due to decreased elongation of lateral roots. In addition such plants exhibit strong chlorosis under both, low and sufficient Fe concentrations and are lethal under normal growth conditions. The *fer* mutant, that was complemented with *AtFIT*, was able to induce Fe deficiency responses and grew as their corresponding tomato wild type plants. *LeFER* gene expression was exclusively present in the root, but not in cotyledons or leaves (Ling et al., 2002). In addition to gene expression studies, LeFER protein abundance was also investigated by using an antiserum, raised against LeFER

(Brumbarova and Bauer, 2005). By using *LeFER* over-expression plant lines, the authors could show that *FER* mRNA expression and FER protein levels were separately regulated, indicating that *LeFER* is regulated post-transcriptionally (Brumbarova and Bauer, 2005). Regarding the model plant *Arabidopsis thaliana*, further work to investigate post-transcriptional regulation of FIT had not been done so far. It was known before that *FIT* expression is induced about 2-3 fold upon low Fe. Expression of *FIT* is focused in the root epidermis (Jakoby et al., 2004). Microarray analysis showed that out of 179 deregulated genes upon Fe deficiency, 72 genes seemed to depend on FIT (Colangelo and Guerinot, 2004). Loss of function *fit* plants suffer from severe Fe deficiency chlorosis, irrespective of Fe supply (Figure 1.1). Such mutant plants are lethal unless they are sprayed with external Fe (Jakoby et al., 2004; Colangelo and Guerinot, 2004). There is no known gene, which could replace or overtake function of *FIT* in *Arabidopsis*. Thus *FIT* seems to have an outstanding function in controlling Fe uptake. Moreover, genes acting upstream of *FIT* to induce *FIT* transcription under Fe deficiency are unknown. The FIT protein has a predicted size of 35 kD and the abundance is regulated on transcriptional and post-transcriptional level (Jakoby et al., 2004, Colangelo and Guerinot, 2004). Previous work could show that FIT over-expressing plants have high FIT mRNA amounts in all tissues under both, high and low Fe supply. However, induction of *FRO2* and *IRT1* took only place under Fe starvation, which indicates a regulation at post-transcriptional level (Jakoby et al., 2004, Colangelo and Guerinot 2004). On which stage this post-transcriptional regulation takes place was unknown. Regulation on mRNA level, translational control, control on protein level in way of protein modification, trafficking or turnover or a combination of these mechanisms could be hypothesised. The fact that FIT belongs to the bHLH family, suggests that interaction with other bHLH proteins and post-translational regulation of FIT could be possible mechanisms to influence FIT action. Such regulatory mechanisms that are acting on bHLH proteins in *Arabidopsis* have been well documented (Huq et al., 2004; Bu et al., 2011; Shen et al., 2007; Khanna et al., 2004; Lampard et al., 2008; Yuan et al., 2008; Long et al., 2010).



**Figure 1.2: Root Fe uptake via the Strategy I in Fe deficient *Arabidopsis thaliana* plants**

When Fe deficiency occurs it has to be relayed to the root epidermis. Once the signal has arrived at the root epidermis (indicated by a red arrow), the signal must be transduced from the cell surface into the nucleus to induce the transcription and translation of the bHLH transcription factor *FIT* as well as other Fe dependent genes such as the bHLH genes belonging to the bHLH subfamily 1b. Interaction between FIT and other bHLH proteins or further nuclear proteins might be important for the induction of the Fe deficiency response. The presence of FIT results in transcriptional induction of the proton extruder *AHA2*, the Fe reductase *FRO2* and the divalent metal transporter *IRT1*. By concerted action of *AHA2*, *FRO2* and *IRT1* the insoluble Fe<sup>3+</sup> complexes can be solubilised and reduced, to be finally taken up into the root epidermis. Further regulatory mechanisms acting at post-transcriptional and post-translational level can be assumed.

#### 1.2.1.1. The bHLH gene family in *Arabidopsis* and its impact on Fe homeostasis

The bHLH gene family is a large family of transcription factors that are present throughout a wide range of organisms and have been well described in mammalian systems (Atchley and Fitch, 1997; Littlewood and Evan, 1998; Ledent and Vervoort, 2001). All of these proteins contain a characteristic bHLH domain encompassing approximately 60 amino acids. The N-terminal part of this region consists of about fifteen most basic amino acids and is known for DNA binding, recognising the E-Box motif (5'-CANNTG-3'). The C-terminal part of the bHLH domain contains mostly hydrophobic amino acids and builds two alpha helices connected by a loop region (Nair and Burley, 2000). This HLH region is

known to build homo or heterodimers with other bHLH proteins. Depending on the dimer that is build, functionality of the individual bHLH protein can be influenced (Murre et al., 1989; Ferre-D'Amare et al., 1994; Robinson et al., 2000; Littlewood and Evan, 1998). Heterodimerisation of two distinct bHLH proteins can for example be preliminary prior binding to a specific promotor region. Only the combination of both N-terminal bHLH regions from this complex binds then to the DNA (Ma et al., 1994; Shimizu et al., 1997). An example for heterodimerisation was shown by Toledo Ortiz et al. (2003), who investigated the interaction between PIF3 and PIF4, both proteins belonging to the PHYTOCHROME INTERACTING FACTORS (PIF).

*Arabidopsis* encodes most probably 170 bHLH genes (Bailey et al., 2003; Heim et al., 2003; Toledo-Ortiz et al., 2003; Paulet et al., 2010) which were classified in twelve (Heim et al., 2003, the classification of Heim et al. is used in this work) or 21 subfamilies (Toledo Ortiz et al., 2003), respectively. These proteins are involved in versatile processes within the plant, for instance developmental processes like stomatal development (Lampard et al. 2008; Veraud et al., 2011), light perception via the PIFs (Bu et al., 2011; Shen et al., 2007; Khanna et al., 2004), responses to hormonal stimuli for instance from jasmonate and salicylic acid (Liu and Stone 2011; Kang et al., 2003) or the regulation of abiotic stresses, including Fe homeostasis via the bHLH transcription factors FIT (bHLH029) (Jakoby et al., 2004; Colangelo and Gueriot, 2004), PYE (bHLH047) (Long et al., 2010), BHLH038, BHLH039 and most probably also BHLH100 and BHLH101 (Wang et al., 2007). A heterodimerisation between FIT and one of the bHLH transcription factors BHLH038 (ORG2) or BHLH039 (ORG3) has recently been shown in vitro and in leaf protoplasts (Yuan et al., 2008). The genes *BHLH038* and *BHLH039* belong to the subgroup 1b within the bHLH gene family (Heim et al., 2003). Both genes are induced under Fe starvation in roots, but also in leaves (Vorwieger et al., 2007; Wang et al., 2007). Simultaneous over-expression of *FIT* and *BHLH038* or *BHLH039* leads to an increased Fe deficiency response and increased Fe contents also under Fe supply conditions (Yuan et al., 2008). Further characterisation, how and under which conditions this interaction takes place is still missing. Besides *BHLH038* and *BHLH039*, two other members of the subgroup 1b, *BHLH100* and *BHLH101*, are also responsive to Fe deficiency in way of induced transcription. But despite the fact that *BHLH100* and *BHLH101* are transcribed in an Fe dependent manner, further characterisation is missing. Single knock out mutants corresponding to one of these four bHLH genes from the subgroup 1b (*038*, *039*, *100*, *101*), show no phenotype or any alteration on gene expression level, because *BHLH100* and *BHLH101* as well as *BHLH038* and *BHLH039* are supposed to be functionally redundant

(Wang et al., 2007). Therefore it remains to be unknown how these bHLH genes are involved in Fe homeostasis. Downstream genes depending on one of the four bHLH genes from subgroup 1b as well as transcription factors regulating these bHLH genes are still unknown. Moreover, the molecular characterisation of the cross talk between FIT and BHLH038 and BHLH039 remains to be described.

One other member of the bHLH gene family, named *POPEYE* (*PYE*), has recently been uncovered for being involved in Fe homeostasis. Unlike *FIT*, *PYE* seems to be involved in Fe homeostasis, rather than having a direct impact on Fe uptake response. *PYE* regulates the expression of *NICOTIANAMINE SYNTHASE4* (*NAS4*) and the reductase *FRO3*, both being involved in Fe distribution throughout the plant (Long et al., 2010; Klatter et al., 2009; Jeong et al., 2008; Mukherjee et al., 2006). Additionally, *PYE* was shown to interact with *ILR3*. *ILR3* is also related to general metal ion homeostasis and auxin signalling (Rampey et al., 2006). *ILR3* was shown to interact with a putative E3 ligase, named *BRUTUS* (*BTS*). In turn, *BTS* is supposed to negatively affect Fe homeostasis. Because E3 ligases are involved in ubiquitination of proteins, this finding seems to be in good agreement with the assumption that *BTS* could be responsible for the degradation of proteins related to Fe uptake and distribution. However, to clarify these recent findings, more work has to be done to unravel the interaction networks on protein level. On the other hand the recent results by Long et al. (2010) show that further bHLH transcription factors beside *FIT* are controlling Fe homeostasis in the plant. It could be possible that such bHLH transcription factors act in parallel to *FIT* rather than in one common pathway. Besides the *FIT* regulated Fe uptake, also Fe transport and distribution throughout the plant has to be tightly controlled to prevent toxic effects caused by free Fe. Therefore a network of transporters and reductases have to be regulated in way of transcriptional control and protein activity. The involvement of transcription factors such as *PYE*, *FIT* and other bHLH proteins seems to have an extremely important function.

### **1.2.2. Control and regulation of Fe transport, distribution and storage throughout the plant**

Understanding how Fe is transported in the plant and where it is localised can help to identify where the Fe deficiency signalling is initiated. Such signalling cascades may subsequently act on *FIT* at transcriptional and post-transcriptional level to induce Fe uptake from the soil.

Once Fe has been taken up by IRT1 it has to be bound to other components and stored in certain compartments, to prevent toxic events within the cell caused by free Fe. On the other hand, Fe pools can serve as a buffer in times of low Fe availability. Such Fe pools can be available quickly and thus compensate the Fe demand, if necessary. Especially, cortex and epidermis of the root can easily become locations of Fe excess in case of increased Fe uptake. To buffer excessive Fe amounts, ferritins are up-regulated in times of Fe overload. Therefore, ferritins possess an essential role in cellular Fe homeostasis (Harrison and Arosia, 1996). The FER proteins, contain a signalling peptide in their N-terminal region, which targets them to the plastids, where they are supposed to localise within the cell (Lescure et al., 1991; Briat and Lobréaux, 1997; Gaymard et al., 1996; Petit et al., 2001). About 90% of the plants Fe is located at the chloroplasts, where it is needed for the electron transport chain, chlorophyll, heme and [Fe-S] cluster synthesis (Kim and Gueriot, 2007). Thus, the chloroplast could be one place where Fe deficiency is recognised first.

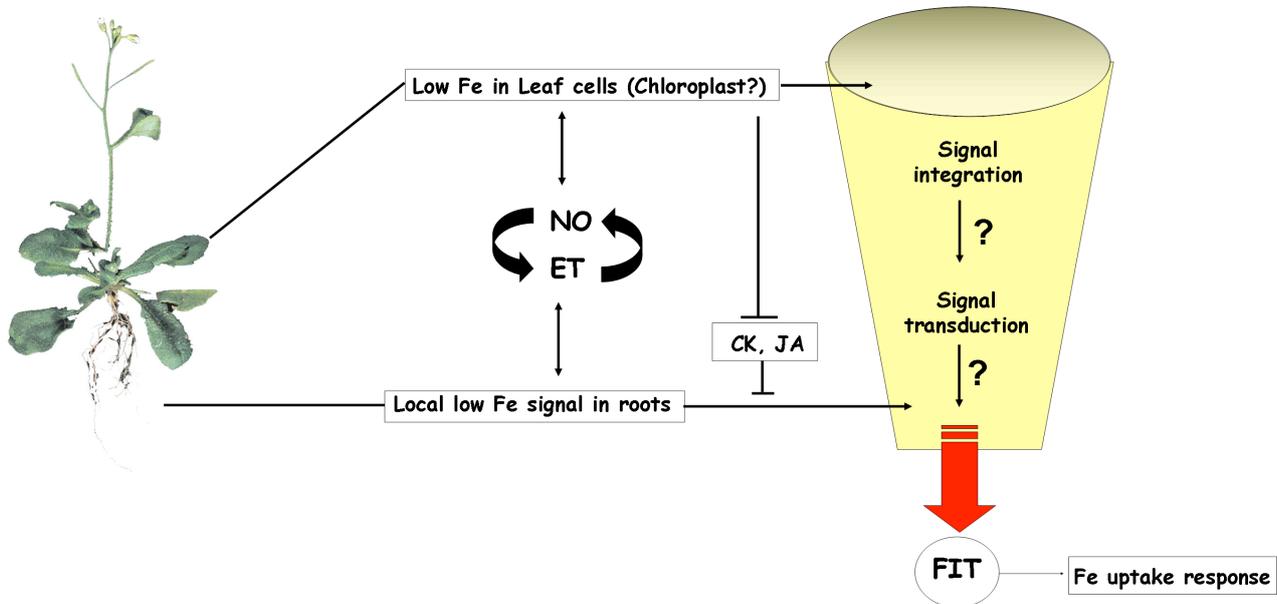
To transport Fe from the root to the shoot, it has to be transported from the outer root cell layers into the vascular bundles, so that it can be transported via the xylem stream to the leaf parenchyma. Xylem loading as well as xylem un-loading seems to be tightly controlled (Stacey et al., 2008; Durrett et al., 2007; Walker and Connolly, 2008). During this transport process every Fe atom has to undergo a series of reduction and oxidation steps until it reaches its destination. For instance citrate or nicotianamine, which function in chelating free Fe, transport ferric Fe through the vascular bundles. The presence of the Fe reductase FRO7 at the chloroplast membrane (Jeong et al., 2008) indicates that the oxidised Fe has to be reduced first prior to its uptake into the chloroplast. Subsequent red-ox steps are thought to control Fe transport and distribution. Within the cell different transporters are located at the cell organelles to maintain proper intracellular Fe distribution (Duy et al., 2007, 2011; Morrissey et al., 2009; Kim et al., 2006; Kushnir et al., 2001; Thomine et al., 2000, 2003; Lanquar et al., 2005; Curie et al., 2000). Expression of the different transporters is often Fe dependent. Thus Fe dependent transcription factors should be necessary to regulate the expression network of these genes. De-regulation of one of the transporters can result in altered Fe deficiency responses, since knock-out as well as over-expression of a transporter can result in altered Fe localisation within the cell. One example is the ferroportin *FPN2*. *FPN2* is an ortholog of the mammalian ferroportins and functions in effluxing Fe from the cytosol into the vacuole (Morrissey et al., 2009). Knock out of *fpn2* resulted in decreased Fe uptake response, due to increased Fe contents in the cytosol. On the opposite, over-expression of *FPN2*, resulted in increased

Fe deficiency responses, because this over-expression led to lowered Fe contents within the rest of the cell (Morrissey et al., 2009). This example shows that Fe levels in the plant cell are tightly controlled and that genes, involved in intracellular Fe homeostasis, have themselves to be tightly regulated by specific transcription factors. Moreover, intracellular Fe levels are somehow linked to the root Fe uptake system. However, the connection and the way this linkage is maintained is still unclear. First, a signalling cascade must transduce the Fe status from the shoot to the root and second, at cellular level, the signal must be detected at the cell surface and has to be transmitted into the nucleus to control transcriptional regulation (Figure 1.2, 1.3). Such a transcriptional regulation implies an involvement of FIT.

### **1.2.3. Sensing Fe deficiency: long distance signalling versus local signalling and the impact of hormones on Fe nutrition**

As described before the rise of an Fe deficiency signal could very likely derive from the plastids in leaf cells, since the chloroplast builds the highest Fe sink within the plant (Kim and Guernot 2007). Independent of where the signal derives from it has to be transmitted to the root, where Fe uptake takes place. Therefore, a shoot-borne long distance signal is proposed for years (Grusak and Pezeshgi, 1996; Schmidt and Schikora, 2001; Vert 2003; Klatte et al., 2009). The existence of shoot-borne signals regulating the root response to Fe starvation has first been identified with two pea (*Pisum sativum*) mutants, named *degenerative leaflets* (*dgl*) and *bronze* (*brz*), both presenting constitutive Fe deficiency responses although they contain high amounts of Fe in their tissues (Kneen et al., 1990; Welch and LaRue, 1990). Reciprocal grafting between *dgl* or *brz* and their parental genotypes indicated that the phenotype of the root is determined by the shoot genotype (Grusak and Pezeshgi, 1996). Further proof came from split-root experiments, which showed that the expression of *IRT1* and *FRO2* is controlled by a local induction from the root Fe pool and through a systemic pathway involving a shoot-borne signal, both signals being integrated to tightly control production of the root Fe uptake proteins. However, the long distance signal could overrule the local root signal (Vert et al., 2003). In addition to a direct putative Fe deficiency signal, the control of Fe homeostasis is also influenced and actively regulated by different plant hormones. It could also be possible that Fe deficient leaf cells trigger hormone bursts to induce Fe uptake in the root and that in addition other hormones could also repress the Fe uptake in times of high Fe supply. An example for cross talk between hormonal stimulation and long distance signalling was described by,

Maurer et al. (2011). The authors could show that a repression of the Fe uptake caused by the plant hormone jasmonate could be overruled by a shoot-borne Fe deficiency signal, which is in accordance with the results by Vert et al. (2003) who showed that the local root signal becomes overruled by the long distance Fe deficiency signal.



**Figure 1.3: Integration of the different Fe signals deriving from the leaves and the roots and the influence of hormones on signal transduction to modulate the Fe uptake response.**

Fe deficiency signals can derive by a local signal in the root or by a long distance signal from the leaf. Chloroplasts seem to be a good candidate for the rise of an Fe deficiency signal, because they contain about 90% of the plant's Fe. The plant hormones Cytokinin (CK) and Jasmonate (JA) act repressive on the Fe uptake response, independent of FIT. The effect of JA and most probably CK can be overruled by a long distance Fe deficiency signal deriving from the shoot. In contrast to the effects of CK and JA, Fe deficiency results in production of the plant hormone ethylene (ET) and the signalling compound nitric oxide (NO). Ethylene and nitric oxide influence themselves and external application of ethylene or nitric oxide results in increased Fe uptake responses. Once an Fe deficiency signal is formed, it has to be transduced to the root epidermis and there, into the nucleus to induce gene expression of *FRO2* and *IRT1* (see Figure 1.2). How this signal transduction and integration of the various signals takes place is so far unknown. There is evidence that FIT as the central key transcription factor in the Fe deficiency response is used as an integrator to receive incoming signals at transcriptional and pos-transcriptional level.

The influence of various plant hormones acting on Fe homeostasis have been described for years. Generally, effects of plant hormones can be either promoting or repressing on Fe uptake. The effect of every plant hormone on Fe homeostasis still has to be analysed in detail, but summarising the current data it can be concluded, that the plant hormones Jasmonate (Maurer et al., 2011) and cytokinin (Séguéla et al., 2008) act repressive on plant Fe nutrition, whereas auxin (Schikora and Schmidt 2001; Chen et al., 2010),

ethylene (Romera et al., 2008; Lucena et al., 2006; Waters et al., 2007; Garcia et al., 2010; Lingam et al., 2011) and the signalling compound NO (Graziano and Lamattina, 2007; Graziano et al., 2002; Chen et al., 2010; Garcia et al., 2010; Garcia et al., 2011) have promoting effects on Fe uptake.

These findings demonstrate that diverse signals enter the root and that these signals are integrated to one common output signal. However, the detailed molecular mechanisms in these signalling pathways are hardly understood. Independent where the Fe deficiency signal derives from and how it may be modulated, it could be possible that FIT as the key transcription factor could be targeted at transcriptional and post-transcriptional level to integrate the different signals deriving from the whole plant (Figure 1.2, Figure 1.3). Still unclear is the question how the Fe uptake response can be repressed in times of Fe resupply (Vert et al., 2003). It could be possible that simple degradation of FIT would result in less transcription of the target genes or that different repressors act in addition to block the promotor target sites of the Fe uptake genes. Joint action of both effects could also be possible. Interestingly, Maurer et al. (2011) could show that the repressive effect of jasmonate acting on the expression of *IRT1* and *FRO2*, seems to act independent of the transcription factor FIT, since a decrease of expression was also observed in *fit* mutant plants (little induction of *FRO2* and *IRT1* at - Fe can also be detected in *fit* plants). Séguéla et al. (2008) could show similar results regarding the plant hormone cytokinin, that represses the Fe uptake response in a *FIT* independent manner as well. Thus FIT seems to have an outstanding function by inducing the expression of *IRT1* and *FRO2*, whereas repression of these two genes can be carried out in bypassing the transcriptional inducer FIT.

As described before, there is also existence of hormonal driven induction of Fe uptake mediated by ethylene (Lucena et al., 2006; Waters et al., 2007; García et al., 2010), auxin (Schikora and Schmidt, 2001; Chen et al., 2010) and by the signalling compound NO (Graziano et al., 2002; Graziano and Lamattina, 2007; Chen et al., 2010; García et al., 2010). In particular, induction of Fe uptake by ethylene and NO rely on the presence of FIT (Lingam et al., 2011; Meiser et al., 2011 (provisionally accepted)) and will be described in the next paragraph.

### 1.2.3.1. The impact of ethylene and nitric oxide on Fe uptake in Arabidopsis

#### • Ethylene

The small gaseous hormone ethylene (ET, C<sub>2</sub>H<sub>4</sub>) has a deep impact on Fe homeostasis (Lucena et al., 2006; Waters et al., 2007; Lingam et al., 2011). Ethylene is produced when Fe deficiency occurs, (Romera et al., 1999; Li and Li, 2004; Zuchi et al., 2009) and this can be artificially triggered by applying the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). As a result, morphological growth responses of Fe deficient plants take place (Romera and Alcantara, 1994; Schmidt et al., 2000) and in addition gene expression of the Fe uptake machinery is induced (Lucena et al., 2006; Waters et al., 2007; García et al., 2010). This effect could be reversed by application of ethylene inhibitors (Romera and Alcantara, 1994; Garcia et al., 2010; Lucena et al., 2006). Therefore, it can be concluded that ethylene is able to promote up-regulation of Fe acquisition responses (Figure 1.3). One possibility to transduce ethylene related incoming signals into the nucleus is a MAP kinase signalling cascade. As a result the transcription factor ETHYLENE INSENSITIVE 3 (EIN3) and its homolog EIN3-like1 (EIL1) bind to the DNA to initiate the transcription of certain ethylene response factors (ERF) (reviewed by Guo and Ecker, 2004). EIN3 stability is post-translationally controlled by the two F-box proteins EBF1 and EBF2. Protein abundance of EIN3 is tightly controlled by proteasomal degradation via the 26s proteasome pathway as a result of the interaction with SCF<sup>EBF1EBF2</sup> (Guo and Ecker, 2003; Potuschak et al., 2003; An et al., 2010).

Lingam et al. (2011) could recently show that EIN3/EIL1 can physically interact with FIT to induce the Fe deficiency response (Lingam et al., 2011). This interaction was found in a yeast two-hybrid screen and was confirmed by bimolecular fluorescence complementation (BiFC) in transiently transformed tobacco leaves. Moreover, they could show by using a polyclonal antibody raised against the c-terminal part of FIT that FIT protein stability is decreased in *ein3/eil1* double knockout mutants, indicating a stabilising effect of EIN3/EIL1 on FIT, via direct physical interaction. Similar to the ubiquitin/proteasome mediated degradation of EIN3 (Guo and Ecker, 2003; Potuschak et al., 2003) also FIT protein amounts seemed to be regulated via the 26s proteasome, because application of the proteasome inhibitor MG132 could restore FIT protein amounts in *ein3/eil1* knockout mutants (Lingam et al., 2011). This interaction between EIN3/EIL1 and FIT is a first proof where FIT was shown to be an integrator for signal transduction, to adapt Fe deficiency responses.

Besides ethylene a second signalling compound, namely NO does influence Fe uptake (Graziano et al., 2002; Graziano and Lamattina, 2007; Chen et al., 2010; García et al., 2010; Meiser et al., 2011 (provisionally accepted)). Recently it was discussed that NO and ethylene might not act in a hierarchical order rather than acting on each other to generate one common output signal (García et al., 2011; Figure 1.3). This output signal could then be transferred on FIT. However, direct evidence for such a molecular linkage is still missing.

- **Nitric oxide (NO)**

NO, comprises important functions in bacteria, animals and humans. NO as a plant signalling compound was first discovered to be produced after bacterial or viral infection (Delledonne et al., 1998; Durner et al., 1998). The nature of NO as a small, un-charged and diffusible molecule, makes it to a feasible signalling compound. NO presents different redox-related states, each of which is associated with specific reactions. These forms of NO include the uncharged nitric oxide (NO), nitroxyl anion (NO<sup>-</sup>) and the nitrosonium cation (NO<sup>+</sup>) (Stamler et al., 1992). NO reacts with metals to form metal-NO complexes, which then react with thiol groups to form S-nitrosothiols (e.g. S-nitrosocysteine, S-nitrosogluthatione and S-nitrosoalbumin) (Watts and Richardson, 2002). Today it is known that NO is involved in a wide range of mechanisms, such as germination, growth and development or reaction to biotic or abiotic stresses, including Fe deprivation (Feechan et al., 2005; Lindermayr et al., 2010; Sun et al., 2006; Desikan et al., 2004; Wendehenne et al., 2004; Delledonne, 2005; Graziano and Lamattina, 2007, Graziano et al., 2002, Chen et al., 2010; Garcia et al., 2011; Murgia et al., 2002; Arnaud et al., 2006). In contrast to common signalling molecules, NO as a diffusible gas and based on its chemistry, NO is unlikely to interact with a single defined receptor (Stamler et al., 1992).

Synthesis of NO in plants is not completely understood. There is evidence for both, a reductive and an oxidative pathway (reviewed in Moreau et al., 2009). S-nitrosogluthatione (GSNO) is a general storage and transport form for NO in animals and in plants (Zhang and Hogg, 2004). GSNO reductase can generate NO by breaking GSNO, thus having an impact on plant defence mechanisms by increasing or lowering the amount of free NO. This was shown by GSNO reductase mutants and reductase over-expression plants (Rusterucci et al., 2007).

A more concrete connection in NO signalling shows the ability of NO to trigger MAP kinase signalling pathways (Kumar and Klessig, 2000; Pagnussat et al., 2004) and the increase of

cyclic guanosine monophosphate (cGMP) and  $\text{Ca}^{2+}$  levels (Durner et al., 1998; Klessig et al., 2000; García-Mata et al., 2003; Wilson et al., 2008). It is known from mammalian cells that NO can regulate the production of cGMP by interacting with the Fe ion, present in the haem group of guanylate cyclase (Russwurm et al., 2004). Interestingly, in plants an increase of NO can also increase the cGMP levels (Durner et al., 1998). Hence, a NO derived signalling cascade mediated by the second messenger cGMP could be present in plants as well.

Interestingly there is also a connection between ROS and NO. ROS can also function as signalling molecules (Mittler et al., 2011). NO and superoxide anions can readily react with each other to form peroxynitrite ( $\text{ONOO}^-$ ) (Wilson et al., 2008). Although this substance is very toxic to many cells, plant cells seem to be somewhat resistant to it (Delledonne et al., 2001). Peroxynitrite can also function as a nitrosylating agent (Lindermayr and Durner, 2009).

The biological action of NO seems to be carried out mostly by post-translational modifications in way of metal nitrosylation (e.g. on haemoglobin or cytochrome), S-nitrosylation on cysteines or tyrosine nitration (Besson-Bard et al., 2008; Lindermayr and Durner 2009; Leitner et al. 2009; Moreau et al., 2009), whereas the main post-translational modification in plants, seems to take place via S-nitrosylation (Moreau et al., 2009). Lindermayr et al (2005) could identify over 100 Arabidopsis proteins that are targets for S-nitrosylation, extracted from GSNO treated cell cultures or NO-treated plants (Lindermayr et al. 2005). One very interesting example for protein S-nitrosylation was shown by analysing S-adenosylmethionine synthetase (SAMS). S-adenosylmethionine (SAM) is the precursor of ethylene biosynthesis. Ethylene was just explained before as an important component to regulate Fe homeostasis. S-nitrosylation of a cysteine, located next to the substrate binding site of SAMS (isoform 1) results in its inhibition (Lindermayer et al., 2006). As a consequence of decreased SAMS activity, ethylene biosynthesis will be inhibited due to less SAM. This example visualises the close connection of NO and ethylene and its possible impact on Fe homeostasis.

Graziano and Lamattina (2007) showed that Fe deficient tomato roots (*Solanum lycopersicum*) started NO production and that scavenging of NO resulted in inability to induce the Fe uptake machinery. Application of external GSNO showed enhanced expression of *LeFER*, *LeFRO1* and *LeIRT1*. Very interestingly NO was unable to induce the expression of *LeFRO1* and *LeIRT1* in the *fer* mutant, which indicates a fundamental function of *LeFER*, regarding NO driven Fe uptake response. This is in accordance with the hypothesis that FER as well as FIT are mandatory to trigger the Fe uptake response.

Similar results were obtained in Arabidopsis. Chen et al. (2010) could show that ferric chelate reductase activity was induced after application of GSNO and reduced after application of the NO scavenger cPTIO. The ferric reductase activity was also reduced in NO synthesis defective mutants. Restoration of the reductase activity in these NO defective mutants could be achieved by external application of GSNO (Chen et al., 2010). Although these results indicate a clear connection between NO and Fe uptake, a detailed molecular linkage (as it was shown for ethylene (Lingam et al., 2011)) that explains the relation between the signalling compound and the physiological output, is still lacking. Also in the light of NO signalling it can be suggested that FIT serves as an integrator, so that the different signals can be modulated at one common gate, namely FIT (Figure 1.3). It is possible that both transcriptional and post-transcriptional regulation act jointly to adapt Fe uptake to respond to the current plant Fe demand. These hints explain why FIT is the key transcription factor in Fe uptake and encourage to put more effort in understanding the underlying molecular regulatory mechanisms.

#### **1.2.4. Integration of signal transduction by post-transcriptional regulation**

The existence of different hormones acting on Fe uptake does still not answer how the original Fe deficiency signal that originates for example from the chloroplast can be transmitted into the root epidermis and how it can generate hormonal stimulation.

The most prominent response to incoming physiological stimuli such as changing nutritional supply is the adaptation of gene expression. Several transcriptomic attempts had been done in the past to understand global changes on Fe homeostasis (Colangelo and Gueriot 2004; Dinneny et al., 2008). However, such attempts rarely mirror final regulatory action which is carried out mainly by proteins. The origin of altered gene expression is caused by differentially regulated transcription factors at protein level that induce or repress gene expression. Protein translation of transcribed genes and protein activation, regulation and stability is also controlled by proteins. Because proteins execute physiological responses that can be measured at transcriptional level, the inspection of regulatory mechanisms acting at protein level (in particular regulation of transcription factors) is highly important to understand gene expression networks and global Fe homeostasis. Due to protein modifications cells are able to adapt order, timing and combinations of protein action by adding and removing different post-translational modifications, depending on the current demands. Compared to transcriptional adaptation, direct protein regulation is a powerful tool how cells can respond in a much faster way.

Protein regulation such as post-translational protein modifications open an enormous repertoire of regulatory options to react on the versatile incoming signals. Therefore it is important to investigate FIT protein regulation to understand how plant Fe uptake is regulated.

Specific post-transcriptional regulation in respect to Fe homeostasis could be attested by investigating FERRITIN 2 (FER2) and IRT1. FER2 was found to be post-translationally regulated in response to metal content, which was shown by using different mutants impaired in internal Fe distribution and storage (Arnaud et al., 2006; Ravet et al., 2009a; Ravet et al., 2009b). Regarding IRT1, Connolly et al. (2002) showed that plants over-expressing IRT1, contained IRT1 protein only in the root, irrespective of Fe supply, indicating post-transcriptional control of IRT1. In addition to this post-transcriptional control, two lysine residues of the large intracellular loop of IRT1 were important for proper IRT1 turnover. These lysine residues could serve as ubiquitin attachment sites to regulate IRT1 at post-translational level (Kerkeb et al., 2008). Ubiquitination of IRT1 was finally proven by Barberon et al. (2011). Moreover, the authors found that IRT1 is regulated by intracellular trafficking and that it is degraded in the vacuole in a proteasome independent pathway (Barberon et al., 2011).

The examples of FER2 and IRT1 demonstrate that signals, regulating Fe uptake and Fe homeostasis, can be integrated by way of Fe-dependent post-transcriptional regulation. Therefore regulation of FIT at post-translational level could also be suggested. However, still unclear is the connection to the original Fe deficiency signal and its transmission into the root. There might be a close connection between post-translational protein regulation and its respective signalling cascades entering the cell. General signalling pathways such as MAP kinase signalling and/or second messengers such as calcium and cGMP could also be feasible to regulate Fe uptake. This assumption is supported by the finding that NO, which is involved in regulating Fe uptake, can trigger MPK and calcium dependent signalling pathways (Durner et al., 1998; Klessig et al., 2000; García-Mata et al., 2003; Wilson et al., 2008).

It is interesting to note that ROS signalling has been proposed not only as a local signalling mechanism but also as a long distance signal from the root to the shoot (Miller et al., 2009; reviewed in Mittler et al., 2011). Because the Fe deficiency long distance signal is still not identified, it is very interesting that ROS has been shown to act as such a long distance signalling molecule. Oscillating ROS patterns have also been reported in particular for root hairs (Monshausen et al., 2007). The way how a long distance ROS signal could be transferred into a stimulus specific signal is still under investigation, but

different possibilities have been proposed by Mittler et al. (2011). Moreover, ROS could be linked to calcium and protein phosphorylation networks (Kobayashi et al., 2007; Ogasawara et al., 2008). Hence, ROS could function as an upstream activator to trigger second messengers and hormonal stimulation. A connection between Auxin, NO and ethylene in respect to ROS has been summarised by Mittler et al. (2011). Because especially NO and ethylene have important functions in regulating the Fe uptake response, a combination of ROS, second messengers and hormones seem to act in concert to regulate the Fe uptake in plant roots. An increase of ROS could be one possible mechanism to initiate (and integrate) an Fe deficiency signal. The rise of a ROS pulse could have final impact on gene expression and on protein regulation, by influencing the action of transcription factors.

#### **1.2.4.1. Post-transcriptional modifications of transcription factors in plants**

Proteins can be controlled by various and complex mechanisms. For example final protein assembly, protein activity, localisation or stability can be directly controlled at protein level. Regarding post-translational modification in plants, a wide range of different modifications has been reported (reviewed in Tootle and Rebay, 2005). Here, three of the major modifications that have further relevance in this work are presented.

##### **• Ubiquitination**

The role of ubiquitination for protein interaction, internalisation and degradation is known for many years and has been demonstrated also in plants (reviewed in Mukhopadhyay and Riezman, 2007; Gohre et al., 2008; Gimenez-Ibanez et al., 2009; Barberon et al., 2011). Mechanistically three enzymes are required to carry out ubiquitination of target proteins. The ubiquitin activating enzyme E1 binds the c-terminal glycine of ubiquitin at its carboxyl group under ATP hydrolysis. The ubiquitin molecule is covalently bound via a thiolester intermediate on a sulphur residue of a cystein, present in E1. In a second step the activated ubiquitin is transferred to a cystein of an ubiquitin-conjugating enzyme E2 (UBC). The transfer of ubiquitin from E2 to a  $\epsilon$ -NH<sub>2</sub> group of a lysin of the target protein is subsequently catalysed by an ubiquitin-protein ligase enzyme E3.

In the case of poly-ubiquitination at one lysin, the c-terminus of the next ubiquitin is covalently bound to one of the seven lysines present in the previously bound ubiquitin. Proteins marked for degradation by ubiquitin can be degraded by the 26S proteasome

containing ATP dependent proteases. Ubiquitin itself is not degraded by the 26S proteasome and can be reused in the cell (Ciechanover et al., 1998). Substrate specificity of ubiquitinating enzymes is reached by different E2 and E3 enzymes, which recognise specific target proteins (Ciechanover et al., 1998; Hershko and Ciechanover, 1998).

Recognition of proteins for ubiquitination can depend on different reasons. On the one hand mis-folding of a protein can result in a degradation signal, on the other hand certain modifications such as phosphorylation can be a molecular switch to initiate ubiquitination. Ubiquitination as a consequence of phosphorylation has been well documented by investigating the light perception pathway in Arabidopsis. Phytochrome interacting factors (PIFs) are negatively acting transcription factors. These transcription factors, belonging to the bHLH family, are stable in the dark and degraded in response to light. All PIFs except PIF7 are phosphorylated and subsequently ubiquitinated prior their degradation (Shen et al., 2007, 2009; Al-Sady et al., 2006).

Fe related ubiquitination in plants was found by investigating the lysine 63-linked ubiquitin conjugase, UBC13, that was identified by its accumulation in response to Fe deficiency in cucumber (*Cucumis sativus*). Two homologues of cucumber *UBC13*, namely *UBC13A* and *UBC13B* have been identified. The double knock out mutant *ubc13aubc13b* shows abnormal Fe deficiency responses, which include the enhanced regulation of *IRT1* and *AHA2*, together with increased Fe reductase activity (Li and Schmidt, 2010). However, direct targets of UBC13A/B have not yet been identified.

As already described before, *IRT1* is also targeted by ubiquitination. Recently, Barberon et al. (2011) showed that *IRT1* is monoubiquitinated at several cytosol-exposed residues and that this post-translational modification controls internalisation/sorting and turnover of *IRT1* to control the *IRT1* pool at the plasma membrane. Moreover the authors could show that ubiquitinated *IRT1* is degraded in the vacuole in a proteasome-independent manner. This example demonstrates further ubiquitin related mechanisms besides proteasome dependent degradation of proteins.

#### • **S-nitrosylation**

Hormonal influence by NO often results in reversible S-nitrosylation of cysteine residues of target proteins (Lindermayr and Durner, 2009; Besson-Bard et al., 2008). The resulting S-nitrosothiols can be removed by several enzymes such as the enzymes GSNO reductase or thioredoxin (Jourdeuil et al., 1999; Feechan et al., 2005; Liu et al., 2001; Tada et al., 2008; Benhar et al., 2008).

The exact mechanisms of S-nitrosylation is still under discussion. Two main pathways have been described by Lindermayr and Durner (2009). (i) a reaction between a thiyl radical and a NO radical (Lancaster, 2006) (ii) nitrosylation of thiols by different nitrogen forms such as peroxyxynitrite, metal-NO complexes (present in haemoglobin or cytochrome c oxidase) or by a nitrosonium cation (Viappiani et al., 2009; Viner et al., 1999; Paolocci et al., 2000; Ferdinandy et al., 2006; Lane et al., 2001; Lindermayr and Durner 2009; Hess et al., 2005).

NO driven S-nitrosylation of proteins can prevent the formation of a disulphide bridge with neighbouring cysteines, thus influencing the three dimensional structure of a protein (Besson-Bard et al. 2008). Moreover, such modifications can be sensors for protein trafficking within the cell and can alter the protein activity (Lindermayr et al., 2010; Tada et al., 2008). So far only a few plant proteins are known to be S-nitrosylated. However, in the recent years, NO received much attention for its involvement in plant physiology. One target protein for S-nitrosylation is NPR-1 (NONEXPRESSOR OF PATHOGEN RELATED GENE 1), the key regulator in salicylic-acid response (Lindermayr et al., 2006; Belenghi et al., 2007; Tada et al., 2008). NPR1 monomers can interact with TGA1 (TGACG motif binding factor 1) to bind to the promoter region of defence proteins. Lindermayr et al. (2010) could show that also TGA1 is a target for S-nitrosylation, which facilitates its nuclear localisation, protects the protein from oxygen-mediated modifications and enhances the DNA binding activity in the presence of NPR1 (Lindermayr et al., 2010).

There is no Fe related S-nitrosylation of any protein known. But since NO is involved in regulating Fe homeostasis, S-nitrosylation could be suggested as one way to regulate Fe homeostasis at post-translational level. Hence, transcription factors such as FIT could be possible targets.

### • Phosphorylation

A very common modification, which is ubiquitous throughout all living organisms is the phosphorylation of proteins, either at tyrosine, serine, threonine or histidine. All four amino acids contain a hydroxyl group that can be covalently linked to a phosphate group by ester bond. Phosphorylation often results in altered protein localisation, stability or interaction (Yoo et al., 2008; Ebisuya et al., 2005). In particular, phosphorylation of transcription factors, can result in altered gene expression of downstream genes (He et al., 2002; Tootle et al., 2005). The phosphorylation of target proteins can result in both stabilisation or destabilisation. In the case of the light in-stable bHLH proteins PIF5 and PIF3,

phosphorylation resulted in ubiquitination and subsequent degradation (Shen et al., 2007; Al-Sady et al., 2006). In contrast, transcription factors such as *HY5* or *HFR1* acting positively in the light perception are degraded in the dark and stabilised in the light. CKII (CASEIN KINASE II) has been shown to phosphorylate the light stable transcription factor *HY5*. Phosphorylation of *HY5* increases its stability (Hardtke et al., 2000). PIF1 was also shown to be phosphorylated by CKII. However, phosphorylation of PIF1 did not result in enhanced stability, but rather in subsequent ubiquitination and degradation (Bu et al., 2011). Therefore phosphorylation of proteins can have different fates.

Often proteins contain more than one phosphorylation site. Depending on the phosphorylation, the activity of the protein may vary. This was shown for the bHLH transcription factor SPCL (SPEECHLESS), which regulates stomatal development in *Arabidopsis* leaves. Lampard et al. (2008) found that SPCL contains five important phosphorylation sites in a specific region, located between the bHLH region and the c-terminal part of the protein. Depending on the specific phosphorylation, the physiological output was either promotion or repression of stomatal development (Lampard et al., 2008). Therefore, a single protein can contain several phosphorylation sites and both, phosphorylation or de-phosphorylation can result in physiological activity.

In addition to simple activation or de-activation phosphorylation can also be an intracellular trafficking signal. For instance the Fe related yeast transcription factor Aft1p is located either in the cytoplasm or in the nucleus, depending on its phosphorylation status (Casas et al., 1997; Curie and Briat 2003).

Depending on the target amino acid, phosphorylation is carried out by serine/threonine (Ser/Thr) specific kinases or by tyrosine (TYR) specific kinases (Stone and Walker, 1995). Further subdivision was made to classify the kinase superfamily into five groups based on phylogenetic analysis: (i) the AGC group consisting of the cyclin nucleotide-dependent family (PKA and PKG), the PKC family and the ribosomal S6 family; (ii) the CaMK group, consisting of calcium/-calmodulin-dependent kinases and the SNF1/AMP-activated protein kinases; (iii) the CMGC group, containing the CDK, the MAPK, GSK-3 and CKII families; (iv) the PTK (Protein Tyrosine Kinase) group; (v) „other“. This group contains unique protein kinases such as CTR1 belonging to the Raf family. CTR1 is involved in the ethylene signalling pathway (Huang et al., 2003). However, it remains worth to mention that some CIPKs apparently have a predicted tyrosine kinase activity (www.ncbi.com, www.arabidopsi.org), which indicates that some Ser/Thr kinases have in addition putative Tyr kinase activities.

The Mitogen-activated protein kinases (MAPK) belonging to the CMGC group, generally function in signalling modules that transduce extracellular stimuli to a range of cellular responses, including the regulation of the activity of transcription factors (reviewed in Fiil et al., 2009). MAPKs are themselves activated by phosphorylation and act independent of second messengers (Widmann et al., 1999; Luan et al., 2008).

The classical signalling mechanism, how MAPK cascades are carried out involve a stimulus triggered activation of a MAPK kinase kinase (MEKK) which phosphorylates a MAPK kinase (MKK or MEK), that in turn phosphorylates a MAP kinase (MPK). As a result of an activated MPK, intracellular localisation, interaction and phosphorylation of transcription factors can be affected, leading to altered gene expression (Fiil et al., 2009). The genome of Arabidopsis encodes about 60MEKK, 10MKKs and 20 MPKs (Ichimura et al., 2002). A high stringency recognition site for MPKs is the motive Pro-X-Ser/Thr-Pro (P-X-S/T-P) (Widmann et al., 1999). Recent studies provide evidence that MAPK cascades are involved in several developmental and stress responses (reviewed in Fiil et al., 2009). For instance the bHLH protein SPCL is targeted by phosphorylation events, which were transduced by MKK4/5 and MPK3 and MPK6 (Lampard et al., 2008).

Moreover the ethylene biosynthesis protein 1-AMINOCYCLO-PROPANE-1-CARBOXYLIC ACID (ACC) synthase 2/6 was shown to be phosphorylated by MPK6, which resulted in increased ethylene signalling (Joo et al., 2008) and in addition EIN3 has also been shown to be regulated by MPKs (Yoo et al., 2008).

As described before, EIN3/EIL1 was shown to interact with FIT (Lingam et al., 2011). This example may indicate that hormonal stimulation and signal transduction in way of MPK signalling could be connected with each other to trigger downstream responses acting on the Fe deficiency response. Such a downstream regulation of the Fe reductase *FRO2* and Fe transporter *IRT1* may be maintained by post-transcriptional regulation of FIT in addition to its transcriptional induction under Fe deficiency. Post-transcriptional regulation of FIT is likely to be influenced and initiated by plant hormones and signalling compounds. Investigating the post-transcriptional regulation of FIT and the impact of plant signalling compounds (such as NO) on FIT abundance is necessary to unravel how Fe uptake is regulated and sensed in plants.

## 2. Aims of this work

Post-transcriptional regulation of *FIT* was suggested by Jakoby et al. (2004) and Colangelo and Guerinot (2004) by using untagged *FIT* over-expression lines. They showed that over-expression of *FIT* resulted in high mRNA amounts but the induction of the target genes *FRO2* and *IRT1* remained unchanged in roots, compared to wild type. In leaves, over-expression of *FIT* could induce ectopic expression of *FRO2* and *IRT1* under Fe deficiency (Jakoby et al., 2004). To understand the regulation of Fe uptake in detail, analysis of *FIT* at protein level is highly demanding. The aim of this work was to investigate post-transcriptional regulation of *FIT*. Fe dependent mechanisms acting on *FIT* should be identified and the ability of *FIT* to regulate the Fe deficiency response was focused in this work. Different tools should be developed to monitor *FIT* protein regulation *in planta*. In order to fulfil our aims, we have defined the following objectives:

### 2.1. Generation and characterisation of HA tagged *FIT* lines

To investigate protein abundance and regulation of *FIT* in respect to Fe, HA-*FIT* fusion gene constructs should be created and used to transform Arabidopsis plants. In order to investigate *FIT* protein abundance *in vivo*, homozygous T3 lines should be generated. The selected HA-*FIT* over-expression lines should be characterised with respect to the regulation of Fe deficiency responses and Fe uptake.

Complementation of *fit-3* mutant plants should be performed to show functionality of the fusion proteins.

### 2.2. Monitoring of *FIT* turnover on protein level to investigate whether and how *FIT* is post-transcriptionally regulated

To understand how the induction of *FRO2* and *IRT1* is regulated, *FIT* protein abundance and turnover in respect to Fe supply should be monitored by western blot experiments. This way it should be analysed if over-expression of *FIT* results in constant *FIT* abundance and if *FIT* translation depends on Fe supply. To investigate protein regulatory mechanisms, the use of inhibitors of protein synthesis and degradation should be established in a reproducible manner. These methods should subsequently be used to investigate *FIT* turnover and stability.

Since NO was shown to affect gene expression of *IRT1* and *FRO2* it should be investigated if FIT protein was affected by NO signalling. By using NO donors and NO signalling inhibitors the influence of NO on FIT protein regulation should be investigated.

### **2.3. *FIT* mutagenesis for identification of post-translational modifications acting on FIT**

Since FIT was likely regulated post-translationally, *in silico* screening and sequence alignment should be used as a tool to identify possible sites in the FIT protein that could be targeted by post-translational modifications. In the case of NO dependent regulation of FIT, possible mechanisms acting on FIT in respect to NO should be proposed, to investigate the hypothesis of NO dependent post-translational regulation in the future. Moreover, phosphorylation as a putative post-translational regulator should be inspected, since this modification is omnipresent in all living organisms and because kinase pathways are very common in sensing external stimuli from the cell surface into the nucleus. Phosphorylation was shown to be important for the regulation of bHLH proteins before (Lampard et al., 2008; Shen et al., 2007; Miura et al., 2011). Since no upstream regulator of FIT is known and since FIT seems to be the major key transcription factor in regulating Fe uptake in Arabidopsis, it could be possible that kinase signalling (and therefore phosphorylation) could be one mechanism acting on FIT to modulate the Fe deficiency response.

In further steps, putative target sites should be identified and altered by PCR mutagenesis to investigate the effects of non-phosphorylatable and phosphomimicking FIT mutants, in way of complementation efficiency, protein abundance and their ability to trigger the downstream responses of FIT. In order to fulfil this aim a set of different FIT constructs should be cloned and transferred into Arabidopsis to generate homozygous T3 lines harbouring the altered FIT gene constructs.

## 3. Material and Methods

### 3.1. Material

#### 3.1.1. Plant material:

- *Arabidopsis thaliana* ecotype Columbia (Col-0) has been used as wild type
- Arabidopsis T-DNA insertion line *fit-3* described in Jakoby et al. (2004).

#### 3.1.2. Bacterial strains for molecular cloning

- *E. coli*, *ccdB* one shot survival T1-Phage resistant cells (Invitrogen)
- *E. coli* INVαF' (Invitrogen) F' endA1 recA1 hsdR17 (rk-, mk+) supE44 thi-1 gyrA96 relA1 φ80lacZ\_M15\_(lacZYAargF) U169 λ-
- *Agrobacterium tumefaciens*: C58C1; GV2260/pGV2260; Cn<sup>R</sup>, Rif<sup>R</sup>

#### 3.1.3. Plasmids

- Non Gateway Vector: pPILY (Ferrando et al., 2000)
  - Size: 4.43 kb
  - Selection marker: Amp<sup>R</sup> transformation control in bacteria
  - Can be used for transient plant transformation via particle bombardment
- Entry Vector for Gateway cloning:
  - pDONR207 (Gateway cloning system, Invitrogen)
    - Size: 5.5 kb
    - Selection marker: *ccdB* suicidal gene: insertion control, Gm<sup>R</sup>: transformation control in bacteria
- Destination Vector for Gateway cloning:
  - pMDC32 (Curtis and Grossniklaus, 2003)
    - Size: 11.7 kb
    - Binary destination vector for over-expression of gene-of-interest
    - Selection marker: *ccdB* suicidal gene: insertion control, Kan<sup>R</sup>: transformation control in Bacteria, Hygromycin<sup>R</sup>: transformation control in plants
  - pMDC83 (Curtis and Grossniklaus, 2003)

- Size: 12.5 kb
- Binary destination vector for over-expression of gene-of-interest-GFP (N-terminal fusions)
- Selection marker: *ccdb* suicidal gene: insertion control, Kan<sup>R</sup>: transformation control in Bacteria, Hygromycin<sup>R</sup>: transformation control in plants
- pAlligator2
  - Size: 11kb
  - Binary destination vector for over-expression of HA<sub>3</sub>-gene-of-interest (C-terminal fusion)
  - Selection marker: *ccdb* suicidal gene: insertion control, Spc<sup>R</sup>: transformation control in bacteria, GFP fluorescence driven by the seed storage promoter At2s3 for transformation control in plants.

#### **3.1.4. Antibodies**

- rat IgG monoclonal anti HA antibody clone 3F10 (Roche) for detection of HA tagged proteins (used 1:1000)
- mouse IgG monoclonal anti GFP antibody clone 7.1 (Roche) for detection of GFP tagged proteins (used 1:500)
- polyclonal goat anti rat horseradish peroxidase secondary antibody (Sigma Aldrich) for detection of anti HA antibodies (used 1:10000)
- polyclonal goat anti mouse horseradish peroxidase secondary antibody (Pierce) for detection of GFP antibodies (used 1:5000)

#### **3.1.5. Software**

- LSM software was used for confocal imaging and picture analysis ([www.zeiss.de/micro](http://www.zeiss.de/micro))
- DNASTAR was used for primer design and alignment ([www.dnastar.com](http://www.dnastar.com))
- NetPhos database for prediction of phosphorylation sites (<http://www.cbs.dtu.dk/services/NetPhos/>)

### 3.1.6. Oligonucleotides

**Table 3.1:** List of oligonucleotide sequences for PCR reactions.

<b>FIT basic cloning</b>	<b>5' to 3' sequence</b>
5'Sall-FIT	GAGTCGACGATGGAAGGAAGAGTCAACGC
3'PstI-FIT	CACTGCAGTCAAGTAAATGACTTGATGAATTCAA
5'attB1_7xHA	GGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCGCCC GGGT
3'attB2_FIT	GGGACCACTTTGTACAAGAAAGCTGGGTTCAAGTAAATGA CTTGATGAATTCAA
5'attB1_FIT	GGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGAAGGAA GAGTCAACGC
att_L1	TGTTTCGTTGCAACAAATTGATGAG
att_L2	TAATGCCAACTTTGTACAAGAAAGCTG
attB1-HA3	GGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCATAACC CATAACGACGT
Seq.primer pAlligator2	GAAGGCGGGAAACGACAATC
3'attB2_STOP_FIT	GGGACCACTTTGTACAAGAAAGCTGGGTTTATCAAGTAAAT GACTTGATGAATTCAA
<b>Primer for genotyping</b>	<b>5' to 3' sequence</b>
FIT-rev1	5'CATACAACACTGCATCTCCAACAA3'
FIT 3' AtFRUN3'	5'-GGTCCCATCATTATTCGTAG-3'
PyroseqAtfer 5'bHLH	5'-TTGAAGCTTCTTTAAACTCT-3'
FRU 5'166-187	5'-CCCTGTTTCATAGACGAGAAC-3
5'35s	5'-ATCCCACTATCCTTCGCAAGACCC-3'
<b>Primer for Mutagenesis</b>	<b>5' to 3' sequence</b>
FlmS221A-1	TATCAATCCTCCTGCAGCCAAAAAATCATTCA
FlmS221A-2	TGAATGATTTTTTTGGCTGCAGGAGGATTGATA
FlmS221E-1	TATCAATCCTCCTGCAGAGAAAAAATCATTCA
FlmS221E-2	TGAATGATTTTTTTCTCTGCAGGAGGATTGATA
FlmY238F-1	GAGGAGAAAGGGTTTTITGTGAGATTGGTGTGT
FlmY238F-2	ACACACCAATCTCACAAAAACCCTTTCTCCTC
FlmY238E-1	GAGGAGAAAGGGTTTGAAGTGAGATTGGTGTGT
FlmY238E-2	ACACACCAATCTCACTTCAAACCCTTTCTCCTC
FlmSS271AA-1	CAGAACTCTAACCTAGCCGCTCCTTCTCCGGACA
FlmSS271AA-2	TGTCCGGAGAAGGAGCGGCTAGGTTAGAGTTCTG
FlmS272E-1	AACTCTAACCTAAGCGAACCTTCTCCGGACACA
FlmS272E-2	TGTGTCCGGAGAAGGTTTCGCTTAGGTTAGAGTT
FlmY278F-1	CCTTCTCCGGACACATICCTCTTAACATATAACC
FlmY278F-2	GGTATATGTTAAGAGGAATGTGTCCGGAGAAGG
FlmY278E-1	CCTTCTCCGGACACAGAGCTCTTAACATATAACC
FlmY278E-2	GGTATATGTTAAGAGCTCTGTGTCCGGAGAAGG

## 3.2. Methods

Standard methods such as PCR, Western blot analysis, enzymatic DNA digestion and DNA and protein gel electrophoresis were performed according to manufacturer`s instructions and standard protocols (Sambrook et al., 1989).

### 3.2.1. Plant material and growth conditions

For physiological assays seeds were surface sterilised as in Jakoby et al. (2004).

- In the 2-week growth system, plants were grown for fourteen days on square plates placed at 21°C/19°C and 16 h light, 8 h dark cycles (long-day condition) in plant chambers of *CLF Plant Climatics*. For Fe deficiency treatment, plants were transferred to 0 µM Fe (- Fe) Hoagland agar plates containing 50 µM ferrozine, for three days.
- In the 6-day growth system seeds were directly germinated on 50 µM (+ Fe) or 0 µM Fe (- Fe) Hoagland agar medium and were grown at long-day conditions.
- In the hydroponic growth system plants were grown to the age of 4 weeks in hydroponic ¼ strength Hoagland medium containing 10 µM Fe (+ Fe), then transferred for one week to 10 µM Fe (+ Fe) or 0 µM Fe (- Fe) as described in Klatte et al., (2009).

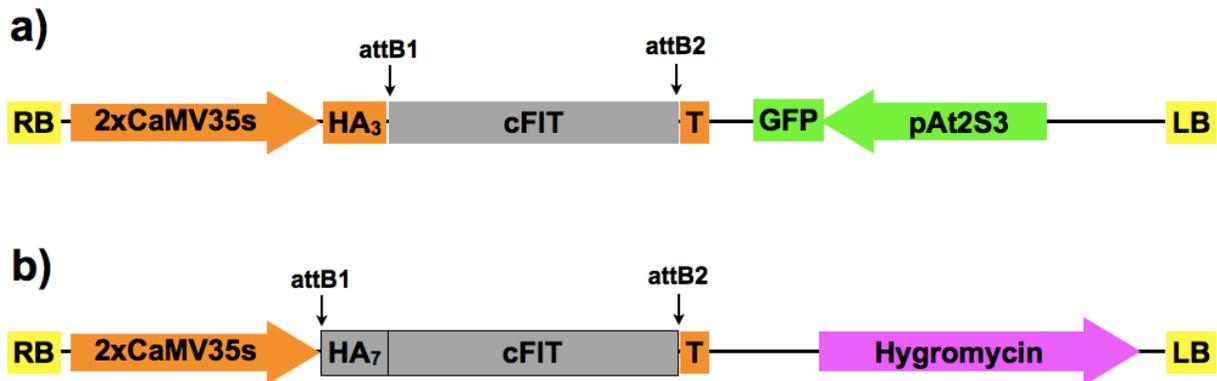
Following Hoagland salt concentrations have been used for the preparation of Hoagland medium: 0.1875 mM MgSO<sub>4</sub> x 7 H<sub>2</sub>O, 0.125 mM KH<sub>2</sub>PO<sub>4</sub>, 0.3125 mM KNO<sub>3</sub>, 0.375 mM Ca (NO<sub>3</sub>)<sub>2</sub>, 12.5 µM KCL, 12.5 µM H<sub>3</sub>BO<sub>3</sub>, 2.5 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. pH has been set to 6.0.

### 3.2.2. Generation of gene constructs

#### 3.2.2.1. Generation of HA-FIT

Two different HA-FIT over-expression constructs were generated. First, *FIT* cDNA was amplified and flanked by attachment sites B1 and B2. The PCR product was gel purified and introduced in a BP reaction into pDONR207 by Gateway cloning (based on homologues recombination) (Figure 3.3a). Empty gateway vectors contain a suicidal gene between the attachment sites that will be exchanged by the gene of interest during the BP reaction. Hence, only bacteria that contain a vector with gene of interest are viable. The FIT sequence was then transferred (by LR reaction) from the entry vector into the binary

destination vector pAlligator2 to obtain a p2xCaMV35S::HA<sub>3</sub>-FIT fusion construct (Figure 3.1a, 3.3a).



**Figure 3.1: T-DNA containing HA<sub>3</sub>-FIT and HA<sub>7</sub>-FIT gene constructs**

a) FIT cDNA was cloned in frame behind the HA<sub>3</sub> tag of the binary destination vector pALLIGATOR2. GFP expression, driven by pAT2s3 can be used for selection of transgenic dry seeds. b) FIT was fused with an HA<sub>7</sub> tag at the N-terminal part (by sub-cloning into pPILY vector) and transferred into the Gateway cloning site of the binary destination vector pMDC32. Selection marker in transgenic plants is Hygromycin. Both gene constructs are driven by a 2xCaMV35s promoter. Transcription is stopped by a terminator (T). Attachment sites (attB) are indicated. The inserted DNA fragment (that was transferred into the Plasmid by Gateway cloning) is represented in grey. Right (R) and left (L) border (B) of the T-DNA is indicated in yellow. The complete T-DNA was transferred into the plant by *Agrobacterium* mediated infection.

Second, *FIT* cDNA was amplified by PCR flanked by Sall and PstI restriction sites and inserted by restriction-ligation into pPILY, harbouring a HA<sub>7</sub> sequence for N-terminal fusion. The obtained HA<sub>7</sub>-FIT construct was PCR amplified and flanked by attachment sites B1 and B2 and then transferred by Gateway cloning (in a BP reaction) into pDONR207 and subsequently into the binary destination vector pMDC32 (in a LR reaction) to finally obtain a p2xCaMV35S::HA<sub>7</sub>-FIT fusion construct (Figure 3.1b, 3.3a). HA<sub>3</sub>-FIT and HA<sub>7</sub>-FIT were both verified by PCR, enzymatic digestion and sequencing (Diplomarbeit, Johannes Meiser). Both destination vectors were transferred into *Agrobacterium tumefaciens* strain GV2260 (containing pGV2260). Tobacco leaves were transiently transformed for verification of expression and translation (by Western Blot analysis) prior to Arabidopsis transformation (Figure 3.3b)

### 3.2.2.2. Generation of FIT-GFP

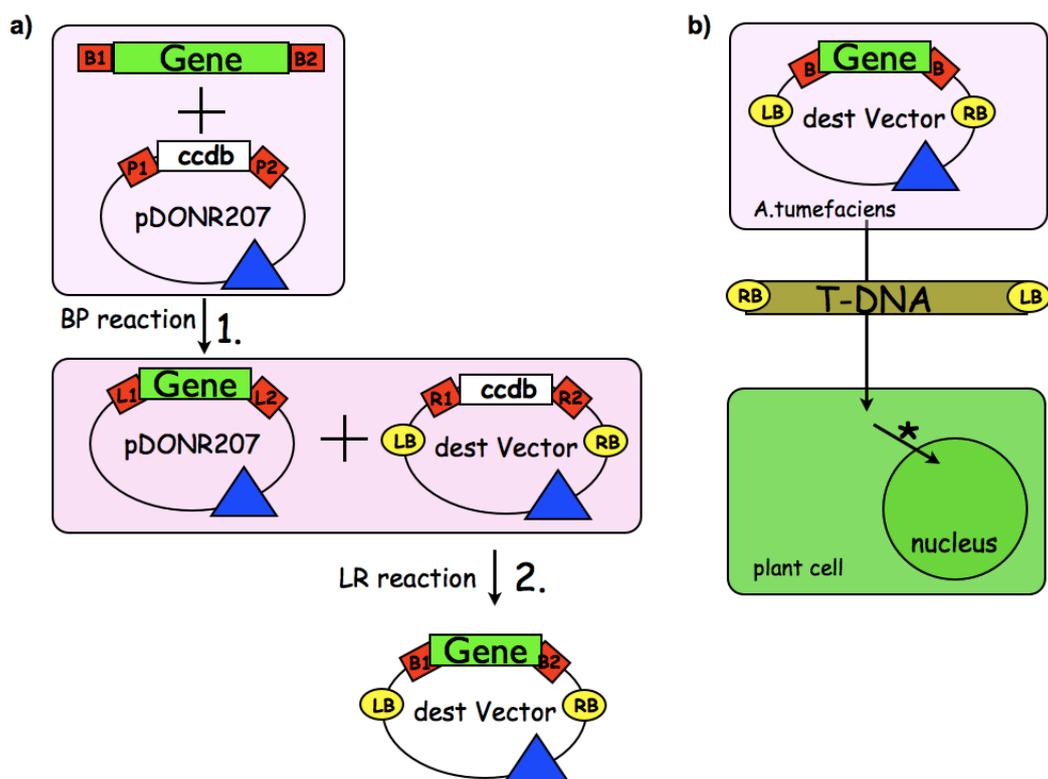
Generation of FIT-GFP fusion proteins was similar to the HA<sub>3</sub>-FIT construction, with slight modifications: genomic *FIT* DNA (instead of cDNA) was PCR amplified and flanked by attachment sites B1 and B2 and introduced by Gateway cloning into pDONR207 (in a BP

reaction). The FIT gene was transferred into the binary destination vector pMDC83 (in a LR reaction), harbouring a Gateway cloning site for c-terminal GFP fusions (Figure 3.2, 3.3a). The gene construct was transferred into *Agrobacterium tumefaciens* strain GV2260 (containing pGV2260) for subsequent plant transformations. Tobacco leaves were transiently transformed for verification of expression prior to Arabidopsis transformation (Figure 3.3b).



**Figure 3.2: T-DNA containing the FIT-GFP gene construct**

Genomic FIT DNA was amplified from genomic DNA and flanked by attachment sites for Gateway cloning. The PCR product was cloned into pDONR207 and then into pMDC83 to obtain genomic FIT fused to GFP at its c-terminal end. The FIT GFP construct is driven by the 2xCaMV35s promoter. Transcription is stopped by a terminator (T). The Hygromycin resistance (magenta) can be used for selection of transformed plants. Attachment sites (attB) are indicated. The inserted DNA fragment is represented in grey. Right (R) and left (L) border (B) of the T-DNA is indicated in yellow.



**Figure 3.3: Schematic illustration of the Gateway cloning strategy and *Agrobacterium* mediated plant transformation**

Gateway cloning is based on homologues recombination, by using commercially available „BP clonase“ and „LR clonase“ enzymes, respectively. a) To obtain the gene of interest with flanking attachment sites B1 and

B2, specific primers, containing the attachment sites B1 and B2, respectively were used to amplify the gene of interest in a PCR. This PCR product can then be transferred in a BP reaction (first reaction) into the entry vector (in this case pDONR207 was used). The vector contains an antibiotic resistance (represented as a blue triangle) that can be used as selection marker in bacteria. In addition, all Gateway vectors contain a suicidal gene (ccdb) between the attachment sites. This suicidal gene will be replaced by the gene of interest in case of successful recombination. Hence, only bacteria containing the vector with the gene of interest are viable. After the BP reaction the attachment sites are changed to specific „L sites“. In a second reaction (LR reaction), the gene of interest can be transferred from the entry vector into the binary destination vector. The empty destination vector contains also the suicidal gene, as well as an antibiotic marker for selection in bacteria. In the destination vector the Gateway cloning site is located within the T-DNA (for detailed T-DNA description see Figure 3.1, 3.2), which is flanked by right and left border (yellow circles). b) The destination vector containing the gene of interest can be transformed into *A. tumefaciens*. *A. tumefaciens* has the ability to transform plant cells, by transducing the T-DNA (present in the binary destination vector) into the plant cell. In case of transient transformation the T-DNA is present in the cytoplasm and will be transcribed and translated for a certain time, before the T-DNA will be degraded by plant defence mechanisms. In case of stable transgenic transformation, the T-DNA will be transduced into the nucleus (indicated by an asterisk above the arrow) and will be integrated into the host genome.

### 3.2.2.3. Mutagenesis

For FIT mutagenesis the pDONR207 vector containing genomic FIT DNA, was used as template. Mutations were introduced via PCR (primers are listed in table 2.1).

**Table 2.2: Thermoprofile of PCR:**

Initial denaturation	95°C	30''	
Denaturation	95°C	30''	
Annealing	55°C	1'	25x
Elongation	72°C	9'	
Final Elongation	72°C	7'	

To digest the input DNA without mutation (the input DNA contains methylations, the newly amplified DNA strands contain no methylations), 1µl DpnI was added to each PCR reaction and incubated for 1 h at 37°C. DpnI is a restriction endonuclease that recognises specifically the motif 5'-GATC-3' with a methylation at the adenine. Only methylated DNA strands are recognised and digested. As a result, all input DNA (without mutation) should have been digested by DpnI, whereas the newly amplified DNA (with mutation) was not recognised by DpnI. After enzymatic digestion, the PCR samples were separated in a 1 % agarose gel by electrophoresis. The obtained DNA separated at 5.5 kb and was excised

and re-isolated by gel extraction. The re-isolated plasmids were transferred into *E.Coli* (INVαF') by heat shock. The transformed bacteria were selected on gentamycin. Grown bacteria clones were inoculated in liquid culture and the plasmids were isolated. Correct mutations were verified by sequencing. Correctly mutated FIT versions, were subsequently transferred from pDONR207 into the binary destination vector pMDC83 (in a LR reaction). The destination vector harbouring the *FIT* gene was then transferred into *Agrobacterium tumefaciens* which were used for plant transformation.

### **3.2.3. Plant transformation**

#### **3.2.3.1. Transient tobacco transformation**

*Agrobacteria* were grown in a 3 ml LB culture over night at 28°C, 225 rpm containing acetosyringone (activates the *Agrobacteria* for plant infection), rifampicin, carbenicillin and the respective antibiotic of the binary vector (kanamycin in the case of pMDC vectors and spectinomycin in the case of the pAlligator2 vector). The bacteria were pelleted and taken up in infiltration medium (10 mM MgCl, 10 mM MES and 100 µM Acetosyringone). The bacteria were incubated for at least 1 hour at room temperature prior to infiltration with a 1 ml syringe into tobacco leaves (Figure 3.3b). 48 hours after transformation, leaves were used for analysis.

#### **3.2.3.2. Generation of stable transgenic Arabidopsis plant lines**

The transformation of *Arabidopsis thaliana* plants (ecotype Col-0 and *fit-3*) was performed following the floral dip method (Clough and Bent, 1998). *Agrobacteria* were grown in a 3 ml LB pre-culture over night at 28°C, 225 rpm containing rifampicin, carbenicillin and the respective antibiotic of the binary vector (kanamycin in the case of pMDC vectors and spectinomycin in the case of the pAlligator2 vector). The next day 500 ml main culture containing acetosyringone and the same antibiotics as the pre-culture, were inoculated by the pre-culture. The main culture was incubated at 28°C, 225 rpm until the bacteria had a optical density of 0.8 - 1. The bacteria were pelleted and taken up in infiltration medium (10 mM MgCl, 10 mM MES and 100 µM Acetosyringone, 1 % Sucrose, 0.5 % Silwet). The bacteria were incubated for at least 1 hour at room temperature prior to plant transformation (Figure 3.3b). Four week old Arabidopsis plants having closed floral buds were dipped for 30 seconds into the respective *Agrobacteria* suspension and were placed back into the growth room at long day conditions. Plants were covered with a plastic hood

for the next 24 h to increase humidity. Plants were grown for two more weeks at long day conditions, prior to plant harvest. Plants were dried at room temperature and dried seeds were harvested. Selection of transgenic seeds was based on GFP fluorescence in the case of pAlligator2 (transgenic seeds were showing GFP fluorescence, due to the *GFP* gene present in the T-DNA (Figure 3.1a)) and based on hygromycin resistance in the case of the pMDC transformants. (Figure 3.1b, 3.2) (seeds were germinated on + Fe Hoagland agar plates containing 15  $\mu$ M Hygromycin). Hygromycin blocks the protein biosynthesis, therefore root elongation of non resistant plants was inhibited (Harrison et al., 2006). Transgenic plants were selected by PCR (primers are presented in Table 3.1) and Western blot and multiplied to the T3 generation for homozygosity. For functional complementation analysis the *fit* mutant (Jakoby et al., 2004) was transformed with these constructs in the same manner.

#### **3.2.4. Gene expression analysis**

Gene expression analysis was performed by reverse transcription-quantitative real-time PCR as described in (Wang et al., 2007; Klatte et al., 2009). Briefly, DNase-treated RNA was used for cDNA synthesis. SYBR Green I-based real-time PCR analysis was performed using ExTaq RT-PCR (TaKaRa) in a “My IQ single colour real-time PCR detection system” (Biorad, USA). For each gene, the absolute quantity of initial transcript was determined by standard curve analysis. Absolute expression data were normalised against the averaged expression values of the internal control gene EF1BALPHA2 (EF). Primer sequences are published in Wang et al., (2007).

All steps of the established RT-qPCR were performed according to recommendations for accurate RT real-time quantitative PCR (Marco Klatte and Petra Bauer 2008, Plant signal Transduction, Methods in Molecular Biology, Issue 479).

#### **3.2.5. Metal measurement**

For metal determination plant material was harvested, dried over night at room temperature (RT) and for 1 d at 120°C in the oven. Roots were washed with 100 mM Ca (NO<sub>3</sub>)<sub>2</sub> before harvest to eliminate metal residues on the roots, deriving from the growth medium. For determination of metal contents dry plant material was powdered with an Ahart mortar. Metal contents were finally determined per g plant dry weight by graphite furnace atomic absorption spectrometry (GF AAS) at the Leibniz Institute für neue Materialien (INM, Saarbrücken).

### **3.2.6. Western Immunoblot analysis**

Western blot was done following the standard procedure described in Sambrook et al., (1989). Total protein extracts containing HA-FIT protein were separated in 10% SDS gels, total protein extracts containing FIT-GFP protein were separated in 8% SDS gels. For blocking and antibody treatments the SNAP ID system (Millipore, USA) was used according to the manufacturer's instructions. Use of antibodies is described in the material section. For detection of the Horse radish peroxidase coupled secondary antibody, the ECL Kit (Amersham, USA) was used. Films for detection of chemiluminescence were used from Amersham, USA.

### **3.2.7. Pharmacological treatments**

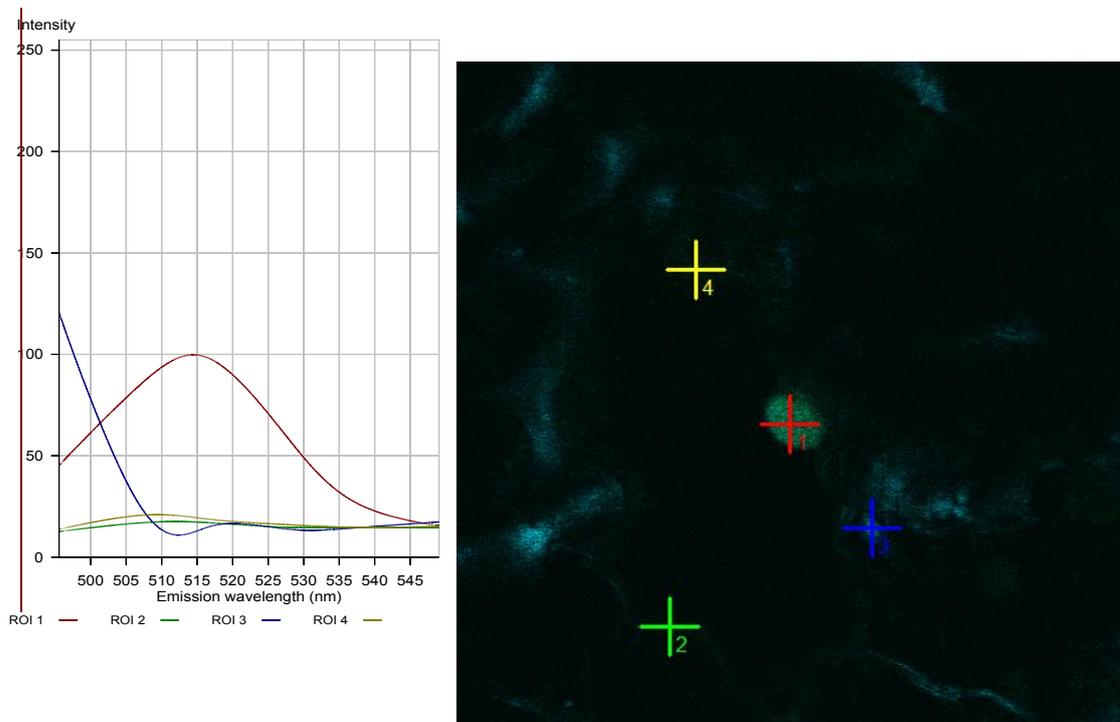
- For protein translation inhibition experiments using cycloheximide (CHX), plants were grown in the hydroponic growth system. Plants were transferred to liquid Hoagland medium containing 50  $\mu$ M cycloheximide (Sigma Aldrich) (1:1000 dilution from 50 mM stock solution dissolved in DMSO) and incubated for 1 hour. Roots and leaves were harvested either directly after treatment (0 time point) or roots were washed and transferred to fresh Hoagland medium for one to eight hour time points after the treatment as indicated. Roots and leaves were harvested separately and frozen in liquid nitrogen until further processing. Plants deriving from the 6-day agar growth system were transferred into six well plates containing the respective liquid Hoagland medium (+ or - Fe) with 50  $\mu$ M cycloheximide. Seedlings were incubated to the same time points as described above.
- Nitric Oxide (NO) experiments were conducted using the 6-day growth assay. 5 day-old seedlings were transferred to fresh 50  $\mu$ M or 0  $\mu$ M Fe Hoagland agar medium, containing as treatments 25  $\mu$ M NO donor S-nitrosoglutathione (GSNO, was synthesised as reported (Stamler and Loscalzo, 1992)) or 1 mM cell-permeating NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, Sigma-Aldrich), respectively. After 24 hour treatments, roots were harvested and further processed.
- For MG132 treatment 6 day old seedlings were incubated for 2.5 hours in liquid Hoagland medium with 42  $\mu$ M MG132 (1:1000 dilution from 42 mM stock solution diluted in DMSO) and subsequently quick frozen in liquid nitrogen for western blot or directly used for confocal imaging, respectively.

### 3.2.8. Confocal imaging

For confocal microscopy of FIT-GFP fusion proteins, a LSM510 confocal microscope was used (located at the department of Pharmacy, Saarland University).

Excitation wavelength: 488, detection filter: 500-530 nm.

For verification of the authenticity of the GFP, a lambda scan was performed and GFP signals were specifically detected at the expected wavelength of 510-520 nm (Figure 3.4).



**Figure 3.4: Lambda scan of FIT-GFP in transiently transformed tobacco leaf cells.**

The red cross (number 1) indicates the position where GFP signal was detected, showing an expected detection at 510 - 520 nm (left side image, red graph). Three randomly selected spots (green (number 2), blue (number 3), yellow (number 4)) were selected in the cell in addition to show that the detection of the GFP signal was specific for the location where GFP was detected.

## 4. Results

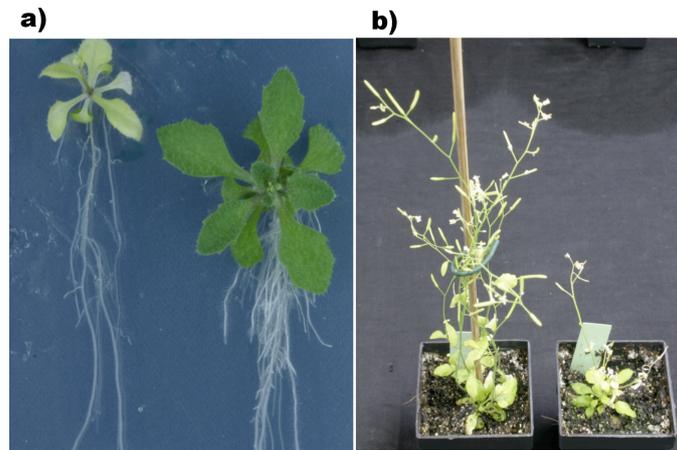
### 4.1. Investigation of FIT protein abundance and regulation (published in Meiser et al., 2011 (provisionally accepted))

#### Generation and characterisation of HA tagged FIT lines

The first hint about post-transcriptional regulation of FIT was provided by the observation that in FIT over-expression (FIT Ox) transgenic plants, up-regulation of Fe deficiency genes took place under – Fe but not under + Fe (Jakoby et al., 2004; Colangelo and Gueriot 2004). This observation was explained by either differential FIT protein abundance in response to Fe supply or by differential FIT protein activity. To monitor FIT protein abundance in respect to Fe supply, we generated hemagglutinine (HA) - tagged FIT Ox transgenic lines. 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 HA tag using specific commercial monoclonal antibodies (Lee et al., 2007). Two types of transgenic lines, harbouring either a triple HA tag (p2xCaMV35S::HA<sub>3</sub>-FIT, 10 lines) or a septuple HA tag (p2xCaMV35S::HA<sub>7</sub>-FIT, 8 lines) were generated.

Functionality of the HA constructs was confirmed in functional complementation experiments by transforming *fit* mutant plants with the respective constructs. *fit* seedling plants expressing HA<sub>3</sub>- or HA<sub>7</sub>-tagged FIT protein were fully complemented in their shoot appearance to wild type phenotype (Figure 4.1, shown for HA<sub>3</sub>-FIT). During the advanced reproductive stage complemented *fit* plants expressing HA<sub>3</sub>- or HA<sub>7</sub>-tagged FIT protein still showed a reminiscent leaf chlorosis. However, the transformed *fit* plants grew significantly better than the parent *fit* mutant plants without the transgene and were able to produce viable seeds without supplementation of Fe chelators. The functional role of *FIT*, namely regulation of Fe acquisition in roots, was restored by complementation. The reappearing leaf chlorosis in complemented *fit* plants can be explained by altered metal homeostasis due to ectopic expression.

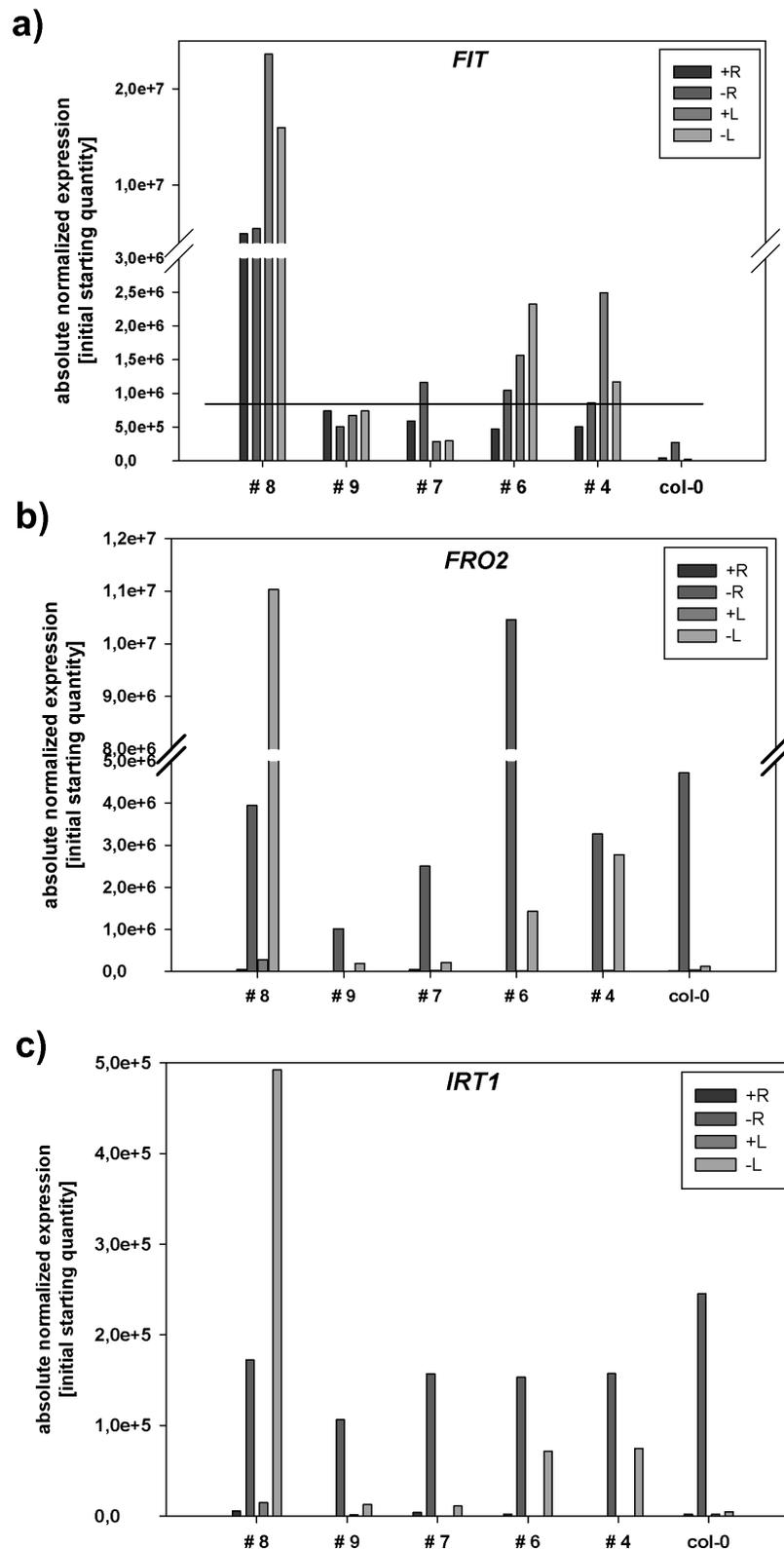
To further investigate functionality of the HA<sub>3</sub>- or HA<sub>7</sub>-tagged FIT protein downstream Fe deficiency responses were evaluated by reverse transcription-qPCR (Figure 4.2). For this purpose, three HA<sub>3</sub>-FIT Ox lines (named HA-FIT 4, HA-FIT 6, HA-FIT 9) and two HA<sub>7</sub>-FIT Ox lines (named HA-FIT 7, HA-FIT 8) were grown in the 2-week growth system and exposed to + or - Fe, respectively.



**Figure 4.1: Functional complementation of *fit* mutants by a HA<sub>3</sub>-FIT Ox transgene**

(a) Phenotypes of seedlings. left, original *fit* parent; right, *fit* complemented by HA<sub>3</sub>-FIT Ox; (b) Phenotypes of flowering plants. left, *fit* partially complemented with HA<sub>3</sub>-FIT Ox; right, original *fit* parent.

In these five HA-FIT Ox lines it was found that the amount of *FIT* transcripts, representing the internal *FIT* and the *FIT* Ox transcripts varied from line to line. Compared to Col-0 a clear over-expression was measured for all HA-FIT Ox lines. Line HA-FIT 8 had very strong over-expression of *FIT* in roots and leaves (ca. 120 times increase in + Fe roots, ca. 20 times increase in – Fe roots compared to the level of Col-0, respectively; ca. 1300 times increase in + Fe leaves, ca. 2700 times increase in –Fe leaves compared to the level of Col-0, respectively (Figure 4.2)), while lines HA-FIT 6 and HA-FIT 4 had weak over-expression in roots and intermediate levels of over-expression in leaves (ca. 12 times increase in + Fe roots, ca. 4 times increase in – Fe roots compared to the level of Col-0, respectively; ca. 90-140 times increase in + Fe leaves, ca. 200-400 times increase in – Fe leaves compared to the level of Col-0, respectively (Figure 4.2)). The lines HA-FIT 9 and HA-FIT 7 had weak *FIT* over-expression in roots and in leaves (ca. 14-17 times increase in + Fe roots, ca. 4 times increase in – Fe roots compared to the level of Col-0, respectively; ca. 15-40 times increase in + Fe leaves, ca. 50-120 times increase in – Fe leaves compared to the level of Col-0 (Figure 4.2)).



**Figure 4.2: Gene expression analysis of different HA-FIT Ox lines**

Reverse transcription-qPCR analysis of (a) FIT, (b) FRO2 and (c) IRT1; Col-0, two independent HA7-FIT lines (#7: HA-FIT 7, #8: HA-FIT 8) and three independent HA3-FIT lines (#4: HA-FIT 4, #6: HA-FIT 6, #9: HA-FIT 9) were analysed. Plants were grown in the 14-day agar growth system and exposed to + Fe (+) or – Fe (-). Roots (R) and leaves (L) were harvested separately and processed for experiments. The horizontal line (fictive line for visualisation) in (a) represents the threshold of FIT expression level, which was needed to obtain ectopic expression of IRT1 and FRO2 in leaves.

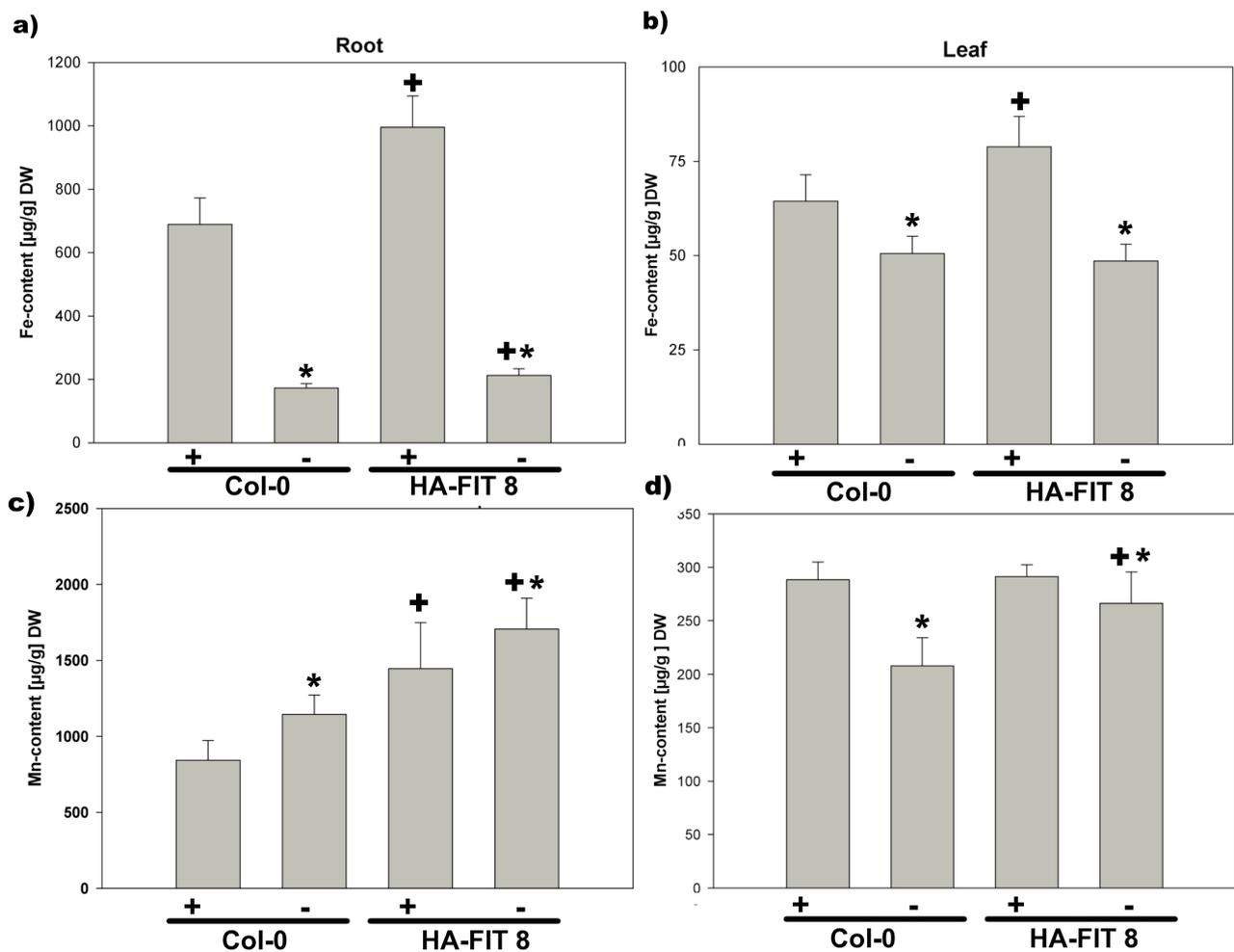
Interestingly, the expression levels of *FRO2* and *IRT1* in response to Fe deficiency were similar in the HA-FIT roots as in roots of wild type (Figure 4.2). Upon Fe deficiency, *IRT1* and *FRO2* were induced at least 30 times compared to + Fe (Figure 4.2). Therefore, these results confirmed that over-expression of *HA-FIT* did not consequently result in an over-expression of *IRT1* and *FRO2* in roots.

Previously, the effect of *FIT* over-expression in leaves was independently published by Jakoby *et al.* (2004) and Colangelo and Guerinot (2004). Jakoby *et al.* (2004) had noticed ectopic expression of *FRO2* and *IRT1* upon - Fe in leaves but not upon + Fe, whereas Colangelo and Guerinot (2004) did not observe any ectopic expression of downstream genes in leaves. This apparent contradiction was not clarified until today. When testing leaf expression in these five HA-FIT Ox lines we found that some of the HA-FIT lines, namely HA-FIT lines 4, 6 and 8, had clear ectopic *IRT1* and *FRO2* expression upon - Fe but not upon + Fe in leaves (Figure 4.2), whereas HA-FIT lines 7 and 9 did not show any ectopic induction of *FRO2* and *IRT1* (Figure 4.2). Untransformed Col-0 plants did not show *FRO2* and *IRT1* gene expression in leaves. The three lines with ectopic *IRT1* and *FRO2* expression in leaves at - Fe had intermediate to high *FIT* over-expression levels in leaves, while the two lines without ectopic expression had weak *FIT* over-expression levels in leaves.

We conclude from these results, that besides the obligatory - Fe condition a minimum threshold level of *FIT* gene expression needed to be reached in leaves, to obtain ectopic expression of *FRO2* and *IRT1*. The ectopic gene expression of *IRT1* and *FRO2* observed in HA<sub>3</sub>- and HA<sub>7</sub>-tagged *FIT* Ox lines reconfirmed functionality of the transgene. In addition strong *FIT* Ox lines such as HA-FIT 8, enabled investigation of *FIT* regulation in leaves independent from root factors. HA-FIT 8 and HA-FIT 9 were selected for further investigations and reverse transcription-qPCR experiments were therefore repeated to confirm our findings. Briefly, induction of *FRO2* and *IRT1* took only place under - Fe independent of the *FIT* amount and levels of *FRO2* and *IRT1* were similar to the ones in wild type in both HA *FIT* Ox lines (Figure 4.4c, d).

To test if over-expression of *FIT* had an effect on the metal contents in HA-FIT Ox plants, metal measurements were performed with HA-FIT 8 plants. Roots and leaves of plants were harvested after five weeks of growth in the hydroponic system. Because *IRT1* transports also other divalent metals such as Mn and Zn (Eide *et al.*, 1996), the Mn content was measured in addition to the Fe content (Figure 4.3). In wild type plants the Fe content of leaves and roots was decreased in response to Fe deficiency. Leaves and roots

of HA FIT 8 plants contained more Fe under Fe supply than the untransformed controls. Similar amounts were found in leaves under Fe starvation and slightly increased amounts in HA FIT 8 roots under Fe starvation. Mn contents were similar in leaves of HA FIT 8 and Col-0 upon + Fe but increased in HA FIT 8 plants upon – Fe. Mn contents were higher in roots of HA FIT 8 than in Col-0. These results indicate that FIT over-expression resulted in partially increased Fe and Mn uptake (Figure 4.3). The slightly increased Fe content in HA FIT 8 is in accordance with Jakoby et al. (2004) who found also higher Chlorophyll contents in their untagged *FIT* over-expression lines.



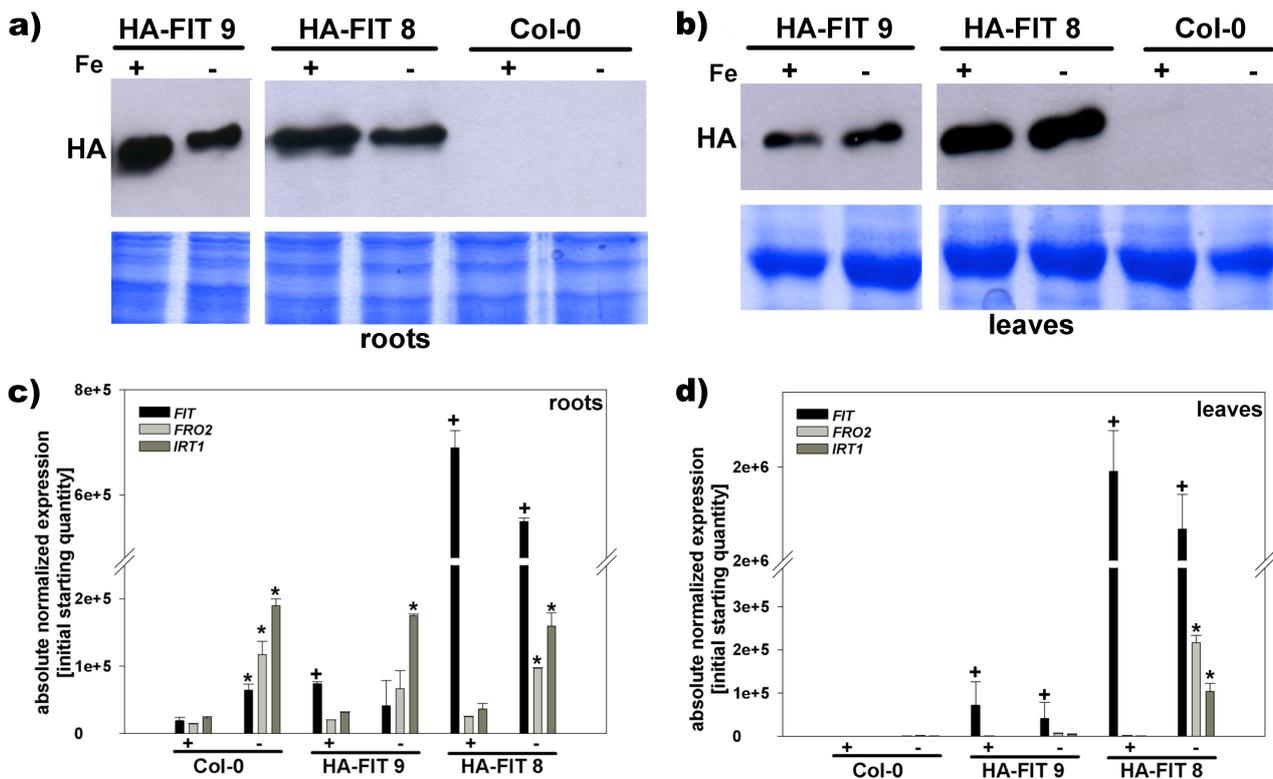
**Figure 4.3: Metal measurement of HA-FIT 8 and wild type (Col-0) plants**

(a, b) Fe and (c, d) Mn contents of (a, c) roots and (b, d) leaves of HA-FIT 8 plants and wild type plants were determined via GF AAS. Unpaired t-test was used to show significant differences ( $p < 0.05$ ). \* indicates significant change versus + Fe of respective plant line ( $p < 0.05$ ); + indicates significant change versus wild type control at respective Fe supply ( $p < 0.05$ );  $n = 5$ .

The increased Mn content was most probably a result of increased passive uptake by IRT1. The finding that *IRT1* and *FRO2* are expressed as in the corresponding wild type

plants (Figure 4.2, 4.4), independent of *FIT* over-expression, could explain that also the Fe contents do not dramatically differ from wild type plants.

To investigate protein abundance of HA-FIT protein, western blot experiments were performed. These experiments revealed that HA-FIT protein was present in roots and leaves of all lines independent of Fe supply (presented for HA-FIT 8 and HA-FIT 9 in Figure 4.4, 4.5), leading to the conclusion that FIT protein activity must be regulated on protein level, because an induction of *IRT1* and *FRO2* could only be measured under Fe starvation.



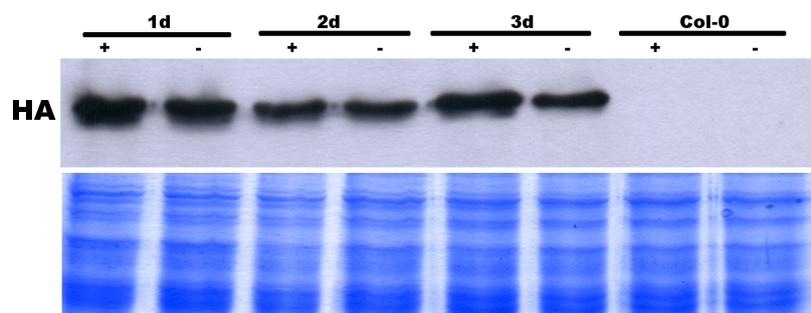
**Figure 4.4: Gene and protein expression analysis of HA-FIT Ox plants**

(a, b) Western blot analysis using anti-HA monoclonal antibody; the Coomassie-stained gel image serves as loading control. Samples were prepared from Col-0, HA-FIT 9 and HA-FIT 8. Plants were grown in the 14-day agar growth system. (c, d) Reverse transcription-qPCR analysis of *FIT*, *FRO2* and *IRT1*; Roots (a, c) and leaves (b, d) were harvested separately and processed for experiments. \* indicates significant change versus + Fe of respective treatment ( $p < 0.05$ ); + indicates significant change versus control at respective Fe supply ( $p < 0.05$ );  $n = 2$ .

In addition a time course experiment with roots of HA FIT 8 plants was performed to monitor HA-FIT protein abundance in respect to Fe deficiency (Figure 4.5). Plants were grown in the 14 day growth system and were harvested after one, two or three days of Fe deficiency and protein content was monitored by western blot experiments and compared to the respective + Fe root samples. All samples contained high amounts of HA FIT

protein. However, after three days of Fe deficiency the HA FIT content decreased, compared to + Fe (to a quantity of 72% compared to the corresponding + Fe), suggesting that HA FIT could be targeted more actively for regulation under - Fe conditions (see also Figure 4.4a and 4.6).

Moreover, a second, slightly smaller form of HA-FIT was present except of the sample that was exposed for three days to Fe deficiency (compare samples of 3d treatment +Fe and - Fe in Figure 4.5), which is the time when Fe deficiency becomes highly induced in Arabidopsis (Thimm et al., 2001). The disappearance of the second smaller protein band of HA FIT could be evaluated as a first hint for post-translational regulation of FIT in respect to Fe supply. A post-translational modification on FIT could result in a different gel mobility of FIT, compared to un-modified FIT.



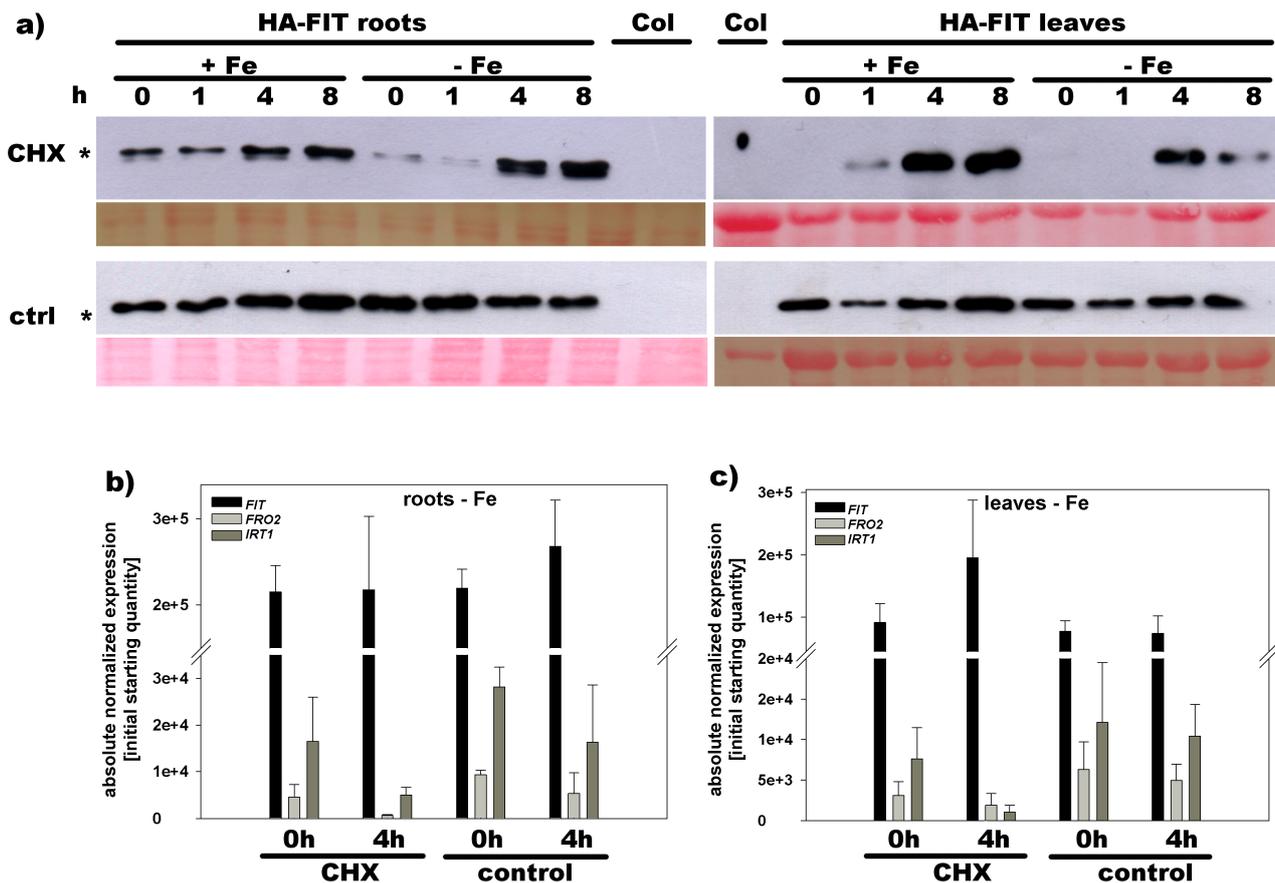
**Figure 4.5: Regulation of FIT protein in response to Fe supply**

FIT protein was detected by Western blot using anti HA antibody. Columbia-0 plants were used as negative control and show the specificity of the antibody. Plants were grown in the 6 day agar growth system and were transferred to Fe deficient medium for one, two or three days, respectively; the Coomassie-stained gel image represents the loading control. HA indicates the expected size of the HA-FIT protein, that was detected with the anti HA antibody.

Because *HA-FIT* was expressed by the strong 2xCaMV35s promotor, we were also interested in FIT protein abundance in wild type plants. Towards this end, an affinity purified polyclonal antibody against the c-terminal part of FIT was generated in our lab by Sivasenkar Lingam. Using this antibody we were able to show, that FIT was detectable under - Fe conditions but not under + Fe conditions (data presented in Lingam et al., 2011; Meiser et al. (2011) (provisionally accepted); see also Figure 4.7a). Having the results from the HA FIT Ox and the wild type plants we could conclude that in wild type plants FIT protein is only present in detectable amounts under - Fe conditions and that in addition, activity of FIT protein must be controlled at post-translational level, in respect to Fe supply, because over-expressing *HA FIT* plants contain HA-FIT protein under both + Fe and - Fe (Figure 4.4, 4.5).

## FIT protein abundance is controlled by active turnover

To investigate whether, despite of high protein abundance, HA-FIT protein was controlled by a turnover, the effect of cycloheximide (CHX) on HA-FIT protein was tested. HA-FIT plants (HA-FIT 8) were grown under + and – Fe in the hydroponic growth system. After an one hour treatment with CHX that inhibited protein translation, plant samples were harvested either immediately after the treatment (time point 0), or plants were retransferred to medium without CHX and harvested one hour, four hours and eight hours after retransfer to + or – Fe medium (time points 1, 4, 8).



**Figure 4.6: Effect of cycloheximide (CHX) on HA-FIT protein and gene expression**

(a) Western blot analysis using anti-HA monoclonal antibody in response to + or – Fe and + CHX or – CHX (ctrl), respectively. Ponceau-S staining served for loading control. The asterisk indicates the expected size of the HA-FIT protein. (b, c) Reverse transcription-qPCR analysis of FIT, FRO2 and IRT1 at – Fe in response to + or - CHX (control); HA-FIT 8 plants were grown in the hydroponic growth system. Roots (a (left side), b) and leaves (a (right side), c) were harvested separately and processed for experiments. After the one hour incubation at + or – CHX, samples were harvested directly after the treatment (0 time point) or one hour, four hours and eight hours after retransfer to + or - Fe growth medium without CHX (1, 4 and 8 h time points); col, root sample of untransformed Columbia-0 plants served as negative control. No significant changes could be detected in (b) and (c). n = 2.

Western blot experiments showed that up to one hour after the CHX treatment (time points 0 and 1), HA-FIT protein bands were less abundant than at four to eight hours after the treatment (time points 4 and 8) in both, roots and leaves (Figure 4.6). Very interestingly, in the – Fe root samples protein bands were less abundant than in the + Fe root samples, namely by a factor of three at time point 0 and by a factor of six at time point 1 (Figure 4.6 a). In the non-CHX treated controls, HA-FIT protein levels varied only slightly. In leaves, no such striking difference of HA-FIT protein bands could be observed upon CHX treatment between + and – Fe samples (compare Figure 4.6 a).

The findings of the CHX experiment allow the conclusion that indeed FIT was rather unstable and regularly degraded, so that the constitutive protein levels (observed in Figures 4.4, 4.5) were achieved through constant re-synthesis of new protein. In roots grown at - Fe, the degradation of HA-FIT was stronger, compared to + Fe, so that a decreased stability of FIT under – Fe conditions or alternatively less degradation of FIT under + Fe, can be inferred. This difference between + and – Fe seemed to be a root specific mechanism, since such a difference could not be observed in leaves. It was then interesting to determine whether CHX treatment also had affected Fe deficiency gene expression under – Fe. Using reverse transcription-qPCR, gene expression was measured at time point 0, where HA-FIT protein was reduced or not detectable and at the four hour time point, where HA-FIT had accumulated again similar to the control. *FIT* gene expression was at a constant high level in roots and leaves in both Fe supply conditions, irrespective of CHX treatment, as expected due to over-expression (Figure 4.6 b and c).

Gene expression of *IRT1* and *FRO2* was only induced under - Fe (+ Fe data not shown, presented as supplemental Figure in Meiser et al. (2011) (provisionally accepted), compare with Figure 4.2, 4.4). Although we could observe a tendency in three independent experiments that the expression of *IRT1* and *FRO2* was slightly reduced at the four hour time point of CHX treated plants, such changes were not significantly different compared to the control conditions, concluding that small amounts of FIT were still sufficient to trigger the Fe deficiency response.

In addition to the HA-FIT experiments we were interested in testing the effect of CHX on FIT and its downstream targets in wild type plant roots (Figure 4.7). Towards this end wild type plants were grown, treated and harvested as described before for HA-FIT 8 plants. In non-CHX-treated wild type roots FIT protein was detectable at – Fe at both time points (Figure 4.7a), but not at + Fe. Which was in accordance with previous findings (Lingam et al., 2011, Meiser et al., (2011) (provisionally accepted)). In CHX treated + Fe grown Col-0 roots FIT protein bands were not detectable. Upon – Fe, FIT protein was not detectable at

time points 0 and 4 after CHX treatment. Therefore the re-synthesis of FIT protein in Col-0 roots was blocked by CHX treatment, which confirmed FIT protein turnover as observed for HA-FIT. A difference to the HA-FIT results was that at the four hours time point FIT protein was still below the detection limit, most probably because of weaker expression compared to the over-expression condition in HA FIT 8 plants.

Moreover, the effect of CHX on gene expression was also tested in wild type roots. Expression of *FRO2* and *IRT1* was found up-regulated by - Fe compared to + Fe (Fig 4.7c, d). A high amount of FIT protein was therefore not required to induce *FRO2* and *IRT1* at the 0 and 4 h time points (in the presence of CHX). *FRO2* was expressed at higher level at - Fe upon CHX than in the control at the 0 hour time point, whereas the corresponding mRNA levels of *FRO2* at the four hour time points were not different (Figures 4.7c). *FRO2* seemed to be over-induced in the presence of CHX rather than repressed due to low FIT protein. Very interestingly, *FIT* gene expression was up-regulated upon + Fe by CHX treatment at the 4 h time point versus the control (Figure 4.7b). This finding suggests that a repressor protein might have suppressed *FIT* transcription at + Fe in the control. This repressor protein might have been susceptible to CHX treatment (and presumably to - Fe). This would explain why CHX treatment resulted in a de-repression of the *FIT* gene. Moreover, this repressor effect was specific for *FIT* since *IRT1* and *FRO2* expression were not affected by CHX at + Fe (4.7c,d).

Because *FRO2* expression was induced after CHX treatment rather than repressed (Figure 4.7c), we were interested in investigating the protein activity of *FRO2* and therefore performed an Fe reductase assay with wild type plants (Fig 4.7e). Plants were again treated for one hour with CHX and either re-supplied to standard Hoagland medium or used directly after the treatment for measuring the reductase activity. CHX treatments were started with a four hour delay to use all plants simultaneously. As expected the control plants grown at -Fe Hoagland medium had a significantly higher reductase activity than the corresponding + Fe grown plants, confirming an induction of reductase activity upon Fe deficiency. CHX treated plants grown on + Fe medium, generally did not show a significant difference to the control plants, most probably because *FRO2* is not much regulated under these conditions. However, Fe deficient plants, treated with CHX had a significantly higher reductase activity than the - Fe control plants, indicating a correlation between the increased *FRO2* expression (Figure 4.7c) and the corresponding protein activity (Figure 4.7e). At the four hour time point, - Fe plants showed decreased reductase activity that was not significant different to the activity of + Fe plants. To draw conclusions on the delayed reduction of the reductase activity at the 4 h time point, further experiments

might have to be done. Because regulation of *FRO2* was not the focus of this work, it remains to be clarified in the future. However, we were able to show that induction of *FRO2* expression after CHX application correlated with induced reductase activity. Therefore increased reductase activity could probably be due to higher *FRO2* protein levels rather than enhanced *FRO2* activity. Perhaps a negative regulator preventing excessive reduction of Fe at - Fe conditions, was also affected by CHX. At the four hour time point, this regulator was again restored and repressed *FRO2* activity. Because the interplay of inducer (like FIT) and repressor was disturbed due to CHX treatment at this time point the reductase activity was probably below the - Fe control level.

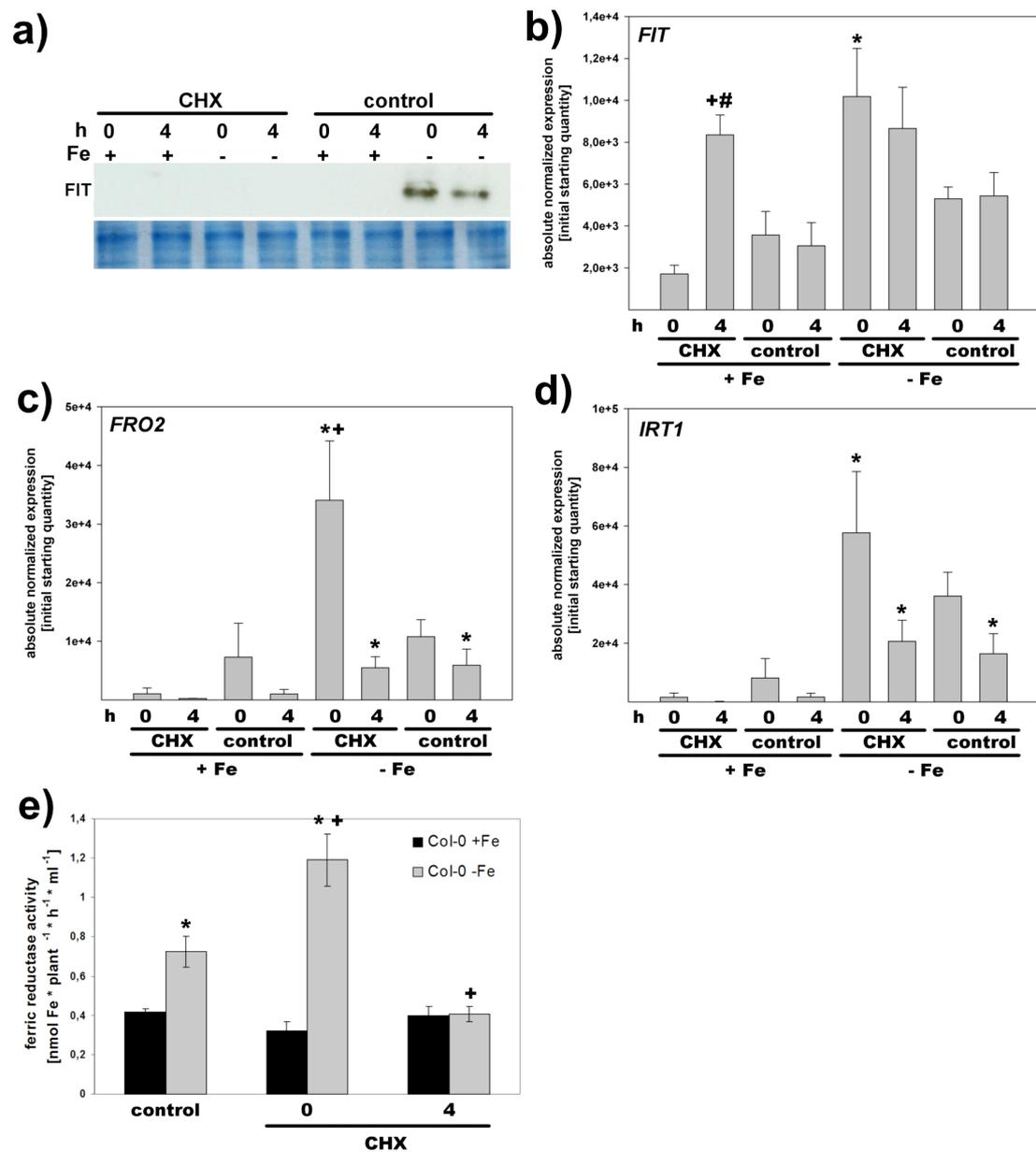


Figure 4.7: Effect of cycloheximide (CHX) on FIT protein abundance and downstream responses in wild type plants

(a) Western blot using anti-FIT-C polyclonal antiserum. For the western blot experiment with the anti-FIT-C polyclonal antiserum, the harvested material was used for protein extraction and final protein samples were provided to Sivasenkar Lingam who performed the western blot experiment. (b,c,d) Reverse transcription-qPCR analysis of (b) FIT, (c) FRO2 and (d) IRT1 in roots. Col-0 plants were grown in the hydroponic growth system at + or – Fe and treated for one hour with + CHX or - CHX (control), respectively. After the one hour incubation at + or – CHX, samples were harvested directly after the treatment (0 time point) or four hours after retransfer to + or - Fe growth medium without CHX (4 h time points). \* indicates significant change versus + Fe of respective treatment ( $p < 0.05$ ); + indicates significant change versus control at respective Fe supply ( $p < 0.05$ ); # indicates significant change versus the 0 h time point of the respective treatment ( $p < 0.05$ )  $n = 2$ .

(e) Reductase assay in respect to Fe supply and CHX treatment. Plants were grown for six days on + Fe (black bars) or - Fe (grey bars) Hoagland agar plates. CHX treatment was performed in liquid Hoagland medium as described above. Untreated control plants (C) were kept in liquid Hoagland medium without CHX. CHX treatments were started with 4h delay, so that all plants could be used simultaneously for reductase assay. Three plants were used per sample. \* indicates significant change versus + Fe of respective treatment ( $p < 0.05$ ); + indicates significant change versus control at respective Fe supply ( $p < 0.05$ );  $n = 5$ .

In summary four major points can be concluded from these experiments. First, *FRO2* expression seemed to be induced after CHX treatment and correlated with FRO2 protein activity. Hence, FRO2 does not seem to be subject of protein turnover, since its activity was induced after CHX treatment. Second, FIT protein levels were negatively affected by CHX treatment in HA-FIT Ox and in wild type plants, suggesting turnover control of FIT. In roots, the effect was stronger at – Fe than at + Fe. Third, reduction of FIT abundance, conferred by CHX, did not result in significantly lowered expression of *FRO2* and *IRT1*, suggesting that small amounts of active FIT protein were sufficient to trigger Fe deficiency responses. Fourth, *FIT* gene expression was up-regulated in + Fe wild type roots upon CHX treatment, indicating that FIT gene expression might be repressed at + Fe by a repressor that is susceptible to CHX.

### **Nitric oxide induces FIT stability by counteracting 26S proteasome dependent degradation**

The above results suggested that FIT abundance was controlled and that FIT was subject of a turnover control at protein level. The question arose which plant signalling factors may affect FIT protein abundance. Previous reports showed that nitric oxide (NO) positively affects Fe deficiency responses in tomato and Arabidopsis (Besson-Bard et al., 2009; Chen et al., 2010; Graziano et al., 2002; Graziano and Lamattina, 2007). Moreover, ethylene affects Fe deficiency regulation in a similar manner as NO (García et al., 2010; Lucena et al., 2006; Wu et al., 2011) and additionally, FIT protein stability is also affected

by ethylene (Lingam et al., 2011). Therefore, we asked whether NO might also control FIT protein stability.

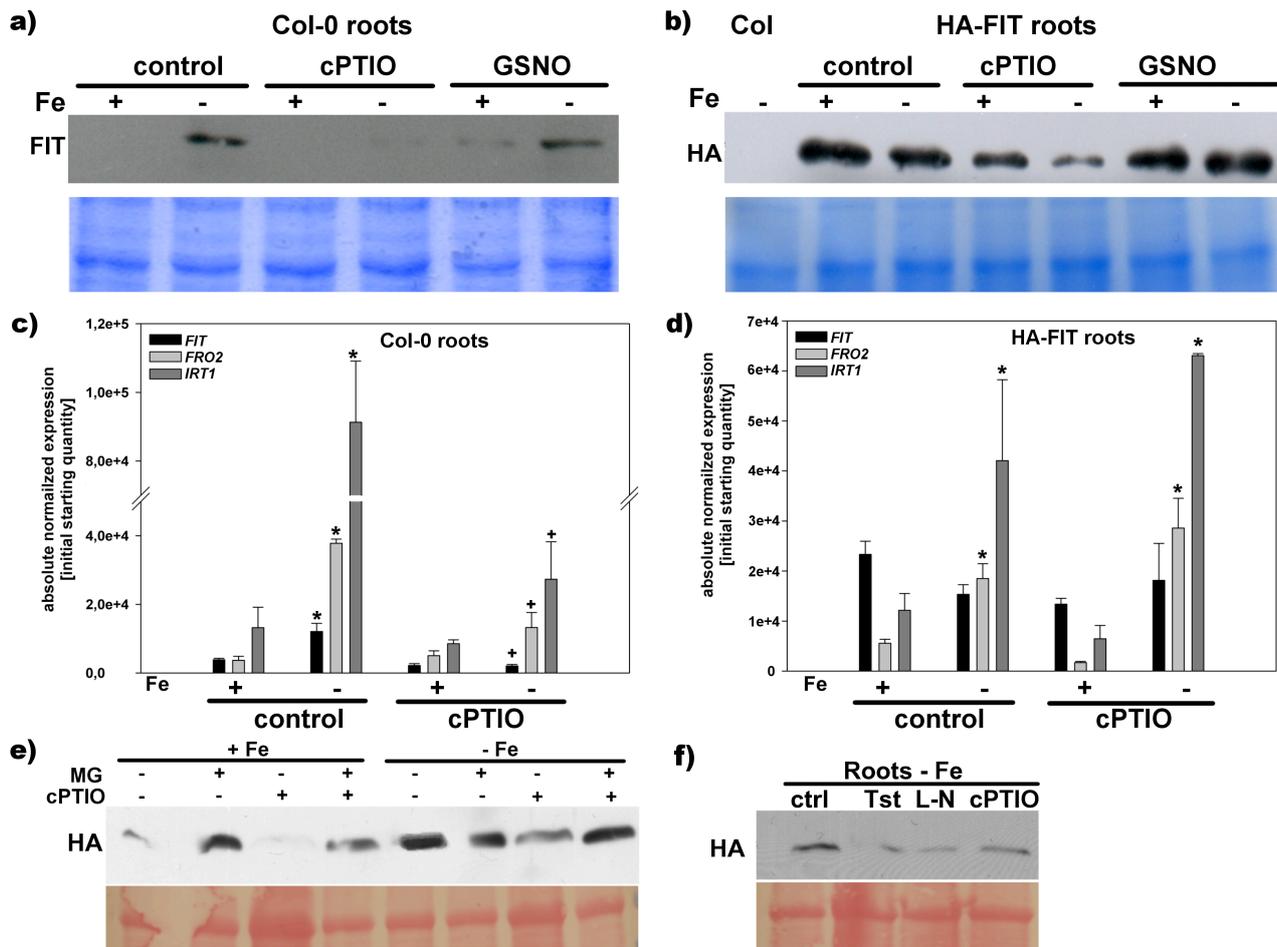
To test this possibility, we grew HA-FIT 9 plants in the 6-day growth system that we found best suitable for NO pharmacological treatments with NO scavenger cPTIO. We selected cPTIO, because it was described as a common plant inhibitor for NO in the literature (Chen *et al.* 2010, Graziano and Lamattina 2007). FIT protein could be detected in - Fe wild type roots and HA-FIT protein could be detected in control roots at + and - Fe (Figure 4.8a, b), which was in accordance with our previous results (Figure 4.4, 4.5, 4.7).

cPTIO treatment caused a strong down-regulation of FIT protein to 2 % at - Fe, compared to control roots, suggesting that inhibition of NO signalling resulted in reduced FIT protein abundance (Figure 4.8a). Treatment with the NO liberating substance GSNO did not affect FIT protein abundance at - Fe. On the other hand, addition of GSNO to + Fe plants resulted in detectable amounts of FIT protein (Figure 4.8a). This finding suggests that NO promoted FIT protein accumulation, while inhibition of NO prevented it. We could confirm these results using HA-FIT plants (HA-FIT 9). cPTIO treatment resulted in a decrease of HA-FIT protein (to 30 % at - Fe and to 50 % at + Fe versus controls).

Addition of GSNO did not increase HA-FIT levels beyond the levels of the controls (Figure 4.8b). We further confirmed the effect of cPTIO on FIT protein by testing additional NO inhibitors, namely Tungstate and L-NAME. We found that all three NO inhibitors reduced HA-FIT protein at - Fe, namely to 40 % (Tungstate), 30 % (L-NAME) and 50 % (cPTIO) versus the controls (Figure 4.8f). Thus, inhibition of NO indeed decreased FIT protein accumulation. We then investigated whether cPTIO treatments had affected the expression of Fe deficiency genes in wild type plants and HA-FIT plants grown as just described. In wild type control roots exposed to + or - Fe, gene expression was as expected and corresponded to the results, described in the previous paragraphs (Figure 4.2, 4.4): *FIT* was induced threefold, whereas *IRT1* and *FRO2* were at least eightfold induced by - Fe (Figure 4.8c, d). The same was observed for HA-FIT plants except that *FIT* was over-expressed compared to wild type. cPTIO application resulted in a decreased expression of *FIT*, *FRO2* and *IRT1* gene expression in - Fe wild type roots compared to the - Fe control (Figure 4.8c). On the other hand, cPTIO treatment had no effect on gene expression in HA-FIT plants (Figure 4.8d). Apparently, the remaining pool of HA-FIT protein in the transgenic over-expression plants was sufficient to trigger *FRO2* and *IRT1* induction.

The decrease of HA-FIT by cPTIO clearly shows that HA-FIT protein regulation cannot be explained merely by a reduced transcriptional activation due to cPTIO but that reduced NO

due to cPTIO affected HA-FIT at protein level, because *HA-FIT* is constitutively expressed due to the control of the 2x35s promotor.



**Figure 4.8: Effect of nitric oxide on FIT protein abundance and gene expression**

All plants were grown in the 6-day agar growth system at + and - Fe. (a) FIT protein in roots of wild type (Col-0) plants, untreated (control), treated for 24 hours with 1 mM cPTIO (cPTIO), treated for 24 h with NO donor GSNO; FIT protein was detected by Western blot using anti-FIT-C polyclonal antiserum (upper image); Coomassie-staining represents the loading control (lower image). (b), HA-FIT in roots of HA-FIT 9 plants, treated and grown as in (a). Coomassie-staining represents the loading control (lower image). (c, d) Reverse transcription-qPCR analysis in (c) wild type and (d) HA-FIT roots treated with or without cPTIO. \* indicates significant change versus + Fe of respective treatment ( $p < 0.05$ ); + indicates significant change versus control at respective Fe supply ( $p < 0.05$ );  $n = 2$ . (e) HA-FIT abundance in roots of HA-FIT 9 plants grown at + or - Fe and treated as indicated for 24 h with 1 mM cPTIO (cPTIO) and four hours with 100  $\mu$ M MG132 (MG); HA-FIT protein was detected by Western blot using anti-HA monoclonal antibodies (upper image); Ponceau S was used as loading control (lower image). (f), HA-FIT protein abundance in roots of - Fe HA-FIT 9 plants, untreated (ctrl), treated with 1 mM Tungstate (Tst), 1 mM L-NAME (L-N) and 1 mM cPTIO, showing that several NO inhibitors caused reduction of HA-FIT protein amounts. Ponceau S was used as loading control (lower image). Plant growth and treatment was performed in co-work with Sivasenkar Lingam. The work with the anti FIT-C antibody for detection of FIT protein in a) was performed by Sivasenkar Lingam.

It was then interesting to further investigate the mechanism by which NO sensing could prevent FIT protein degradation. Towards this end, we incubated cPTIO-treated HA-FIT plants with the common proteasome inhibitor MG132. In this experiment, HA-FIT was reduced to 50 % at – Fe and to 6 % at + Fe upon cPTIO treatment compared to the controls (Figure 4.8e). When cPTIO-grown seedlings were treated with MG132, FIT protein levels were restored at – Fe and + Fe (Figure 4.8e).

Hence, we conclude that upon inhibition of NO signalling, FIT protein was more susceptible to degradation by the proteasome. Therefore, application of proteasome inhibitors could result in a restoration of FIT protein levels after cPTIO treatment. We propose that NO promotes FIT protein stability by inhibiting proteasomal degradation of FIT.

#### **4.2. Identification of putative phosphorylation sites in FIT (manuscript in preparation)**

Because FIT is targeted by post-translational regulation we were interested in identifying regulators acting on FIT at post-translational level. Besides the stabilising effect of nitric oxide on the one hand, a second (de-stabilising) regulator was most probably acting on FIT to target FIT to the 26S proteasome. Such a de-stabilisation could act in respect to Fe supply, since turnover of FIT was stronger under - Fe conditions (Figure 4.6a). Turnover of transcription factors is very important in cells. This way cells remain responsive to incoming signals and can turn off transcriptional induction if not needed anymore. In many cases, post-translational modification in form of phosphorylation is associated with the regulation of protein activity and also its subsequent degradation (Shen et al., 2007; Lampard et al., 2008; Miura et al., 2011; Yoo et al., 2008; Han et al., 2010). Towards this end we searched for putative phosphorylation sites in the c-terminal part of FIT to investigate if phosphorylation could be one possibility for an Fe dependent regulation of FIT. The c-terminal part of FIT has been used for interaction studies in yeast two-hybrid screens and was shown to be the regulatory domain of FIT (Lingam et al., 2011). Moreover, complementary work in our group, has recently proven that FIT is phosphorylated, confirming our hypothesis that phosphorylation could be an important post-translational mechanism of FIT regulation (unpublished data).



respective DNA sequence (top). Framed in red are the amino acids and the base triplets that were mutagenised. The respective primers are presented in the material and method part (Table 2.1).

We were following three different strategies to find putative phosphorylation sites (Figure 4.9). (i) First, the C-terminal part of the FIT amino acid sequence was aligned with the C-terminal part of the ortholog LeFER (Ling et al., 2002; Bauer et al., 2004) to identify conserved amino acids, that could be putative targets of phosphorylation. (ii) Second, we used the public available database NetPhos (<http://www.cbs.dtu.dk/services/NetPhos/>) to make an *in silico* prediction regarding putative phosphorylation sites in the c-terminal part of FIT. (iii) We compared the FIT sequence with phosphorylation sites of other bHLH proteins that were known to be phosphorylated, such as SPCL (Lampard et al., 2008). To make a selection on putative phosphorylation sites in FIT we took the results from all three strategies in account, trying to narrow down on sites that gathered the results from more than one strategy. Being aware of the fact that further positions could be targeted for a phosphorylation we selected four positions within the FIT c-terminal part that could be most likely a target for phosphorylation (Table 4.1, Figure 4.9). In the c-terminal part of FIT we found two conserved tyrosines (Y238, Y278), one PAS (S221) domain, which was similar to a common phosphorylation target site (PXS/TP) (Widmann et al., 1999) and one region that was identical to one of the phosphorylation target sites in the bHLH protein SPCL (SS271/272) (Lampard et al., 2008). All four selected positions were found positive by prediction with NetPhos. Hence, these four positions could be phosphorylation sites and were therefore selected for further analysis. One phosphomimicking (that pretends a phosphorylation by exchanging the selected amino acid to a glutamate) and one non-phosphorylatable (that makes this position non-phosphorylatable, by exchanging the selected amino acid to an alanine (in the case of serine) and to a phenylalanine (in the case of tyrosine)) mutant form of each position was generated by PCR mutagenesis, using genomic *FIT* DNA. In total, we generated eight mutant forms of *FIT*, each fused to GFP at its C-terminal part (Table 4.1). In the case of S271S272, we decided to make a double mutation to create the non-phosphorylatable form S271AS272A (from now on named SS271AA), to exclude the possibility that either the one or the other serine could still be phosphorylated. In the case of the opposite phosphomimicking mutation we mutagenised S272 to S272E (Table 4.1). Genomic *FIT* and cDNA *FIT* was also fused to GFP. These two constructs were used as wild type control.

**Table 4.1: Overview of the generated gene constructs.**

FIT mutagenesis constructs are named in the following system: The first letter indicates the amino acid, that was subject of mutagenesis. The number indicates the position of the amino acid in the FIT amino acid sequence beginning at the first amino acid from the FIT full sequence. The letter behind the number indicates the amino acid, that replaced the selected amino acid. FIT without additional codes indicates unmutated (wild type) FIT. For interaction studies with bHLH039, HA-bHLH039 and bHLH039-GFP had also been cloned. All generated FIT constructs were tested for expression in tobacco. Functional constructs were subsequently transferred in wild type (Col-0) plants and *fit* mutant plants (*fit-3*). Homozygous plant lines should be generated by selfing into the T3 generation and selection via selection marker and genotyping (progress is described by showing the current generation). Y: Tyrosine, S: Serine, F: Phenylalanine, A: Alanine, E: Glutamate, gDNA: genomic DNA, cDNA: copy DNA,

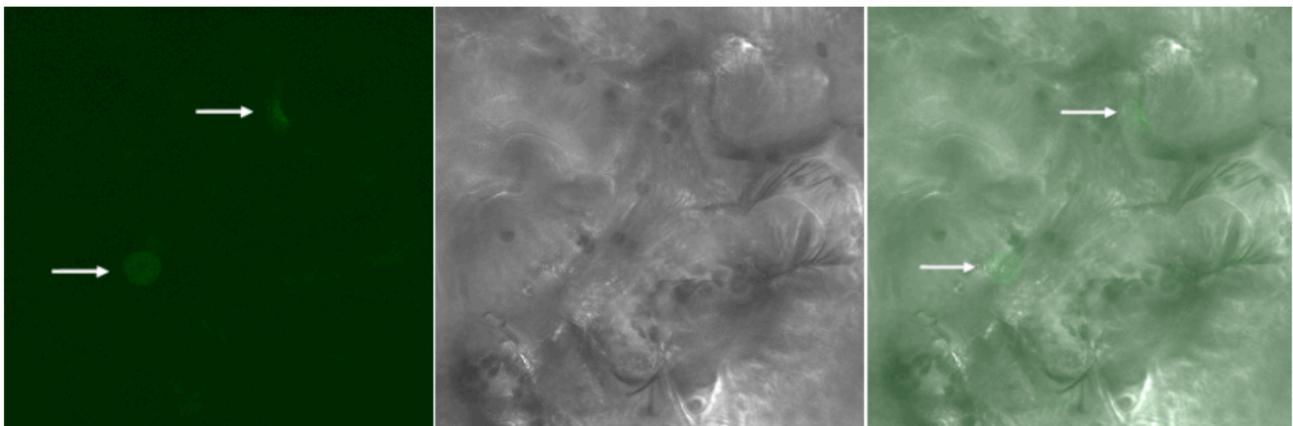
Name	Construct	Successful Test in Tobacco	Genotype	Status
S221	S221A-GFP	Yes	Col-0	T2
			<i>fit-3</i>	T1
	S221E-GFP	Yes	Col-0	T2
			<i>fit-3</i>	T1
Y238	Y238F-GFP	Yes	Col-0	T3
			<i>fit-3</i>	T3
	HA <sub>3</sub> -Y238F	Yes	Col-0	T3
			<i>fit-3</i>	T3
	Y238E-GFP	Yes	Col-0	T3
			<i>fit-3</i>	T3
HA <sub>3</sub> -Y238E	Yes	Col-0	T3	
		<i>fit-3</i>	T0	
SS271/2	SS271AA-GFP	Yes	Col-0	T3
			<i>fit-3</i>	T2
	S272E-GFP	No	Col-0	T0
			<i>fit-3</i>	T0
Y278	Y278F-GFP	Yes	Col-0	T2
			<i>fit-3</i>	T0
	Y278E-GFP	Yes	Col-0	T3
			<i>fit-3</i>	T3
FIT	FIT-GFP	Yes	Col-0	T1
			<i>fit-3</i>	T3
	HA <sub>3</sub> -FIT	Yes	Col-0	T3
			<i>fit-3</i>	T3
bHLH039	bHLH039-GFP	Yes	Col-0	T2
	HA <sub>3</sub> -BHLH039	Yes	Col-0	T3

To investigate the interaction ability of the mutated forms of FIT with known FIT interaction partners such as bHLH039 and bHLH038 (Yuan et al., 2008) or EIN3 (Lingam et al., 2011)

we fused bHLH039 to an HA and a GFP tag, respectively. With this tool we had the opportunity to perform co-immunoprecipitation studies between the mutated FIT versions harbouring a GFP tag and HA<sub>3</sub>-bHLH039. Such experiments have not been performed so far and could be useful to further characterise how the specific interaction between FIT and its interaction partners functions at molecular level.

#### **4.2.2. Analysis of FIT mutant forms in respect to intracellular localisation and protein abundance in transiently transformed tobacco leaves**

To confirm functionality of the constructs, tobacco leaves were transiently transformed with each construct. With the transient transformation we had a fast tool to screen each mutant version in tobacco leaf cells, regarding intracellular localisation using confocal imaging on the one hand and general protein abundance using western blot analysis on the other. For tobacco transformation, *Agrobacterium tumefaciens* cultures containing the respective plasmid were injected into the leaf mesophyll of tobacco leaves. 48 hours after the transformation, leaf pieces were harvested and used for confocal microscopy and western blot experiments.



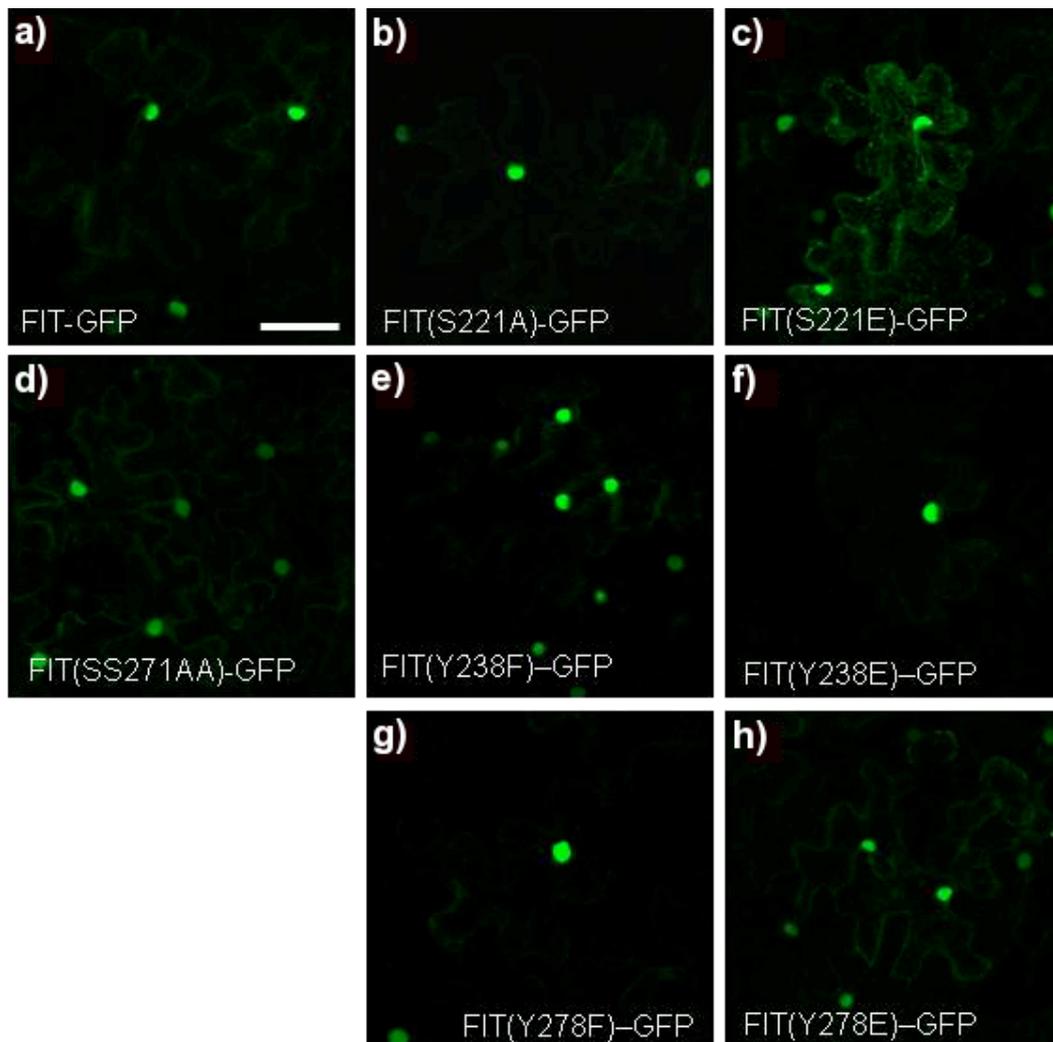
**Figure 4.10: Transient transformation of tobacco leaf cells with FIT-GFP.**

GFP fluorescence was detected with a confocal microscope at 500-530 nm and signal identity was confirmed with a lambda scan (Figure 3.4). GFP fluorescence on the left, bright field in the middle, merged on the right side. The arrows indicate GFP fluorescence present in the nuclei of two neighbouring cells.

#### **• Analysis of intracellular localisation of FIT in transiently transformed tobacco leaves**

To test the quality of the fused GFP tag, we transformed tobacco leaves with FIT-GFP to investigate the FIT-GFP protein localisation (Figure 4.10)

As expected, FIT-GFP was localised in the nucleus (Figure 4.10), whereas the cytoplasmic portion of detected GFP was rather little. The GFP signal authenticity was analysed and confirmed by lambda scan (see Material and Methods, Figure 3.4).

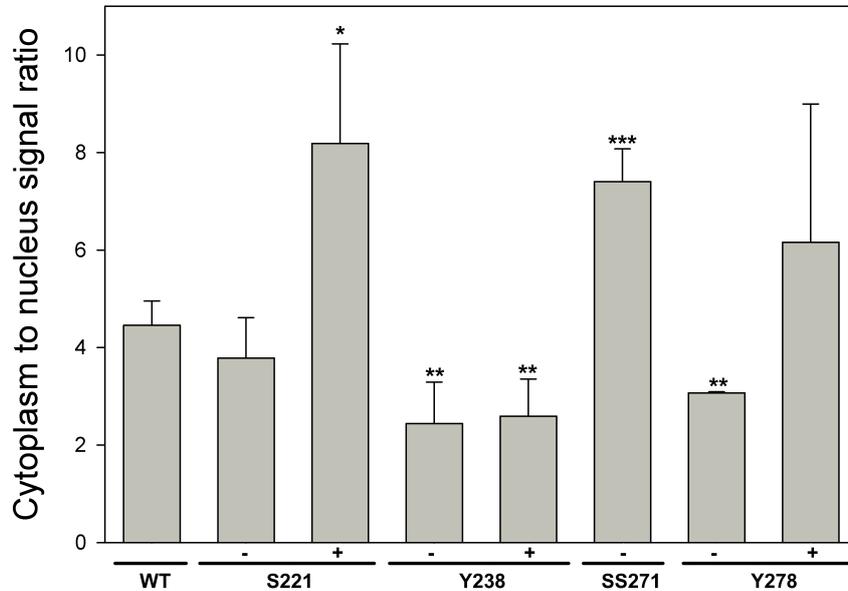


**Figure 4.11: Transient transformation of tobacco leaf cells with (a) wild type FIT-GFP and (b-h) mutated FIT-GFP constructs.**

GFP fluorescence was observed by confocal microscopy. Authenticity of true GFP was checked by lambda scans (Figure 3.4). All transformations were done at least three times. One representative image was selected for presentation. The bar in a) indicates 50µm, all images were taken with the same magnification.

To analyse the individual mutated forms, each construct was transferred into tobacco leaves to investigate their localisation pattern. Figure 4.11 shows representative images of all constructs, gathered from three independent experiments. The different mutant forms clearly differed in their cellular localisation in respect to nucleus and cytoplasmic localisation. Because the transformation efficiency of the individual cells in transiently transformed tobacco leaves differs from cell to cell, we selected representative cells and

made Z-stacks of these cells to measure the complete GFP signal of one cell. By using Image J software we were able to quantify the complete GFP signal intensities from the cytoplasm and the nucleus of the respective cell. Using these two values we calculated the corresponding ratio for subsequent comparison (Figure 4.12).



**Figure 4.12: Cellular localisation of mutated FIT-GFP constructs, compared to wild type FIT-GFP (WT) in transiently transformed tobacco leaf cells.**

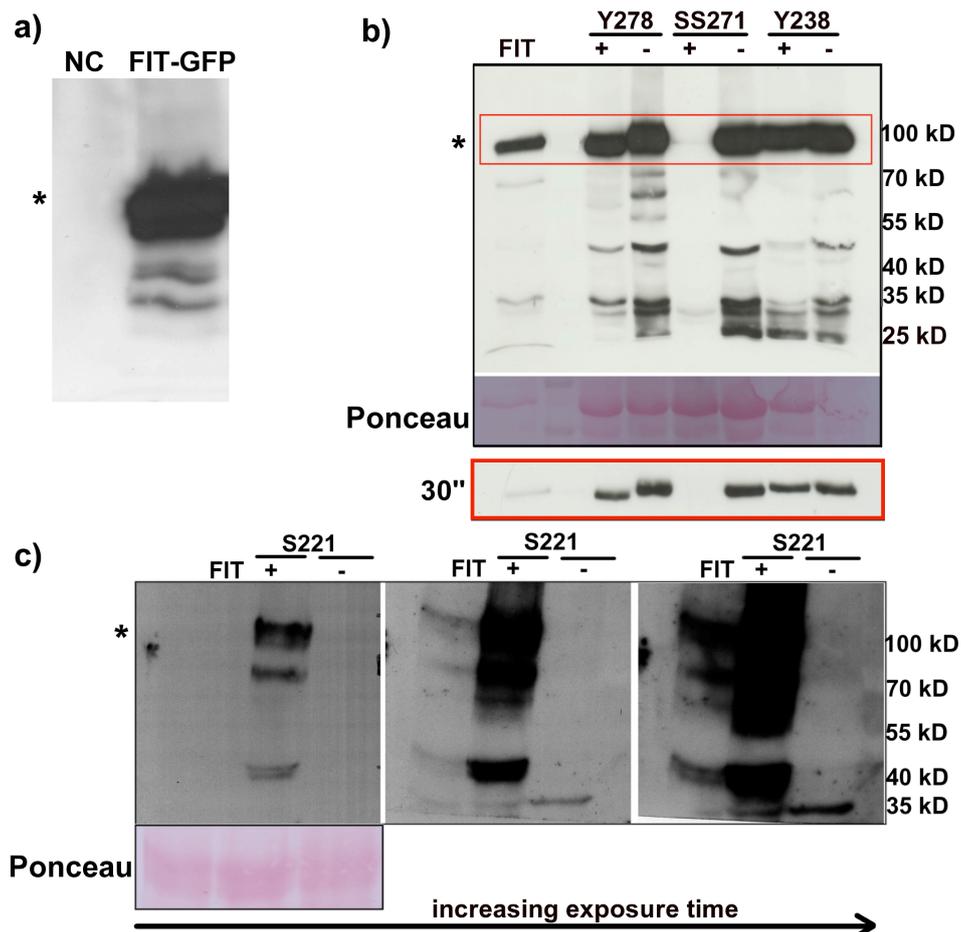
To detect full GFP signal of one cell, z-stacks of the individual cells were made and full projections of the cells were performed to obtain the complete GFP signal from one cell. The cytoplasm to nucleus GFP signal ratio was calculated for each construct. For quantification of the cytoplasm and nucleus GFP signal intensity, respectively, the IMAGE J software was used. The total signal intensity of one single cell was detected and subsequently used for the ratio calculation. Mean value and standard deviation was calculated from three individual experiments. Statistical significant differences compared to the wild type FIT-GFP signal ratio was calculated using the unpaired T-test. \*  $p < 0.01$ ; \*\*  $p < 0.005$ ; \*\*\*  $p < 0.0001$ . Bars without asterisk indicate no significant difference ( $n = 3$ ).

Out of these measurements, we were able to draw conclusions on the individual mutations with respect to cellular localisation. The mutated form FIT<sup>S221E</sup> and FIT<sup>SS271AA</sup> had significantly higher cytoplasmic localisation compared to FIT-GFP, whereas FIT<sup>Y238E</sup>, FIT<sup>Y238F</sup> and FIT<sup>Y278F</sup> had stronger nuclear localisation compared to FIT-GFP. FIT<sup>S221A</sup> and FIT<sup>Y278E</sup> were not significantly different compared to FIT-GFP. The mutated form FIT<sup>S271E</sup> did not show any GFP signal.

These results gave first hints that the selected mutations did affect FIT by way of cellular localisation in comparison to un-mutated FIT-GFP.

- **Analysis of FIT protein abundance in transiently transformed tobacco leaves**

Besides intracellular localisation of the different FIT forms, we were also interested in general protein abundance of the mutated FIT versions and performed therefore western blot experiments. To test the specificity of the GFP antibody, tobacco leaf samples transformed with FIT-GFP and untransformed tobacco leaf samples were processed and analysed by western blot. The anti GFP antibody was of high specificity and did not show any unspecific bands (Figure 4.13a).



**Figure 4.13: Western Blot analysis of transiently transformed tobacco leaves with wild type FIT-GFP and different mutated FIT-GFP constructs.**

(a) Western Blot with FIT-GFP and untransformed tobacco as negative control (NC) to show specificity of the antibody. High exposure time was applied, to see all possible unspecific bands in the negative control. (b) Western Blot with FIT-GFP, FIT<sup>Y278E</sup>(+), FIT<sup>Y278F</sup>(-), FIT<sup>S272E</sup>(SS271 +), FIT<sup>SS271AA</sup>(-), FIT<sup>Y238E</sup>(+), FIT<sup>Y238F</sup>(-). The most prominent band was detected at around 100 kD (indicated with an asterisk, framed in red in b). A second picture with less exposure time (30 seconds) was made for better differentiation of the size differences within the 100 kD area (lowest picture in b, framed in red). (c) Western Blot with FIT-GFP and FIT<sup>S221E</sup>(+) and FIT<sup>S221A</sup>(-). Presented are three different exposure times of the same Western Blot (increasing exposure time from left to right). To detect the wild type FIT-GFP bands the film had to be

exposed in a way that the bands of FIT<sup>S221E</sup> were already over-exposed. Ponceau-S staining in b) and c) (respective lower image) was used as loading control. + indicates the phosphomimicking mutation of the respective position, - indicates the non-phosphorylatable mutation of the respective position.

In the following experiments we analysed the different GFP-tagged FIT mutant forms (Figure 4.13b, c). First of all, we found that two forms namely FIT<sup>S272E</sup> and FIT<sup>S221A</sup> contained no detectable amounts of GFP fusion protein. In the case of FIT<sup>S272E</sup> this was reasonable since we could not detect GFP fluorescence by microscopy. This protein version of FIT was either not translated or extremely rapidly degraded. Also re-cloning of this construct did not result in any GFP signal. In the case of FIT<sup>S221A</sup>, it must have been out of other reasons such as quick protein turnover, because GFP fluorescence was detected before (Figure 4.11b). To clarify this, further experiments have to be done in the future. The opposite mutation FIT<sup>S221E</sup> seemed to have very high protein abundance, above the level of wild type FIT-GFP (Figure 4.13c). All other mutated versions could be detected by western blot and we could conclude three major points (Figure 4.13b,c): First, the amount of free GFP expected at 27 kD was only small, leading to the conclusion that all generated constructs seemed to be fully expressed and translated into protein, suggesting that the GFP signals observed at the confocal microscope were FIT-GFP fusion proteins, rather than free GFP. Second, we detected more than one specific protein band in all forms that were analysed, including the un-mutated FIT-GFP. The most prominent band of each construct was around 100 kD, which was above the expected size of 63 kD. Therefore, either the real protein mobility differs in our PAGE conditions or the proteins were modified which then resulted in a higher size (Shui et al., 1992). Un-mutated FIT-GFP also showed a weak band at the expected size around 63 kD, therefore a general modification on all FIT forms could be one possible explanation. Further experiments have to be performed in the future to unravel possible modifications. All other specific bands below the expected size of 63 kD could be degradation products. Especially between FIT<sup>Y278E</sup> and FIT<sup>Y278F</sup> we could observe that smaller protein forms below 63 kD differed in their intensity, (taking in account, that both lanes were equally loaded (see corresponding Ponceau-S stain below the western blot image)) (Figure 4.13b)). Therefore we suggested that the respective mutations could possibly result in different protein stabilities or degradation efficiencies, respectively. Third, compared to the un-mutated FIT-GFP the mutated forms differed in size within the most prominent band appearing at 100 kD, leading to the conclusion that the different mutations affected the gel mobility of the protein (see also short exposed film in Figure 4.13b (lowest image)). The strongest difference

could be observed by mutating tyrosine 278. The phosphomimicking mutation FIT<sup>Y278E</sup> clearly had higher gel mobility than FIT<sup>Y278F</sup>. In addition FIT<sup>Y278F</sup> separated above the wild type FIT-GFP. FIT<sup>SS271AA</sup> seemed to have the same gel mobility than FIT-GFP. The mutant forms FIT<sup>Y238E</sup> and FIT<sup>Y238F</sup>, showed both again slightly higher size than FIT-GFP.

Summarising the results from the transient tobacco leaf transformations, it can be concluded, that the different mutations, resulted in different effects compared to the wild type FIT-GFP, such as altered intracellular localisation, protein abundance and protein size.

#### **4.2.3. Analysis of stable transgenic Arabidopsis plant lines harbouring mutated FIT forms**

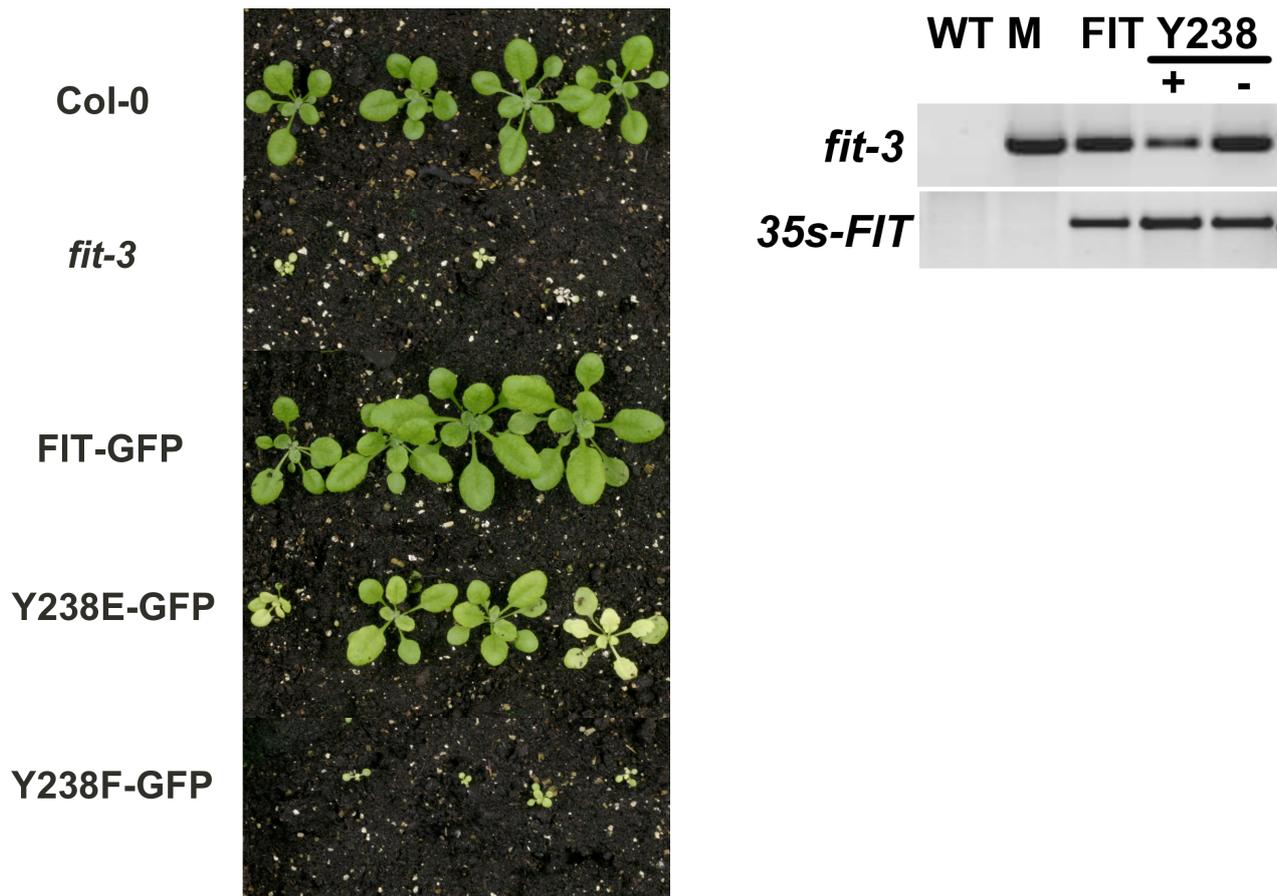
All constructs that were successfully tested in transient tobacco transformations were subsequently used for transformation of Columbia-0 and *fit-3* mutant plants to generate stable transgenic Arabidopsis plant lines (Table 4.1). Generation of the stable transgenic Arabidopsis lines was performed by using the same *Agrobacteria* clones (harbouring the same vectors), that were used for the tobacco transformation. Only by using transformed Arabidopsis plants we were able to analyse FIT in the root tissue and to confirm the results obtained from tobacco results. Moreover, tobacco plants can hardly be grown under controlled Fe deficiency conditions.

With these stable transgenic lines we wanted to investigate four major points. First: The ability of the individual FIT mutant form to rescue the *fit* knock-out mutant should be investigated. It could be possible that the introduced mutation results in functionally impaired FIT so that this specific form is unable to rescue the mutant phenotype. Second: Protein abundance should be investigated *in vivo* by confocal imaging, to investigate if the results obtained by transient tobacco transformations could be confirmed. Third: Protein abundance of each mutant form should also be investigated by western blot experiments. Fourth: Downstream activation of *FRO2* and *IRT1* should be investigated by real-time RT-qPCR to analyse functionality of the different FIT forms.

#### **• Complementation assay of stable transgenic Arabidopsis plant lines**

At the end of this work, two mutant forms (FIT<sup>Y238E</sup> and FIT<sup>Y238F</sup>) and wild type FIT-GFP have been successfully transferred into Columbia-0 and *fit-3* and homozygous seeds could be generated. With these three lines complementation assays have been performed to

investigate their ability to rescue the *fit-3* mutant. All plants were germinated and grown for two weeks on soil to investigate their phenotype (Figure 4.14).



**Figure 4.14: Complementation assay**

FIT-GFP, FIT<sup>Y238F</sup>-GFP and FIT<sup>Y238E</sup>-GFP were each transferred in *fit-3* mutant background by floral dip in order to test their ability to complement the defective *FIT* gene. Homozygous plant lines were generated by selfing and were confirmed by PCR (right side image) and confocal microscopy (Figure 4.15, 4.16). For PCR verification, the *fit-3* specific T-DNA insertion was amplified by PCR (top) and the T-DNA containing 2x35s::FIT-GFP was verified in a second PCR (bottom). Columbia-0 (Col-0, WT) plants served as positive control, *fit-3* mutant plants (M) served as negative control. FIT-GFP (FIT); Y238E-GFP (+); Y238F-GFP (-).

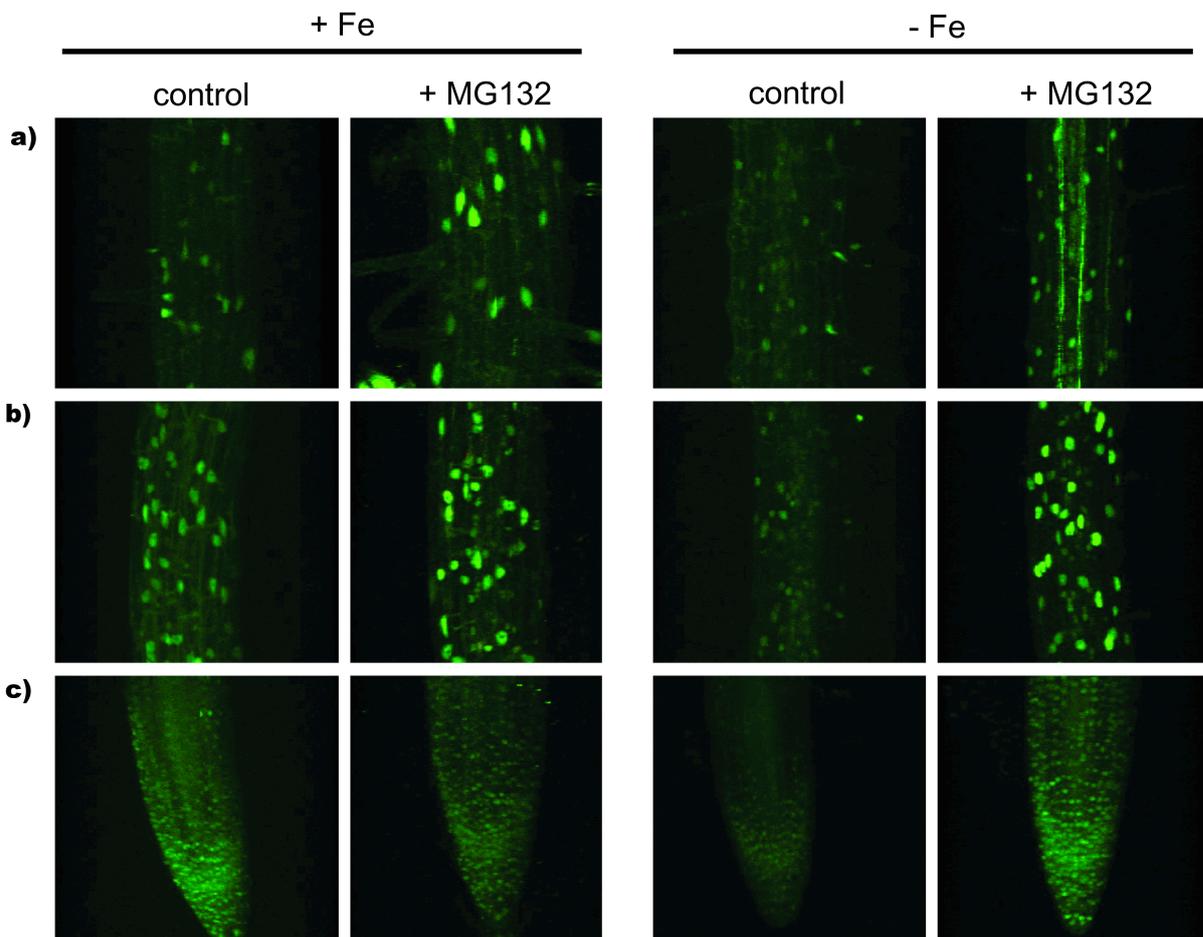
As a control Columbia-0 plants were grown as wild type plants and *fit-3* plants were grown as a negative control. FIT-GFP/*fit-3* could fully complement the *fit-3* mutant phenotype, showing that the FIT-GFP construct was fully functional and able to complement the knocked out *FIT* gene. FIT<sup>Y238E</sup> partially complemented the *fit-3* mutant, resulting in plants that were similar in size compared to the wild type control. However, they still displayed a weak leaf chlorosis. The opposite mutation FIT<sup>Y238F</sup> could not complement the mutant and grew not significantly better than the homozygous *fit-3* plants. The genotype of each line was verified by PCR. In a first reaction the T-DNA harbouring 2x35s::FIT-GFP was amplified (untransformed wild type and *fit* plants did not contain this T-DNA) and in a

second PCR the existence of the T-DNA that disrupts the endogenous *FIT* gene (which results in the *fit* mutant) was tested (Figure 4.14, right side). Only the untransformed wild type plants did not contain a disrupted *FIT* gene. Hence, improved plant phenotypes compared to the *fit* mutant plants must be due to the newly introduced T-DNA. Alterations in the FIT amino acid sequence seemed to affect the ability of FIT to rescue the mutant phenotype. These results gave first hints, that the introduced mutations resulted in impaired FIT function and that depending on the mutation such an impairment was differentially severe. The GFP tag did not seem to influence FIT function, since unmodified FIT-GFP could restore the wild type phenotype.

- **Localisation of FIT mutant forms in roots of stable transgenic Arabidopsis plant lines by confocal microscopy**

In further experiments we investigated the cellular localisation of the different FIT forms in the transformed Arabidopsis plants by using confocal microscopy. With these experiments we wanted to investigate, if the intracellular localisation of the FIT mutant forms from the tobacco transformations (Figure 4.11, 4.12) was coherent with the ones from Arabidopsis. We took all images with the same settings, to be able to compare the images with each other. Since heterozygous plant lines gave GFP fluorescence already, we started to work with the currently available T2 plants. First, we grew (homozygous) FIT-GFP/*fit-3* plants for six days on + and - Fe medium to analyse wild type FIT-GFP abundance in Arabidopsis roots in respect to Fe supply.

Because it was previously shown that FIT might be degraded by the 26S proteasome (Figure 4.8; Meiser et al., (provisionally accepted); Lingam et al., 2011; Sivitz et al., 2011), we treated plants with the proteasome inhibitor MG132 to analyse if differences in protein abundance could be observed, compared to untreated control plants. We analysed three zones in each root to cover the root tip, the elongation zone and the root hair zone. FIT-GFP could be detected in every root zone under Fe supply as well as under Fe deficiency, which was in accordance with the previously obtained results from the HA-FIT plants (Figure 4.4, 4.5). FIT-GFP was mostly localised to the nucleus. The portion of cytoplasmic FIT-GFP was only minor (Figure 4.15). As expected, the application of MG132 resulted in increased GFP signal intensities. This increase seemed to be more prominent on Fe deficient plants (Figure 4.15) which was in accordance with our CHX results, where we documented enhanced turnover of HA-FIT in Fe deficient roots (Figure 4.6).



**Figure 4.15: FIT-GFP abundance in respect to Fe supply and in the presence of the proteasome inhibitor MG132 in complemented *fit-3* plants.**

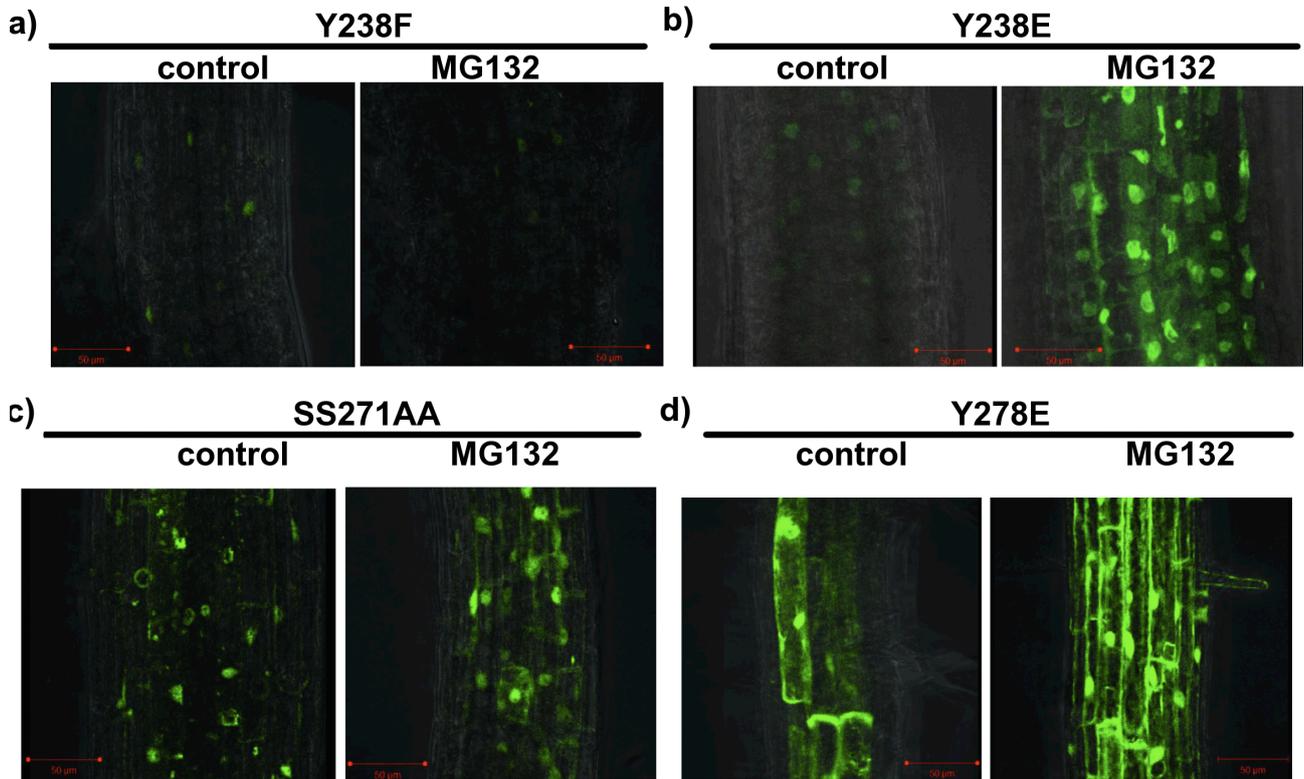
GFP signals were detected by confocal microscopy in three different root zones of 6 day old seedlings grown on + Fe or - Fe, respectively. (a): root hair zone, (b): elongation zone, (c): root tip. For MG132 treatment plants were transferred for 2,5 h to liquid Hoagland medium with MG132 or without (control), prior analysis. All images are z-stacks, representing a full projection image of the root. All images are representative images (n=5).

In further experiments, we grew all available mutagenised FIT forms from the T2 generation (Table 4.1) in the same way as it was done for FIT-GFP, to perform similar analysis. Because the seedlings stay viable in this experiment we were able by using this method to select T2 positive plants displaying the GFP signal while simultaneously performing initial experiments with the individual lines to obtain more results to characterise the different mutant forms, including the MG132 treatment. All four lines that were investigated displayed clear GFP signals. An increase in GFP signal intensity could be observed for FIT<sup>Y238E</sup>, FIT<sup>SS271AA</sup> and FIT<sup>Y278E</sup> but not for FIT<sup>Y238F</sup> (Figure 4.16). Interestingly, the two forms, FIT<sup>SS271AA</sup> and FIT<sup>Y278E</sup>, that had higher cytoplasmic to nucleus ratio in transient tobacco transformations, displayed a similar localisation pattern in

Arabidopsis roots. In the case of FIT<sup>Y278E</sup>, where this difference in localisation was not significantly different (in tobacco experiments, compared to wild type FIT-GFP) the high cytoplasmic portion of GFP signal was even better visible than in the case of FIT<sup>SS271AA</sup> (Figure 4.16d, compare with Figure 4.11, 4.12).

The two opposing forms FIT<sup>Y238F</sup> and FIT<sup>Y238E</sup> did not show a high cytoplasmic portion of GFP signal in the control conditions. Here, the GFP signal seemed to be focused at the nucleus, which was again in accordance to the tobacco results, where these two forms had higher nuclear localisation compared to wild type FIT-GFP (Figure 4.16a, b, compare with Figure 4.11, 4.12). After MG132 treatment no striking difference in GFP signal intensity could be observed for FIT<sup>Y238F</sup>, but for FIT<sup>Y238E</sup>. In FIT<sup>Y238E</sup> plants, the cytoplasmic portion of GFP signal increased after the addition of MG132, most probably due to over-accumulation of FIT-GFP. The different levels of GFP signal between the different plant lines could be explained by different expression levels as it was shown before for the different HA-FIT plant lines (Figure 4.2). Such differences in basic expression level will presumably end up in different GFP intensities. However, the difference in protein abundance caused by MG132 within the same plant line must be due the application of the proteasome inhibitor, since such plants derive from the same origin having equal expression levels.

Summarising the results obtained with the mutagenised FIT forms, we can conclude that the results from transient tobacco transformations and the ones from stable transgenic Arabidopsis plants were complementing each other. The introduced mutations indicate that the selected amino acids are likely to be involved in FIT protein control. Phosphorylation and de-phosphorylation of FIT at specific amino acids could result in altered FIT localisation and stability. These alterations in localisation and stability could thereby influence the biological functionality of FIT, ending up in an altered Fe uptake response. For detailed description of the molecular functions of these amino acids, further experiments have to be performed in the future. In the case of FIT<sup>Y238F</sup> it could be possible that un-phosphorylated tyrosine 238 is not affected by MG132 treatment. Interestingly FIT<sup>Y238F</sup> was also unable to complement the *fit-3* mutant. Overall these preliminary data are very promising, since they could be reproduced by different experimental approaches.



**Figure 4.16: Effect of MG132 on protein abundance of different mutagenised FIT-GFP constructs in *fit-3* mutant background (T2 plants).**

All plants were grown for 6 days at + Fe. For MG132 treatment, plants were transferred for 2,5 h to liquid Hoagland containing MG132 or not (control). All images are z-stacks, representing a three-dimensional image of the root. One representative image is presented each (n = 5). The red bar represents 50 μm. For quantification of the individual GFP signals, higher magnification is necessary. Reliable quantification data could not be generated so far. Further improvement of the method will be done in the future.

## 5. Discussion

In this work regulatory mechanisms acting at protein level upon a key transcription factor of the Fe deficiency response were investigated. FIT protein was the subject of a turnover control at both + and – Fe conditions and was susceptible to proteasomal degradation. The turnover control took place in a stronger manner at – Fe than at + Fe. Nitric oxide (NO) was identified as an internal signal for achieving full-level FIT protein accumulation. NO counteracted the proteasomal degradation of FIT and presumably acted in a similar manner as ethylene (Lingam et al., 2011). The FIT protein activity depended mainly on Fe supply. A small pool of active FIT protein was found sufficient to trigger downstream Fe acquisition responses at - Fe. In addition to NO and ethylene we present evidence that phosphorylation of FIT may be a further regulator, that could regulate FIT activity.

### **High level expression of *FIT* in Fe deficient leaves leads to ectopic expression of *FRO2* and *IRT1***

By detailed analysis during the generation of the HA-FIT plants we could resolve that ectopic activation of *FRO2* and *IRT1* in leaves of *FIT* over-expression plants depended on the amount of *FIT* transcript. Only above a certain *FIT* transcription threshold level, activation of downstream genes took place. We suggest that this level was more likely reached when *FIT* expression was driven by a double CaMV 35S promoter rather than a single promoter in FIT Ox lines (compare with Colangelo and Guerinot, 2004 and Jakoby et al., 2004). Additional factors needed for FIT protein activation by – Fe must therefore be present in leaves as well. The requirement for high *FIT* gene and FIT protein expression levels in leaves seems in apparent contrast to the situation in roots where obviously a small pool of FIT is sufficient to trigger Fe acquisition responses. We interpret the failure to activate low levels of FIT protein in leaves in a way that perhaps these additional activation mechanisms have a low affinity for FIT in leaves. Moreover such additional ectopic effects of FIT could result in altered metal distribution within the leaves. It could be possible that due to the ectopic expression of *FRO2* and *IRT1* and perhaps other transporters and reductases in the leaf cells (like *FRO3*, *VIT1*, *NRAMP3,4*, *OPT3*) are also deregulated, which would result in a mis-localisation of Fe throughout the leaf cells. This could explain, why the complemented HA-FIT/*fit-3* plants show a weak leaf chlorosis in the advanced reproductive stage, whereas they look like wild type in the younger stage (Figure 4.1). During the reproductive stage metals are re-mobilised from the leaves and transported to the flower organs and the developing seed (Klatte et al., 2009). Metal measurements

(Figure 4.3) did hardly show alterations in Fe or Mn content, whereas such measurements do not mirror intracellular variations in metal localisation. Also during the generation of the FIT-GFP/fit-3 plants, slight leaf chlorosis during the reproductive stage could be detected. Since HA-FIT was also turned-over in leaves and was able to induce downstream genes, it can not be ruled out that FIT can overtake certain functions also in leaves. However, further experiments would have to be performed to clarify this. A first attempt could be to measure the expression of further genes like *FRO3* or *VIT1* to investigate if their expression is affected by *FIT* over-expression.

### **FIT activity is controlled at multiple steps from transcription to active FIT protein**

Due to our combined analysis of FIT protein regulation in wild type plants on one side and in HA-FIT over-expression plants on the other side, we could discriminate multiple regulatory mechanisms acting upon FIT protein. The wild type situation allowed uncovering transcriptional and post-transcriptional regulation. To investigate the endogenous FIT abundance, Sivasenkar Lingam generated a polyclonal affinity-purified antiserum, directed against the C-terminal peptide of FIT (Lingam et al., 2011; Dissertation Lingam, in preparation). This antiserum allowed us to detect FIT in wild type plants. The over-expression plants allowed us to confirm that indeed post-transcriptional effects took place, since the regulation of FIT protein abundance could be followed by uncoupling it from the transcriptional control, because *HA-FIT* was driven by the 2xCaMV35s promotor instead of the native *FIT* promotor. The findings about the multiple mechanisms that confer FIT activation are summarised in Figure 5.1.

The first control step in FIT activation takes place at transcriptional level. *FIT* gene expression is induced at – Fe (represented in Figure 5.1a, wild type situation; compare with (Colangelo and Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2005). *FIT* induction requires transcription factors acting upstream of *FIT*. Positive regulators must be themselves activated by – Fe. Alternatively, a negative regulator might suppress *FIT* transcription at + Fe. Elimination of the repressor at – Fe would equally result in an induction of *FIT*. In this respect it is interesting that application of cycloheximide (CHX) at + Fe resulted in increased *FIT* gene expression. An explanation for the observation is that CHX destroyed the repression at + Fe. The presumptive repressor of *FIT* at + Fe might be subject of a protein turnover control. It is appealing to speculate that the turnover control of the *FIT* repressor responds to Fe so that the repressor may be effectively diminished upon – Fe.

As a second regulatory step FIT was controlled by post-translational turnover control (Figure 5.1a, b). Inhibition of protein translation led to a decrease of FIT protein abundance irrespective of *FIT* transcription. FIT protein must undergo cycles of degradation and re-synthesis. In wild type plants, turnover of FIT was detectable only at – Fe. Yet, upon CHX treatment which resulted in induced *FIT* gene expression at + Fe to the same level as at – Fe, FIT protein was still not detectable. Because any synthesised FIT protein would again be turned over. From our studies using HA-FIT plants we can confirm that turnover of FIT took place at + and – Fe in roots and leaves, whereby it was most pronounced upon – Fe in roots. Increased turnover of FIT at – Fe can be explained by a better recognition as a substrate for protein degradation, for example due to a specific modification of FIT, present at – Fe only. It is not uncommon for transcription factors that their degradation occurs, after they have been modified and activated (for example by phosphorylation) (Gampala et al., 2007; Shen et al. 2007, Shen et al. 2009). The physiological reason for switching off active transcription factors is to allow cells to continuously remain responsive to incoming signals. A third level of regulation took place at the level of protein activity (Figure 5.1a, b). The output of FIT activity was measured as induction of *IRT1* and *FRO2*. Abundance of FIT protein was not found proportional to the level of activity of FIT protein. Despite of elevated levels of FIT/HA-FIT protein in over-expression roots, a general increase of FIT activity was not found, compared to the wild type. On the other hand, CHX treatment, which caused a down-regulation of FIT protein abundance, could not significantly affect the downstream responses. The activation of FIT was therefore not compromised by CHX treatment. Obviously, low amounts of active FIT protein were sufficient to trigger full *IRT1* and *FRO2* induction. On the other hand, FIT activity was constrained at a maximum level that could not be exceeded by increasing FIT/HA-FIT protein amounts. The activation of FIT was most probably a bottleneck. This bottleneck could have been achieved through limitation of enzymes that may confer specific post-translational modifications needed for FIT activity or through a limitation of FIT interaction partners such as bHLH038 and bHLH039 (Yuan et al., 2008). It could also be possible, that only after post-translational modification of FIT the heterodimerisation between FIT and bHLH038/039 can take place. One example is the brassinosteroid responsive transcription factors BZR1 and BZR2, that are controlled by GSK3 kinase mediated phosphorylation. Only after phosphorylation they can interact with 14-3-3 proteins (Gampala et al., 2007).

If FIT has to be modified first or if such a modification has to be removed prior to FIT action, is so far unclear. Both could be possible, which could be observed by investigating the transcription factors regulating photomorphogenesis. In this case the light stable

transcription factors become phosphorylated prior to activation and are stabilised after phosphorylation. However, the light in-stable PIF transcription factors, become ubiquitinated and degraded after phosphorylation (Shen et al., 2007; Al-Sady et al., 2006). The mechanisms acting at the regulation of photomorphogenesis, show that there is no clear dogma if phosphorylation results in a stabilisation or de-stabilisation of a protein. Moreover these results show that phosphorylation of a protein can be obligate to initiate ubiquitination and subsequent degradation via the 26 s proteasome.

### **Nitric oxide stabilises FIT by reducing proteasomal degradation**

As shown previously, cPTIO caused a decrease of Fe deficiency gene expression (Besson-Bard et al., 2009; Chen et al., 2010; Graziano et al., 2002; Graziano and Lamattina, 2007). At transcriptional level NO can therefore induce *FIT*, *IRT1* and *FRO2*. In addition, we demonstrated here that cPTIO also caused a reduction of FIT protein. cPTIO, a scavenger of NO, caused a reduction of FIT protein levels at + and – Fe. Thus, it can be inferred that NO promoted FIT protein levels. The observed inhibitory effect of cPTIO on FIT protein levels were not merely the result of reduced transcriptional activation for two reasons. First, reduced protein accumulation caused by cPTIO was also apparent in HA-FIT over-expression plants where *HA-FIT* transcription was not regulated by NO and thus not affected by cPTIO. Second, MG132, which inhibits proteasomal degradation, restored FIT protein levels upon cPTIO treatment. These observations suggest that inhibition of NO provoked a stronger proteasomal degradation of FIT. Hence, NO may act to prevent proteasomal degradation of FIT. Proteasomal degradation of FIT has recently also been reported by Sivitz et al. (2011), who showed that FIT-GFP abundance increased upon MG132 treatment compared to untreated controls. The results from Sivitz et al. (2011) confirm our results in the way that FIT is subject of turnover and that it has to be constantly degraded to keep up the induction of *FRO2* and *IRT1*. In addition to these findings we give evidence that the presence of NO is important for FIT stability. It could be suggested that perhaps NO may lead to modifications on FIT that regulate FIT stability and in addition also FIT function.

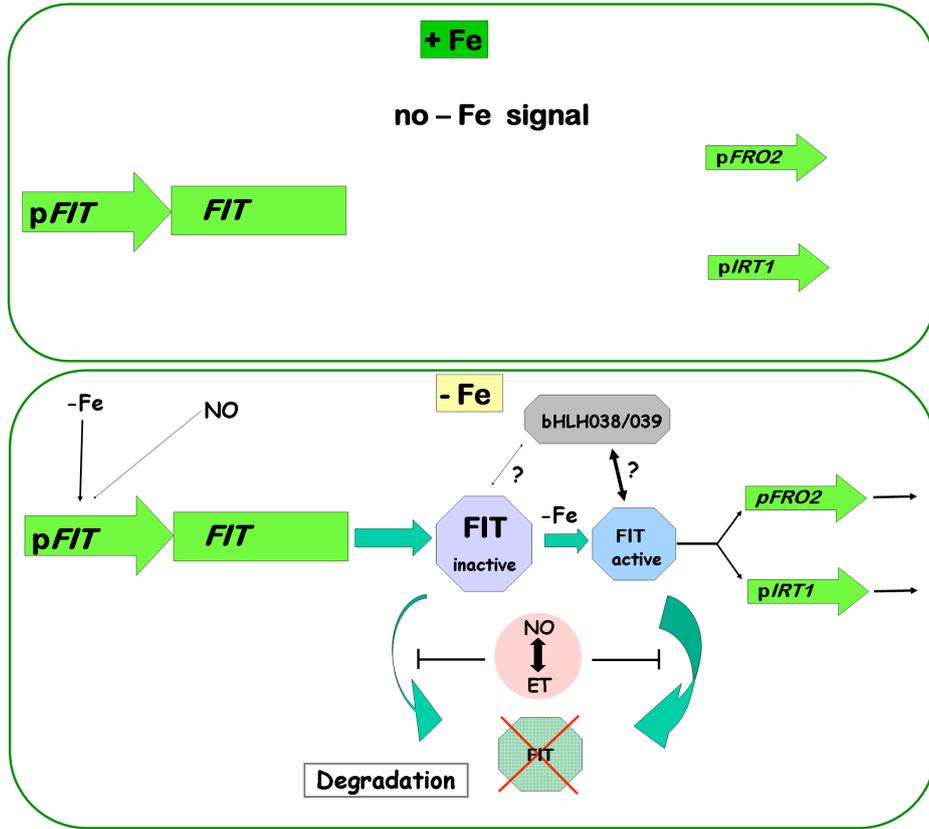
Although principally low amounts of FIT protein are sufficient to trigger FIT downstream responses to full level, this was not the case upon cPTIO treatment in wild type. Perhaps the remaining levels of active FIT were too low in the wild type treated with cPTIO to cause downstream gene induction. In HA-FIT plants treated with cPTIO, the levels of remaining FIT protein were higher than in the wild type, and presumably sufficient amounts of active

FIT were among it. This could be the reason why in HA-FIT plants, cPTIO application did not affect downstream gene expression. NO could be involved in activating and stabilising FIT directly or indirectly. Direct effects could be exerted by way of S-nitrosylation of cysteines (Lindermayr and Durner, 2009; Tada et al., 2008; Lindermayr et al., 2010), while indirect modifications may occur through alterations of enzyme activities occurring as a response to NO. The amino acid sequence of FIT contains three cysteines, whereas two are in the c-terminal part (Figure 4.9 b)) and one is in the n-terminal part of FIT, all three cysteines are conserved to LeFER. Since the C-terminal part of FIT is supposed to be the target for regulation and was used in yeast two hybrid screen before (Lingam et al., 2011), a NO dependent S-nitrosylation of FIT could be likely. If modifications of FIT would occur in response to NO, this could be the explanation why the physiological output differed between untreated controls, CHX and cPTIO application.

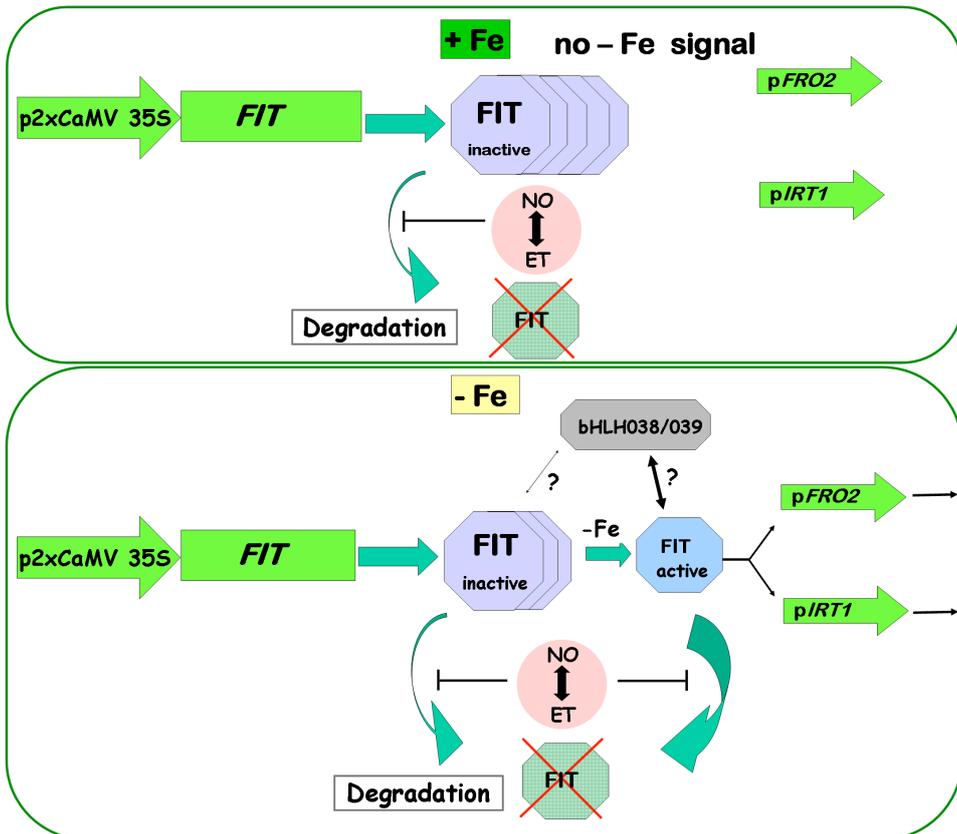
Interestingly three of the four bHLH proteins from the subgroup Ib, namely bHLH038/039/100 contain conserved cysteines (Vorwieger et al., 2007), which could be putative targets of S-nitrosylation as well. A NO dependent regulation of these genes is supported by the findings of Garcia et al. (2010), who showed that the expression of bHLH038 and bHLH039 is increased if external GSNO is supplied. Since transcriptional regulation by NO seems to be in addition to post-translational regulation as it was shown here for FIT, a NO dependent post-translational regulation of the bHLH genes from the subgroup Ib could also be possible. Such a NO dependent regulation of FIT and/or bHLH038/039 could be one possibility to regulate their interaction (Yuan et al., 2008; see Figure 5.1).

The NO effect on gene expression and FIT protein regulation paralleled that of ethylene (Besson-Bard et al., 2009; Chen et al., 2010; García et al., 2010; Graziano et al., 2002; Graziano and Lamattina, 2007; Lingam et al., 2011; Lucena et al., 2006). Ethylene like NO is required for full-level up-regulation of Fe deficiency gene expression and FIT protein abundance. This observation suggests that NO and ethylene act in the same way and perhaps in sequential order. It was recently proposed that a strictly linear relationship between NO and ethylene action may not exist and that they may both promote each other (García et al. 2010, García et al. 2011, Romera et al. 2011). Thus, FIT may be the key regulator in integrating the different incoming signals from hormonal and intracellular triggers (Figure 1.3, 5.1). Limitation of FIT activity seems crucial in plants since FIT activity control may serve to prevent excessive Fe acquisition. Excessive Fe uptake can lead to uncontrolled toxicity effects due to free metals, for instance resulting in radical production through the Fenton reaction.

**a) Wild Type**



**b) FIT over expression**



**Figure 5.1: Model summarising the regulation of FIT activity in wild type and HA-FIT Ox plants by multiple control steps**

(a), Wild type. In + Fe wild type roots, *FIT* induction does not take place. It might be repressed by a negative regulator. Downstream targets of FIT like *FRO2* and *IRT1* are not induced.

In – Fe wild type roots, *FIT* transcription is induced. The presumptive *FIT* repressor protein might be removed by – Fe. Subsequently, FIT protein is produced. Due to a - Fe signal, FIT is activated and promotes induction of *FRO2* and *IRT1*. FIT itself is degraded due to protein turnover (compare this work and (Lingam et al., 2011; Sivitz et al., 2011). Nitric oxide (NO) and ethylene (ET) increase the accumulation of FIT, by counteraction of proteasomal FIT degradation (compare this work and (Lingam et al., 2011).

bHLH038/039 an interacting factor of FIT (Yuan et al., 2008) could regulate FIT activity by additional selective interaction with FIT. On the other hand post-translational modification of FIT could be mandatory to allow this interaction. Conclusively, activation of downstream responses could rely on heterodimerisation. Thus, modification of FIT for subsequent interaction could be the rate limiting step.

(b), HA-FIT over-expression. Transcriptional control of *HA-FIT* is not relevant due to the 2xCaMV 35S promotor. In + Fe HA-FIT roots, HA-FIT is targeted by protein turnover. Nitric oxide (NO) and ethylene (ET) increase the accumulation of FIT, probably by counteraction of proteasomal FIT degradation. In the absence of a – Fe activating signal, *FRO2* and *IRT1* targets are not induced.

In - Fe HA-FIT roots, HA-FIT is activated by a – Fe signal and promotes the expression of *FRO2* and *IRT1*. Further details in (b) are as in (a).

**Phosphorylation as one way for post-translational regulation of FIT**

Besides nitric oxide acting on *FIT* transcriptional regulation and FIT stability, we also propose a post-translational regulation present on FIT, that should be responsible for Fe dependent turnover speed of FIT. Thus, such a modification (or the specific removal of that modification) should result in less FIT stability. By way of *in silico* analysis we predicted four amino acids in the C-terminal part of FIT, being putative targets for phosphorylation. Complementing work from our group, could confirm our hypothesis of phosphorylated FIT (unpublished data). In these experiments, protein samples of HA-FIT plants were treated with phosphatase prior to separation in a two-dimensional polyacrylamid gel and subsequent immunological detection of these gels. Phosphatase treated samples showed a clear difference in the spot pattern compared to untreated controls (remember that the HA antibody is highly specific). Moreover, phosphatase treated HA-FIT proteins showed a shift in their gel mobility, which could be observed in SDS-PAGE experiments. These findings could explain the findings from Figure 4.5, namely that a second smaller form could be detected directly under the main band. Hence, this could be due to different modified FIT forms (different phosphorylation states) present in the protein extract, resulting in slightly altered gel mobilities.

The here presented work shows that the different mutations present in the FIT amino acid sequence, resulted in differential cytoplasm to nucleus localisation ratios. In addition, the corresponding protein abundances differed in their amounts and in their gel mobility or protein size, respectively. A correlation between gel mobility and cellular localisation may be possible. It is tempting that proteins with higher nuclear localisation have lower gel mobility (compare FIT<sup>Y278F</sup>, FIT<sup>Y238F</sup> and FIT<sup>Y238E</sup> with FIT-GFP in figure 3.12 and 3.13). FIT<sup>Y278E</sup> had a smaller protein size than FIT<sup>Y238F</sup> and was not significantly different in localisation compared to wild type FIT-GFP. Why the opposite versions FIT<sup>Y238E</sup> and FIT<sup>Y238F</sup> show the same localisation pattern is not clear so far. It is known from the literature that phosphomimicking protein modifications do not always result in full physiological functionality, because the introduced glutamate with its negatively charged carboxyl group is similar, but still different from a tyrosine covalently coupled to phosphate (Widmann et al., 1999). However, non-phosphorylated proteins are thought to disturb the function of the protein of interest by inhibiting possible phosphorylations at this amino acid. In case such phosphorylation has importance, loss of function effects should be the result, due to the non-phosphorylatable mutation. Taking the results from these phosphatase treatments and the results about the mutated FIT forms, presented in this work into account, strengthen the hypothesis about phosphorylated FIT. Hence, phosphorylation of FIT may influence FIT activity and turnover speed.

The complementation experiments gave first hints in interpreting the possible molecular function of tyrosine 238. FIT<sup>Y238F</sup> was unable to complement the *fit* mutant, leading to the hypothesis that phosphorylation of tyrosine 238 would be obligate for FIT function and maybe its subsequent degradation, since application of MG132 did not show an effect on protein abundance of FIT<sup>Y238F</sup>. On the other hand it could also be possible that FIT<sup>Y238F</sup> is degraded by a proteasome independent pathway. Proteasome independent degradation of ubiquitinated proteins was recently shown for ubiquitinated IRT1, that is degraded in the vacuole (Barberon et al., 2011). In the case of FIT<sup>Y238E</sup>, the *fit* mutant could be partially rescued, because the phosphorylation was mimicked. However, such a mimic could not equally replace the native phosphorylation on FIT, resulting in an intermediate state at physiological activity. Moreover both versions of FIT<sup>Y238</sup> resulted in higher nuclear localisation. This was not the case for the opposite forms of FIT<sup>Y278</sup> and FIT<sup>S221</sup>, which resulted in opposite nuclear to cytoplasmic ratios.

Antagonistic results due to opposite mutations on one specific amino acid seem to be the most logical result as it was the case for serine 221. FIT<sup>S221A</sup> may undergo a very rapid turnover, because it could not be detected by western blot although it gave clear GFP

signals, that could be detected by microscopy. In contrast, FIT<sup>S221E</sup> had very high stability or impaired degradation, respectively, because the protein abundance was above the wild type FIT-GFP level (Figure 4.13c). In this case de-phosphorylation of serine 221 could be a target for FIT degradation. It will be of particular interest to test, if FIT<sup>S221E</sup> and FIT<sup>Y238F</sup> will be able to induce the expression of *FRO2* and *IRT1* in *fit-3* mutant background. Following our model (Figure 5.1), it could be expected that FIT has to be modified (which should result in activation of FIT) prior initiating transcriptional activation of *FRO2* and *IRT1*. Therefore serine 221 and/or tyrosine 238 could be activation sites and simultaneously degradation signals. Depending on the amino acid the modification could be either phosphorylation (in case of Y238) and/or de-phosphorylation (in case of S221). Such activated FIT should likely to be more susceptible for degradation. Degradation of activated FIT would also be in support of the model, presented by Sivitz et al. (2011), who proposed that FIT has to be degraded, after it bound to the promotor regions of target genes. An example for phosphorylation-dependent ubiquitination is the MYC like bHLH transcription factor *ICE1* (*INDUCER OF CBF EXPRESSION1*). It was shown that ICE1 is phosphorylated at S403. Mutated ICE1<sup>S403A</sup> showed enhanced cold resistance and blocked poly-ubiquitination *in vivo* (Miura et al., 2011), therefore de-phosphorylated ICE1 seemed to be the active protein in this case. Very interestingly ICE<sup>S403A</sup> showed over-induction, this means that the de-phosphorylated form of ICE1 can constantly induce downstream responses. The model by Sivitz et al., (2011) proposes that FIT has to be constantly turned over, because activated FIT can only shortly function in transcriptional induction. Their hypothesis is based on MG132 treatments. They show that FIT protein abundance increases, but downstream responses (induction of *FRO2* and *IRT1*) decrease in the presence of MG132. This way they explain that „used“ FIT is unable to induce transcription of its target genes and has to be degraded so that „fresh“ FIT can bind to the promoter region of its target genes. Whether or not this model is true can not be answered with such experiments. The generation of the here presented FIT mutant versions such as FIT<sup>S221A/E</sup> and FIT<sup>Y238F/E</sup> can ultimately clarify how downstream responses of FIT will be influenced (as it was shown for ICE1). These data will help to draw a more reliable and in detail model about FIT regulation. Regarding the here inspected phosphorylation sites, it can be summarised that all four phosphorylation sites that were inspected, showed results that were different to wild type FIT-GFP and also different to each other, which raises the possibility that all the selected mutations could function in FIT protein regulation. Several phosphorylation sites in one protein with different outputs is not uncommon. The bHLH protein SPCL was also described in having more than one phosphorylation site, each

having different effects (Lampard et al., 2008). Moreover, EIN3 was also reported for being phosphorylated at T174 which resulted in enhanced stability, whereas phosphorylation of T592 led to degradation (Yoo et al., 2008). With the here presented data we can conclude that phosphorylation might be mandatory to regulate FIT function.

What makes a phosphorylation so important for a protein? Covalently coupled phosphates result in conformational changes within the three-dimensional protein structure, which can be important for the functionality of the protein. For example, such a conformational change may be necessary to introduce an interaction with specific interaction partners such as bHLH038/039 (Yuan et al., 2008) or EIN3/EIL1 (Lingam et al., 2011). Introduction of protein-protein interaction after post-translational modification, is common in plants as it was shown for example for BZR1 and BZR2 (Gampala et al., 2007), PIF5 (Shen et al., 2007) or NPR1 (Tada et al., 2008). Perhaps the interaction between EIN3/EIL1 and FIT (Lingam et al., 2011) is only possible if one or both proteins are phosphorylated. Phosphorylation of EIN3 by a MAPK has been reported (Yoo et al., 2008).

In further experiments specific interaction studies with the different FIT mutant forms and interaction partners can be performed by using the bimolecular fluorescence complementation (BiFC) method. Cloning of the different gene constructs into the specific binary destination vectors had been done during this work in parallel. Our aim will be to investigate if there are differences in the interaction efficiency between the different FIT mutant forms with its interaction partners. BiFC has been successfully applied in our lab to prove the interaction between FIT and EIN3/EIL1 (Lingam et al., 2011). With the newly developed mutant forms we can characterise the detailed molecular mechanism that underlies this interaction.

### **Integration of Fe deficiency signals on FIT**

A still open question is the origin of the Fe deficiency signal and its subsequent transduction. After the rise of a signal, it may be relayed by second messengers such as  $\text{Ca}^{2+}$ , cyclic nucleotide monophosphates, inositol polyphosphates, NO or other small molecules (Reddy et al., 2011). In several reports it was shown, that  $\text{Ca}^{2+}$  plays a crucial role in biotic and abiotic stress signalling (Reddy, 2001; Hepler, 2005; McAinsh and Pittman, 2009, DeFalco et al., 2010). Increasing cytoplasmic and/or nuclear  $\text{Ca}^{2+}$  concentrations are the result of perceived stress signals such as, cold, heat, salt, drought, osmotic stress or oxidative stress and trigger transcriptional responses directly or indirectly (reviewed in Reddy et al., 2011). Alterations in  $\text{Ca}^{2+}$  concentrations are specific for certain

stresses and are called  $\text{Ca}^{2+}$  signatures.  $\text{Ca}^{2+}$  binding proteins are thought to bind  $\text{Ca}^{2+}$  ions to sense changes in cellular  $\text{Ca}^{2+}$  concentrations to initiate the generation of appropriate physiological responses (Nakayama et al., 2000; Day et al., 2002; Reddy et al., 2011).  $\text{Ca}^{2+}$  sensors can be grouped into sensor relays and sensor responders (Sanders et al., 2002; Kudla et al., 2010). Sensor relays interact with other proteins to regulate their activity. For instance, the group of calcineurin B-like calcium sensor proteins (CBLs). Whereas animal CBLs interact with phosphatases, plant CBLs interact with a family of protein kinases called CBL-INTERACTING PROTEIN KINASES (CIPKs) (Luan et al., 2002; Luan, 2008). These kinases can then regulate downstream responses by phosphorylating target proteins. One example for such a regulatory network is the phosphorylation dependent activity control of the potassium channel AKT1. Under potassium deficiency, a reactive oxygen species (ROS) pulse is generated that in turn generates a  $\text{Ca}^{2+}$  pulse, which is subsequently detected by a CBL. This CBL interacts with CIPK23 and can phosphorylate AKT1 (Li et al., 2006; Xu et al., 2006; Lee et al., 2007; Luan, 2009; Lan et al., 2010). Phosphorylations are reversible modifications, that can be reversed by protein phosphatases. Lan et al. (2010) showed this for the phosphorylation of AKT1, which can be de-phosphorylated by AIP1, an A-type protein phosphatase 2C member (PP2C). This way one single protein can be regulated by directed phosphorylation and de-phosphorylation. Bioinformatic approaches revealed the existence of 25 CIPKs (Yu et al., 2007, Kolukisaoglu et al., 2004) and 10 CBLs (Kolukisaoglu et al., 2004) in Arabidopsis, resulting in a complex interaction network, that can regulate versatile downstream pathways.

Because FIT seems to be subject of post-translational modification in way of phosphorylation, it will also be of high interest to identify kinases that confer such phosphorylations. Phosphorylation of Y238 and Y278 would probably be performed by different kinases (tyrosine specific kinases) than the ones that would be responsible for S221 and S271 (ser/thr specific kinases). Moreover, it will be interesting to know if also phosphatases exist that remove phosphorylations off FIT, since phosphorylation is a reversible modification. The ROS triggered induction of AKT1 in way of CBL1-CIPK23 driven phosphorylation and counteraction by the phosphatase AIP1 (belonging to the PP2C family) gives an example how phosphorylation and de-phosphorylation can regulate one single protein (Lan et al., 2011). Moreover, this example also documents the relation of ROS signals triggering stress responses to abiotic stress, such as malnutrition. In the case of potassium deficiency a ROS pulse is triggered, that in turn induces the second messenger  $\text{Ca}^{2+}$  and thereby activates the CBL-CIPK pathway. A ROS driven  $\text{Ca}^{2+}$  pulse

as a result of Fe deficiency could also be feasible. Such a regulatory network and the possibility that ROS can function as a long distance signal (Mittler et al., 2011) suggests thereby one possibility how such a signalling could be initiated and relayed. Different phosphorylation states of FIT could be the result of such a signalling cascade, where FIT functions as the receiver of this cascade. In addition to this, ethylene and NO could be thought to adapt and fine tune the Fe deficiency response.

Regarding Fe deficiency, ROS could be generated due to disassembling of the respiratory chain because of lacking Fe atoms that are obligate as cofactors (Zaharieva et al., 2004; Sun et al., 2006). Mittler et al. (2011) postulated that ROS signals can be specific to certain stimuli. Induction of NO, ethylene, MAPK pathways or second messengers such as calcium could be accompanied by a ROS pulse or could be a consequence of it, respectively. Increasing  $Ca^{2+}$  concentrations could result in an activation of a responsive CBL that in turn interacts with a CIPK (like in the case of AKT1) or phosphatase to subsequently transform FIT into a more active state. Inactivation of FIT could be performed by degradation via the proteasome and/or reversed phosphorylation state. Further possibility could be a MAPK-driven phosphorylation cascades acting on FIT. On the other hand NO and ethylene result in enhanced stability of FIT (Lingam et al., 2011; Meiser et al., (provisionally accepted)). This would be in accordance with the initial proposed theory that a network of stimuli act on FIT, which in turn integrates versatile signals to tightly regulate Fe homeostasis.

## **Conclusions**

Protein turnover of transcription factors is prevalent in plants. Post-translational modifications affect the protein interaction capacities of bHLH proteins and their functions (Barnes and Firulli, 2009; Bracken et al., 2003). In plants, functional modifications of bHLH proteins were reported to occur during light perception and development (Han et al., 2010; Kang et al., 2009; Lampard et al., 2008; Park et al., 2008; Shen et al., 2007; Shen et al., 2009). Hormone response transcription factors such as the ones related to auxin signalling are also targets for proteasome action, for example of the ubiquitin 26S proteasome system (Schwager et al., 2007; Vierstra, 2009). The interpretation is that this way cells remain continuously responsive to the incoming signals and reorientation of activity of these transcription factors can be modulated in a flexible manner. Once activated, transcription factors can be rapidly removed from the cell by protein degradation to prevent excessive action.

Our study is among the first to address the control of a key Fe deficiency transcription factor in response to incoming signals such as nitric oxide. Controlled turnover and activity of FIT strengthens the importance of FIT. This work answers the question if FIT is post-translationally regulated and reaches further by asking for the consequences and the type of protein modifications acting on FIT. Phosphorylation and ubiquitination of FIT seems to be involved in FIT turnover and activity control. Moreover, S-nitrosylation could be an other modification acting on FIT by stabilising it. This way plant roots remain responsive to changing Fe availability in the soil as well as changing demands for Fe nutrition during the day and throughout the plant life cycle. It remains to be elusive which signals control the Fe uptake, upstream of FIT. The identification of the kinases that modify FIT would make it possible to get one step upstream towards the origin of the Fe deficiency signal which is one of the ultimate goals in understanding plant Fe nutrition.

## 6. Perspectives

With the here presented data we could identify FIT as a plausible molecular connection for the cross-talk between the plant hormone NO and the Fe deficiency signalling pathway. We gained evidence to support our model for post-translational modification of FIT to adapt the Fe deficiency response. 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 categorised in three main goals.

First, S-nitrosylation of FIT: to answer this question a collaboration with the Institute of Biochemical Plant Pathology, located at the Helmholtz Zentrum München has been initiated recently. The group of Prof. Durner is an expert in investigating S-nitrosylation on proteins. If the in vitro analysis of FIT confirm a S-nitrosylation, mutagenesis of the respective cysteine(s) could be performed to further investigate the effect of such a modification by functional analysis.

Second, ubiquitination of FIT: to prove ubiquitination, FIT should first be immunoprecipitated with the HA or the GFP tag to investigate direct ubiquitination of FIT by specific commercially available antibodies. Such work can be done in a fast way and gives final proof, rather than indirect hints by using chemicals such as MG132. However, the results from the MG132 experiment make it reasonable to check ubiquitination with such an antibody. Moreover, it could be searched for interacting E3-ligases such as F-box or RING finger proteins that confer ubiquitination on FIT. Because the E3-ligase BTS is supposed to negatively affect Fe homeostasis by influencing the PYE-ILR3 network (Long

et al., 2010), BTS could be responsible for the degradation of proteins related to Fe uptake and distribution as well. Therefore the relation of BTS to FIT and perhaps the bHLHs from the subgroup 1b, could be investigated in detail. A more general approach to find candidate genes could be performed by using the available set of data from microarray and databases.

Third, phosphorylation of FIT: this will be the major part of the future work. Once all lines are available homozygous, downstream responses of FIT can be investigated by real-time qRT-PCR and direct phosphorylation of FIT could be proven by *in vitro* kinase assays and/or mass spectrometric analysis to identify and prove phosphorylations at specific positions. In addition it would probably be reasonable to make one FIT version that contains all four non-phosphorylatable sites to inspect if such a FIT mutant is indeed non-phosphorylatable or if other amino acids can be phosphorylated as well. Moreover, the kinases that confer phosphorylations on FIT should be identified. The available data that can be obtained by computer analysis, could help to identify candidates that can then be inspected regarding their ability to interact with FIT. To screen a high number of candidates, the yeast two-hybrid assay could be used.

Obtaining detailed understanding of the molecular mechanisms that control Fe uptake, can help to breed higher quality crop plants to battle nutritional problems worldwide.

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# Curriculum Vitae

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### Dissertation

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### Study and Diploma thesis

10/2002 - 4/2008	Diploma Thesis: Regulation of the protein expression in <i>Arabidopsis thaliana</i> in dependence of the micronutrient Iron. Supervisor: Prof. Dr. Petra Bauer Study of Human- und Molecular biology at the Saarland University, Germany Majors: Biochemistry, Virology, molecular plant biology
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## Publication list

**Meiser, Johannes**; Lingam, Sivasenkar; Bauer, Petra; 2011 Post-translational regulation of the Fe deficiency bHLH transcription factor FIT is affected by iron and nitric oxide, *Plant Physiology* (provisionally accepted)

### Personal Presentations at International Meetings

„Trinational Arabidopsis Meeting“ 2009, Cologne, Germany. **Meiser, Johannes**; Fink-Straube, Claudia; Bauer, Petra. Over-expression of *FIT* results in altered metal contents in Arabidopsis leaves. (Poster)

„International Symposium on Iron Nutrition and Interaction in Plants“ 2010, Budapest, Hungary. **Meiser, Johannes**; Lingam, Sivasenkar; Bauer, Petra. Post-translational regulation of the bHLH transcription factor FIT. (Poster)

„Plant Protein Phosphorylation Symposium“ 2011, Tübingen, Germany (14. - 16. September 2011). **Meiser, Johannes**; Lingam, Sivasenkar; Ivanov, Rumen; Brumbarova, Tzvetina; Bauer, Petra. Post-transcriptional regulation of the iron dependent bHLH transcription factor FIT. (Poster)