Regulation of Fe DEFICIENCY INDUCED TRANSCRIPTION FACTOR (FIT) protein abundance in response to ethylene and nitric oxide

Zur Erlangung des Grades
des Doktors der Naturwissenschaften
der Naturwissenschaftlich-Technischen Fakultät III
Chemie, Pharmazie, Bio- und Werkstoffwissenschaften
der Universität des Saarlandes

von

Brahmasivasenkar Lingam

Saarbrücken

January 2013

Tag des Kolloquiums: 12.06.2013

Dekan:	Prof. Dr. V. Helms
Berichterstatter:	Prof. Dr. P. Bauer
	Prof. Dr. U. Müller
Vorsitz:	Prof. Dr. I. Bernhardt
Akad. Mitarbeiter:	Dr. K. Lepikhov

Abstract

Understanding the regulation of key genes involved in plant iron acquisition is important for breeding Fe-rich staple crops. In Arabidopsis the basic helix-loop-helix protein FER-LIKE FE DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT), a central regulator of Fe acquisition, is regulated by Fe at the transcriptional and posttranscriptional levels. In this study, we investigated FIT regulation in response to Fe supply in Arabidopsis. The plant hormone ethylene promotes iron acquisition, but the molecular basis for this is largely unknown. FIT levels were reduced upon application of ethylene inhibitor aminoethoxyvinylglycine and in the *ein3eil1* mutant. Ethylene signaling by way of EIN3/EIL1 required for full-level FIT accumulation. Treatment with MG132 could restore FIT levels. Upon ethylene signaling, FIT is less susceptible to proteasomal degradation. Hence, ethylene triggers Fe deficiency responses transcriptionally and posttranscriptionally. Besides ethylene, we identified nitric oxide (NO) as a stabilizing stimulus for FIT abundance. Treatment with NO inhibitors caused a decrease of FIT abundance and in the wild type, also a decreased FIT activity. Independent of *FIT* transcription, FIT protein stability and activity, therefore, targets of control mechanisms in response to Fe and NO. This decrease of FIT protein levels was reversed by the proteasomal inhibitor MG132, suggesting that in the presence of NO FIT protein was less likely to be a target of proteasomal degradation.

Zusammenfassung

Um Nutzpflanzen mit erhöhtem Gehalt an Eisen (Fe) zu züchten, ist es notwendig, die Regulierungsmechanismen der Gene der Fe-Aufnahme zu verstehen.

Ein zentraler Regulator der Fe-Aufnahme in Wurzeln von *A. thaliana* ist das basische Helix-Loop-Helix Protein FIT. Dieses wird durch diverse Signale wie z.B. Fe-Bedarf und Hormone auf transkriptioneller und posttranskriptioneller Ebene reguliert. In der vorliegenden Arbeit wurde die Regulation von FIT in Abhängigkeit von Fe und dem Hormon Ethylen, das die Fe-Aufnahme verstärkt, sowie die molekulare Wirkung von Ethylen untersucht. Der Gehalt an FIT Protein nahm bei Gabe eines Ethyleninhibitors sowie in der *ein3eil1* Mutante ab. Der Ablauf des Ethylensignalweges über EIN3/EIL1 ist nötig für den FIT Level. MG132 normalisierte die FIT Expression. Bei eingehendem Ethylensignal ist FIT gegenüber proteasomalem Abbau weniger anfällig, so dass Ethylen die Eisenmangelantworten transkriptionell und posttranskriptionell steuern kann. Zudem haben wir Stickstoffmonoxid (NO) als Stabilisator für das FIT Protein identifiziert. NO Inhibierung führte zu verminderter FIT Akkumulation und, im Wildtyp, verminderter Aktivität. Die FIT Proteinstabilität und –aktivität ist somit abhängig von durch NO und Fe gesteuerte Kontrollmechanismen. Die Abnahme des FIT Proteingehaltes konnte durch den Proteasominhibitor MG132 umgekehrt werden. Dies bedeutet möglicherweise, dass FIT in Anwesenheit von NO weniger dem Abbau durch das Proteasom unterliegt.

List of contents

List of contents

List of contents

1. Introduction

1.1 Importance of iron

Iron is one of the essential micronutrients for all living organisms. In many organisms, Iron (Fe) serves as a cofactor in vital metabolic pathways for instance the electron transport chain of respiration. Plants do have an additional requirement for iron as it is necessary for/in photosynthesis and chlorophyll biosynthesis. Due to its significant role in several biological processes Fe deficiency can cause serious nutritional disorders in organisms. One of such wide spread and common disorders is iron deficiency anemia (IDA), according to WHO (World Health Organization; http://www.who.int/en) four to five billion people of world's population of developed and developing nations are suffering from the IDA. Majority of them subsist on iron poor, plant based diets. In plants insufficient iron can cause leaf chlorosis, stunted growth and ultimately effects to crop yield with poor nutrient quality.

To combat with IDA it is very important to improve the efforts to increase the bio available Fe content in staple foods and crops. Biofortification has wide acceptance as sustainable way of solving this Fe nutrition disorder. Improving our knowledge in understanding various complex mechanisms regulating plant iron homeostasis is important to develop approaches and to design genetically engineered staple crops particularly grown on marginal soils (calcareous, alkaline soils). On the other hand over accumulation, and excess of iron can cause adverse effects by generating cytotoxic hydroxyl radicals via the fenton reaction (von Wirén et al., 1999). In spite of its ubiquitous and presence in generous amounts in soils Fe is not readily bio available for plants because it forms insoluble complexes under aerobic conditions at neutral or alkaline pH (Grotz and Guerinot M.L., 2006). Therefore, plants developed highly sensitive, sophisticated and tightly regulated mechanisms to cope with their nutritional requirement and to maintain the right balance inside the plant body.

1. Introduction

The dynamic process of iron acquisition mechanisms of plants from the soil, iron mobilization, uptake, transport within the plant body and distribution to appropriate targets will be briefed in the following paragraphs.

1.2 Iron acquisition in plants

Upon sensing Fe deficiency, plants induce a set of highly sophisticated, coordinated responses that act in a collective manner to coup the plant to maximize Fe mobilization and uptake from the soil. In order to obtain sufficient iron from the surrounding environment, plants uses two distinct strategies. Based on these strategies plants are divided in two groups with respect to the strategy that they use for iron uptake. These strategies are mainly classified based on the mechanism that they use for the uptake of iron.

1.2.1 Iron acquisition strategies in plants

Since the uptake of Fe should be tightly regulated to maintain the essential levels plants evolved two distinct and specific strategies. These are known as Strategy I and strategy II.

1.2.2 Strategy I Fe uptake

Upon iron deficiency strategy I plants reduces the Fe (III) to Fe (II) prior to absorb. Hence, this strategy is also known as reduction based strategy. Dicots and nongraminaceous plants use this type of strategy to acquire iron for their needs. Although it has been described in many nongraminaceous species, by taking the advantage of various modern available tools, in the model plant Arabidopsis this strategy was very well investigated and characterized.

In general reduction based strategy plants follow a sequential three step process to acquire Fe from soil. First, they activate root H^+- ATPase to extrude protons in order to acidify the soil to increase the solubility of Fe (III), next, they reduce the Fe (III) to Fe (II) by Fe (III)-chelate reductase, which takes place at the plasma membrane of root epidermal cells. In a subsequent third step, Fe (II) will be transported across the membrane with the help of a divalent metal transporter which acts downstream of the Fe (III)-chelate reductase (Eide et al., 1996; Vert et al., 2003). In Arabidopsis AtIRT1 serves as major root transporter that is responsible for the uptake of the reduced iron.

The major steps involved in the strategy I Fe uptake will be explained further in the following sections.

1.2.3 Activation of H^+ ATPase and proton extrusion

In response to Fe starvation, H^+ ATPases will be activated to extrude protons (Schmidt et al., 2003; Santi et al., 2005). Protons extrusion is responsible for the acidification of soil and root interface (Römheld and Marschner, 1986). In Arabidopsis, although H^+ ATPases such as AHA1, AHA2, and AHA7 are induced upon Fe deficiency on root epidermis (Dinneny et al., 2008; Colangelo and Geurinot, 2004), AHA2 is the main root H^+ ATPase than the other two AHAs, and only loss of AHA2 leads to fail or reduced rhizosphere acidification under Fe starvation, hence considered as key player in Fe deficiency (Santi and Schmidt, 2009). Gene expression analysis of +/-Fe grown wild type, *fit* mutant, and FITOx lines suggested that FIT is required for *AHA2* induction but not sufficient alone to induce AHA2 in response to Fe status of the plant (Ivanov et al., 2011).

1.2.4 Iron-chelator reduction

This appears to be a rate-limiting step in Fe acquisition in Strategy I plants (Connolly et al., 2003). Plasma membrane localized ferric chelate reductase encoding gene FRO2 reduces Fe (III) to Fe (II).

In Arabidopsis, characterization of three allelic ferric reductase deficient mutants such as *frd1-1, frd1-2, frd 1-3* of AtFRO2 indicated the essential role of AtFRO2 in Fe reduction. These mutants failed to induce ferric chelate reductase activity upon Fe deficiency (Yi and Guerinot, 1996). Under Fe limiting conditions AtFRO2 is upregulated (Robinson et al., 1999). *AtFRO2 mRNA* was found in root epidermal cells. In addition to its transcriptional regulation, AtFRO2 also regulated at posttranscriptional level (Connolly et al., 2003). *AtFRO2* is one among the eight-member gene family in Arabidopsis.

1.2.5 Iron transport

The reduced ferrous iron can be transported to the root epidermal cells by the divalent metal transporter IRT1 (Eide et al., 1996; Vert et al., 2002), besides Fe, upon Fe starvation, AtIRT1 could coincidently transport Zn, Mn, Cd, Co and Ni (Eide et al., 1996). Similar to AtFRO2, AtIRT1 is also localized on the plasma membrane and highly induced in the root epidermal cells in iron limiting conditions. The function of IRT1 has been demonstrated by characterizing the loss of function mutant *irt1*. *Irt1* mutants are defective in Fe uptake and also impaired to accumulate other metals such as Zn, Mn, Cd, and Co under Fe deficiency (Vert et al., 2002). *Irt1* mutant exhibits chlorotic phenotype and has severe growth defects when grown on soil, which leads to death. Hence, these mutants require external iron supplement for their survival.

In addition to its transcriptional control, *AtIRT1* is also controlled at the protein level. AtIRT1 protein is repressed up on generous iron supply. IRT1 over expression (35S::AtIRT1) transgenic plants constitutively express *AtIRT1 mRNA* irrespective of Fe supply, but AtIRT1 protein accumulates only under Fe deficiency (Connolly et al., 2002). This additional level of regulation is to turn off the Fe uptake machinery when it is not needed.

Recent findings reported that IRT1 protein accumulation is independent of Fe nutrition status/supply (Barberon et al., 2011), which is contradictory to the previous reports by Connolly et al., 2002. However, the authors of Barberon et al., 2011 proposed that this might be due to the effect of N-terminus truncated IRT1 protein expressed by Connolly et al., 2002, this difference might cause the misfolding, degradation and resulting only Fe deficiency specific accumulation of IRT1 protein.

Previously, it was shown that IRT1-GFP fusion protein is localized to plasma membrane, this is in agreement with its attributed function as metal importer (Vert et al., 2002). Conversely, IRT2-GFP fusion protein is localized to intracellular compartments, which hints the possible sequential role of these two proteins in cellular iron transport.

On the other hand, a recent report by Barberon et al., 2011 showed that IRT1 protein localized to *trans*-Golgi network (TGN)/early endosomes. By immunolocalization approach with IRT1 specific antibody they could show that TGN localization of IRT1 but not plasma membrane. However, in the same study, using pharmacological approach they could show that IRT1 cycles to the plasma membrane to perform iron and metal uptake at the cell surface and is sent to the vacuole for proper turnover. It was shown that IRT1 is monoubiquitinated on several cytosol exposed residues *in vivo* and that mutation of two putative monoubiquitination target residues in IRT1 triggers stabilization at the plasma membrane and leads to extreme lethality (Barberon et al., 2011). It was reported that ubiquitination of specific lysine residues of the loop region leads to internalization of ZRT1 protein which is a member of ZIP family transporters as IRT1 (Gitan and Eide, 2000). IRT1 protein poses two lysine residues in its cytoplasmic loop and their mutations to arginine enhanced the IRT1 stability (Kerkeb et al., 2008). However, the recent findings of Barberon et al., 2011 regarding IRT1 localization contradicting to the previous findings of Vert et al., 2002.

1.2.6 Strategy II iron uptake

This strategy is also known as chelation-based strategy. Graminaceous monocots/grasses use this strategy to take up iron from soil. Upon iron limited situation plants belongs to this class of Fe uptake, synthesize mugenic acid (MA) family phytosiderophores(PS) and secrets from the root epidermal cells into the rhizosphere. This serves to chelate with Fe(III) and solubilize, the resulted Fe(III)-PS complexes are then transported into the root epidermis by yellow stripe1 (Zm YS1) transporter, which was identified from maize (von Wirén et al., 1999; Curie et al., 2001).

The chelation strategy is considered more highly efficient than the reduction based strategy (Strategy I) since it is less sensitive to pH. Due to this reason grasses can grow on calcareous soils where dicots cannot grow since they rely on strategy I uptake.

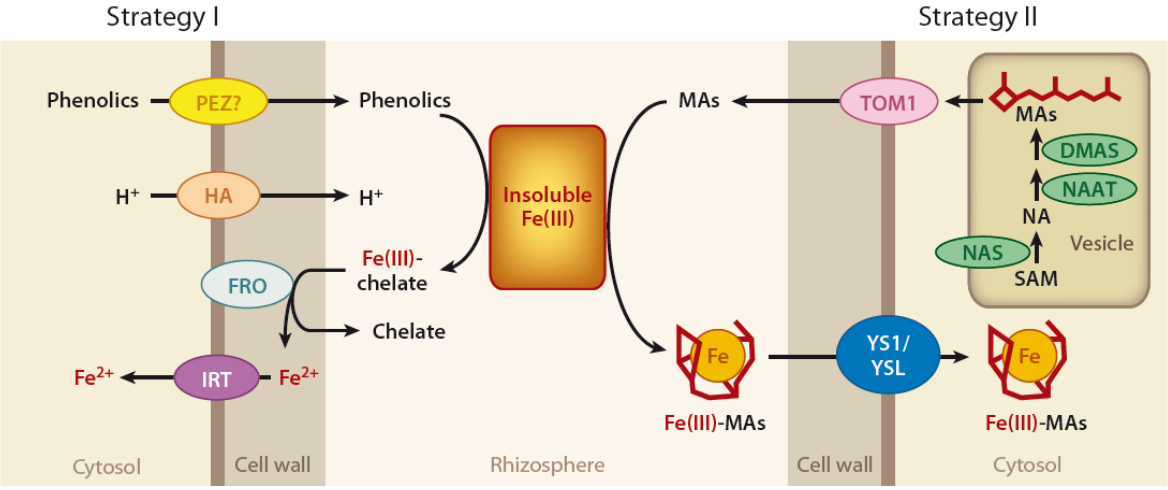


Figure 1.1 Fe acquisition strategies in higher plants (Kobayashi and Nishizawa, 2012)

Strategy I in nongraminaceous plants (left) and Strategy II in graminaceous plants (right). Ovals represent the transporters and enzymes that play central roles in these strategies, all of which are induced in response to Fe deficiency. Abbreviations: DMAS, deoxymugineic acid synthase; FRO, ferric-chelate reductase oxidase; HA, H^+-ATPase; IRT, iron-regulated transporter; MAs, mugineic acid family phytosiderophores; NA, nicotianamine; NAAT, nicotianamine aminotransferase; NAS, nicotianamine synthase; PEZ, PHENOLICS EFFLUX ZERO; SAM, *S*-adenosyl-L-methionine; TOM1, transporter of mugineic acid family phytosiderophores 1; YS1/YSL, YELLOW STRIPE 1/YELLOW STRIPE 1–like.

1.3 Regulation of iron uptake responses in plants

To survive in fluctuating environmental conditions, gene regulation plays a critical role. In Fe deficient or sufficient conditions, plants either induce or suppress several genes that are related to Fe homeostasis. However, upregulation of Fe acquisition associated genes at limited Fe conditions is more pronounced in both strategies (I and II) and the central regulators of these genes were also identified. Details and recent updates of iron response regulation of strategy I Fe acquisition is described in the following sections hence the current study is mainly focused on Arabidopsis which is a strategy I plant.

1.3.1 Regulation in strategy I plants

Recent transcriptomic investigations have targeted to unravel novel regulatory networks engaged in Fe homeostasis in Arabidopsis (Dinneny et al., 2008; Buckhout et al., 2009; Schuler et al., 2011). However, it seems that two distinct networks are involved in Fe acquisition in strategy I plants (nongraminaceous). Most of the Fe acquisition associated components are regulated either via/by FIT regulatory network or via/by POPEYE regulatory network.

1.3.2 Regulation of Fe uptake components of FIT network

The central regulator of strategy I plants (dicot and nongraminaceous) was first identified in *solanum lycopersicum* (tomato). Map based cloning of T3238*fer* mutant, which is impaired in the Fe deficiency response revealed a gene encoding a *BHLH* transcriptional regulator *FER* (Ling et al., 2002). *SlFER* induce upon Fe deficiency and positively regulates Fe deficiency responsive genes such as *IRT1* and *NRAMP1* (Ling et al., 2002; Brumbarova and Bauer 2005). In Fe sufficient condition *FER* expression is repressed in roots at posttranscriptional level.

In Arabidopsis, FIT (FER-like iron deficiency–induced transcription factor) is the functional ortholog of FER and is needed for the regulation of strategy I Fe deficiency response (Jakoby et al., 2004; Colangelo and Guerinot 2004; Yuan et al., 2005). *FIT* is expressed upon –Fe at root epidermal cells where *IRT1* and *FRO2* is also induced. FIT loss of function mutant *fit* is failed to induce *IRT1* and *FRO2*. FIT could regulate Fe uptake components transcriptionally and posttranscriptionally, *FRO2* is transcriptionally controlled by FIT, whereas *IRT1* is regulated at both levels. *fit* mutant exhibits severe growth retardation (Fig. 1.2) and is lethal unless excess of external Fe is supplied (Jakoby et al., 2004; Colangelo and Guerinot 2004). In the present study, we have uncovered how FIT itself is regulated. These findings were described and discussed in detail in results, discussion sections respectively. Moreover, few other important findings about regulatory components of FIT network has been discussed in closely related sections for instance IRT1 regulation was discussed in iron transport section as well.

Figure 1.2 Phenotype of wild type Columbia-0 (left side) and *fit-3* (right side) mutant plant
Plants were grown for six weeks on soil in long day conditions. *fit-3* mutant plants growth retarded and display severe leaf chlorosis and are unable to produce seeds unless supplied with external Fe.

Analysis of FIT overexpression transgenic lines revealed that constitutive *FIT* expression is not sufficient to induce *FRO2* and *IRT1*(Jakoby et al., 2004; Colangelo and Guerinot 2004; Meiser et al., 2011), this might indicate that probably FIT may require an additional binding/interacting partner to form a heterodimer. This heterdimer formation might further leads to the target downstream responsive genes (*FRO2* and *IRT1*). In fact, four bHLH genes namely bHLH38, 39, 100, 101 are induced by Fe deficiency (Yuan et al., 2005; Wang et al., 2007; Yuan et al., 2008). Bimolecular fluorescence complementation experiments showed that FIT interact with bHLH38 and bHLH39. In transgenic plants that overexpress both *FIT* and *bHLH38* or *bLHH39*, *FRO2* and *IRT1* expression was high, and these plants accumulated higher levels of Fe than wild type (Yuan et al., 2008). These findings support the possibility of heterodimer formation of FIT with bHLH38 or bHLH39.

Another key players such as NO and planthormones that influence FIT regulatory network components were discussed in detail in the following corresponding sections.

1.3.3 Regulation of Fe uptake components of POPEYE (PYE) network

In addition to the regulatory network that is controlled and regulated by FIT, a parallel regulatory network that regulated by a bHLH transcription factor (*bHLH047*) called POPEYE (PYE) have gained significant attention in recent times. Cell-type specific high resolution expression profiling of Fe deficient Arabidopsis roots reveled the existence of an alternative gene regulatory network of Fe deficiency response. Interestingly, the members of this regulatory network is present in the stele/vasculature (Dinneny et al., 2006), where as members of FIT regulated network mainly confined to epidermal tissue. From this network PYE and putative E3-ubiquitin ligase named as BRUTUS were further analyzed for their role in Fe deficiency response. PYE might play essential role in root development under –Fe condition. *pye* mutant shows poor growth in –Fe condition. PYE protein is localized to nuclei of all –Fe root cells, indicating that PYE spread across the all root cells after its induction at pericycle cells.

Microarray and ChiP-on-chip analysis indicated that PYE may negatively regulate Fe homeostasis associated genes *FRO3, NAS4* and *ZIF1*. In *pye-1* mutant these genes were highly induced and prolonged at –Fe (Long et al., 2010). *BRUTUS* (*BTS*) is another candidate gene that have similar expression pattern in pericycle cells as *PYE*. *bts* knockdown mutant showed better performance on Fe deficient medium in contrast to *pye* mutant. In Fe deficient conditions *bts-1* showed increased root growth and increased rhizosphere acidification than wild type, suggesting that BTS might function as negative regulator for Fe deficiency response. bHLH proteins often forms heterodimers to trigger/interact their downstream targets (as FIT). Yeast-two-hybrid analysis reveled that PYE and BTR interact indirectly through a PYE homolog (Long et al., 2010). However, it is not yet clear for the biological meaning of their associative induction in pericylce cells, PYE negative regulation of Fe homeostasis related genes and PYE-BTS interaction, some these observations (interaction studies) need to be confirmed in planta.

1.4 Influence of phytohormones in nutrient uptake/nutrient signaling

ABA is considered as stress hormone that is involved in various biotic and abiotic stress responses. The link between ABA levels and nitrogen status in different plant species was well addressed (Signora et al., 2001; Yendrek et al., 2010). ABA also regulates Pi starvation responses and sulfur homeostasis (Ciereszko and Kleczkowski, 2002; Shin et al., 2006). Several findings reported the interaction between auxins and the signaling pathways of nutrients such as nitrogen, phosphorus, potassium, and sulfur (Franco-Zorilla et al., 2004; Ticconi and Abel, 2004; Ashley et al., 2006; Kopriva et al., 2006). Cytokinins have been implicated in various aspects of plant growth and development. The role of Cytokinins (CKs) in the control of various nutrient signaling/homeostasis such as nitrogen, phosphorus, sulfur, and iron has been studied (Maruyama-Nakashita et al., 2004; Sakakibara et al., 2006).

Recently, the role of ethylene in nitrate dependant root growth and development has been identified (Tian et al., 2009). With regard the involvement of ethylene in Pi starvation, it has been identified that ethylene could mediate inhibition of primary root growth and root hair formation (Ma et al., 2003; Lei et al., 2011). Ethylene production is increased and the expression of ethylene biosynthesis genes are induced in potassium (K+) limiting conditions (Shin and Schachtman, 2004). Till date, little is known regarding the influence/role of GA in nutritional starvation responses and is limited to Phosphorus (Pi). It has been demonstrated that GA signaling could modulate PSR gene expression (Jiang et al., 2007).

Knowledge pertaining to Jasmonate (JA) in nutrients signaling/homeostasis is currently limited to potassium and sulfur. JA positively regulates the potassium and sulfur related genes (Maruyama-Nakashita et al., 2003; Rubio et al., 2009).

1.5. Role of plant hormones in modulating Fe deficiency responses

Plant hormones control numerous cellular activities (division, elongation and differentiation), and processes including pattern formation, sex determination, organogenesis, and responses to several abiotic and biotic stress. Hormones are critical signaling molecules that coordinate all aspects of plant growth and defense. As reported previously by several authors that the systemic regulation is involved in the regulation of Fe deficiency responses. Recent studies suggested that various plant hormones modulate Fe deficiency responses either in positive or negative manner. Impact/influence and the role of various plant hormones in the context of Fe deficiency responses will be discussed in the following sections. To date, only influence of plant hormones on FIT regulatory network is known. Hence, the influence of hormones can be considered as the components of FIT network.

In addition, ethylene, auxin, and signaling molecule Nitric oxide act together in modulating the efficiency of FIT dependant Fe uptake components.

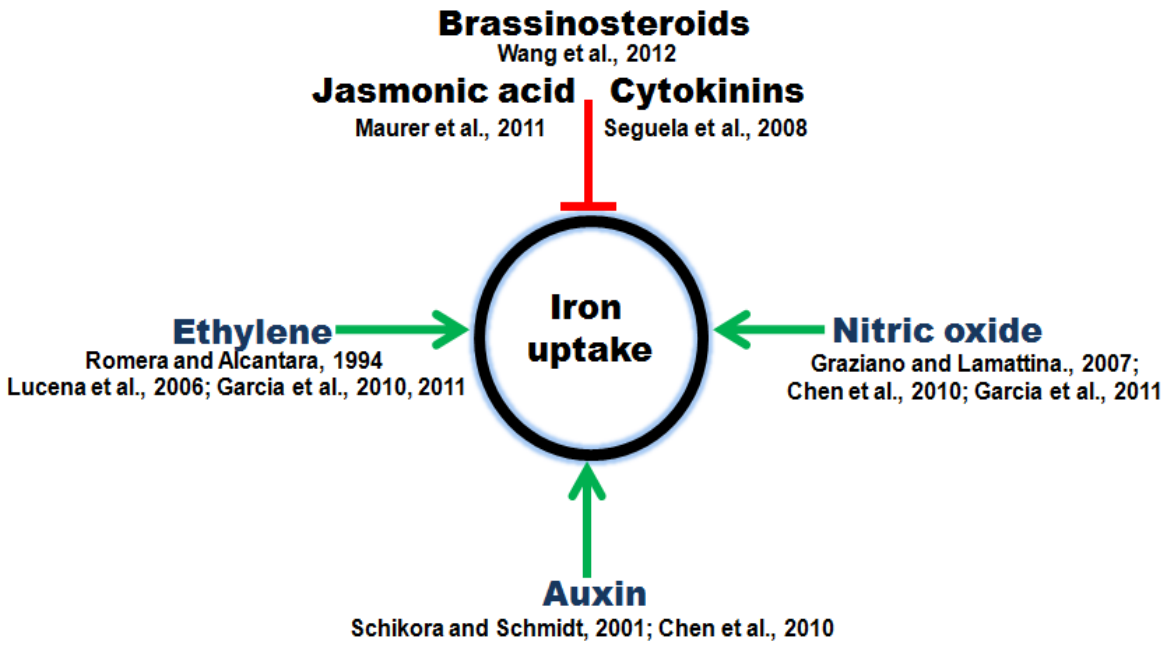


Figure 1.3 Schematic presentation of regulatory effect of planthormones and Nitric oxide on Fe uptake (acquisition genes) in plants
Iron acquisition associated genes are positively regulated by auxin, ethylene and nitric oxide (represented by green arrows). Conversely, brassinosteroids, cytokinins and jasmonic acids negatively regulate the Fe acquisition genes (indicated by red color bar)

1.5.1 Plant hormones that modulate Fe acquisition components in positive manner

1.5.1.1 Auxin

Phytohormone auxin plays critical role in iron deficiency responses in various plant species that belongs to strategy I Fe uptake. Early assumptions pertaining to the role of auxin in Fe deficiency adaptive responses mainly comes from the similarities that are observed from the morphological changes that appears during the exogenous application of auxin resembles to that of plants exposed to Fe deprivation such as formation of dense root hairs in order to increase the surface area to absorb the micronutrients such as Fe as much as possible (Cholodny 1931; Jackson 1960). Auxin is one of the systemic signaling molecules involved in Fe deficiency stress responses (Landsberg E.C. 1984).

Exogenous application of auxin mimics the morphological responses such as enhanced root hair formation and induces the transfer cells in the epidermis (Landsberg E.C. 1986, 1996). Increased auxin production has been observed in the roots of Fe deficient plants (sunflower/*Helianthus annuus*, Römheld and Marschner, 1981), and in Arabidopsis (Chen et al., 2010). Studies by Schikora and Schimidt in 2001 suggested that auxin may require in signaling pathway that mediate the root hair formation under Fe deficiency.

External auxin supply leads to enhance Ferric Chelate Reductase (FCR) activity (Chen et al., 2010; Li and Li, 2004). Analysis of Arabidopsis *yucca* mutants that produce higher auxin revealed that the higher levels of endogenous auxin levels could increase root FCR activity and also induces *FIT* and *FRO2* gene expression. Auxin insensitive mutant such as *aux1-7* is failed to induce FCR activity and also to induce the full level expression of *FIT* and *FRO2*.

However, *axr 1-3* another auxin insensitive mutant did not show any significant difference or they behaved like wild type in their FCR activity and the expression of *FIT* and *FRO2*.

The only difference between these two different auxin insensitive mutants *aux 1-7* and *axr 1-3* is in *aux1-7* mutant basipetal transport of auxin is blocked where as in the case of *axr1-3* mutant although one of the components for auxin sensing is missing basipetal auxin transport from the shoot to root might be functioning (Lincoln et al., 1990). Therefore, it was concluded that the auxin may act as signaling compound that carries the shoot derived Fe deficiency signals to the root for the full level induction of FCR activity (Chen et al., 2010). Most recently findings showed that a local symplastic Fe gradient in lateral roots upregulates AUX1 to accumulate auxin in lateral root apices as a prerequisite for lateral root elongation (Giehl et al., 2012).

1.5.1.2 Ethylene

Ethylene is one among the five basic original phytohormones. Although ethylene has long been considered as the ripening hormone, in contrast to its simple chemical nature ethylene is known for its essential roles in various aspects of plant life that typically contains seed germination to seed production. Ethylene controls seed germination, root initiation, root hair development, flower development, sex determination, fruit ripening, and senescence. Besides that ethylene also plays an important role in regulating responses to several biotic and abiotic stresses (Lin et al., 2009).

Upon ethylene or its metabolic precursor ACC treatment the so-called triple response phenotype (Shortened hypocotyls and roots, radial swelling of hypocotyl and roots and exaggerated apical hook) of etiolated dicotyledonous seedlings is the most typical and research focused ethylene response (Zhu and Guo 2008).

The mutants that show less sensitivity to ethylene or ACC treatment allowed in identifying the components of the ethylene and in some cases mutants that exhibits constitutive triple response phenotype even under normal growth condition (Guzman and Ecker, 1990; Zhu and Guo, 2008). Plenty of ethylene mutants collection from a variety of plant species and data obtained from the analysis of these mutants depicted the detailed role of this plant hormone. Mutant screens served as potential tool to identify a number of genes that are responsible for ethylene biosynthesis, signal transduction, and response pathways and based on epistasis analysis a linear model involving the ethylene components has been built. Besides this, map based cloning and candidate gene characterization of natural ethylene response defective mutants, combined with analysis of gene function, DNA-protein, protein-protein interaction techniques had been employed to identify new components of ethylene signaling (Lin et al., 2009).

1.5.2. Plant hormones that modulate Fe acquisition components in negative manner

1.5.2.1 Brassinosteroids

Brassinosteroids (BRs), as a class of plant polyhydroxysteroids, exist in plants (Noguchi et al., 1999; Divi and Krishna, 2009). BRs considered as sixth class of planthormones. BRs play crucial roles in several developmental processes in plants, including seed germination, root growth, floral initiation and flowering (Sasse, 2003; Divi and Krishna, 2009). Recent reports demonstrated that BRs also participate in the response of plants to biotic and abiotic stresses (Divi and Krishna, 2009). However, the role of BRs in nutrient uptake is largely unknown.

Most recently, BRs have been implicated in the regulation of Fe deficiency responses. These observations suggesting that BRs are likely to play a negative role in regulating

Fe-deficiency-induced *FRO*, expression of cucumber (*Cucumis sativus*) *CsFRO1* and *CsIRT1*, as well as Fe translocation from roots to shoots (Wang et al., 2012). It seems that, JA, BRs and cytokinins may negatively regulate Fe deficiency responsive genes.

1.5.2.2 Cytokinins

Other interesting phytohormones that have an impact on Fe deficiency responses are the cytokinins (CKs). CKs control various growth and developmental processes such as seed germination, cell division, stem cell maintenance, nutrient allocation, leaf senescence, action of auxin. Findings by Seguela et al in 2008 reported that CKs can negatively regulate the Fe deficiency responses. Moreover, it appears to be only a subset of Fe deficiency responsive genes that are confined to the root epidermis such as *FIT*, *FRO2* and *IRT1* are under the control of CKs. Hence, the treatments with CKs causes root growth inhibition it can be implied that CKs influence the Fe uptake by affecting the rate of growth (Seguela et al., 2008). Interestingly, cytokinins acts antagonistically to auxins, the same phenomenon has been observed in the case of iron deficiency response regulation as well.

1.5.2.3 Jasmonic acid

Recently, the role of phytohormone Jasmonic acid (JA) has been reported in response to Fe deficiency responses. JA can negatively regulate Fe deficiency responses by repressing the induction of *FRO2*, and *IRT1* gene expression and also partially *FIT* in Arabidopsis (Maurer et al., 2011). External application of application of the ibuprofen inhibitor of lipoxygenase results an upregulation of *FRO2* and *IRT1* gene expression.

Mutants impaired in JA such as the *jar1-1* which is unable to transform jasmonate into the active jasmonate-Ile, and *coi1-1* defective in jasmonate signaling the expression levels of *IRT1* and *FRO2* were higher than in wild type under Fe deficient conditions, where as FIT levels were not affected in these two mutants suggested that the JA repress *FRO2* and *IRT1* genes independent of *FIT* (Maurer et al., 2011).

1.6 Ethylene in Fe uptake

Several findings suggested the involvement of ethylene in Fe uptake responses in strategy I plants. A strong physiological connection between ethylene and iron deficiency responses in different dicotyledonous plants has been established. Ethylene production is increased under Fe deficiency in several strategy I plants (Romera et al., 1999; Li and Li, 2004; Molassiotis et al., 2005). Treatment with ethylene precursors ACC, Ethophane can mimic morphological growth response of Fe deficient plants (Romera and Alcantara, 1994; Schmidt et al., 2000). Moreover, treatment of several strategy I plants with inhibitors of ethylene synthesis or action greatly decreased their ferric reductase activity, while treatment with precursors of ethylene synthesis enhanced it (Romera and Alcantara, 1994). Furthermore, addition of ethylene precursors can induce Fe deficiency responsive genes such as *IRT1* and *FRO2* (Lucena et al., 2006; Waters et al., 2007; Garcia et al., 2010). Ethylene inhibitors could abolish Fe deficiency responses (Romera and Alcantara, 1994) and can repress *FRO2* and *IRT1* mRNA levels (Garcia et al., 2010; Lucena et al., 2006).

ETHYLENE INSENSITIVE3 (EIN3) and ETHYLENE INSENSITIVE3- LIKE1 (EIL1) are two members out of a small family of plantspecific transcription factors that are activated through the ethylene signaling pathway (Chao et al., 1997). These two proteins that are highly related in their amino acid sequence then regulate a series of ethylene responses from the seedling stage to reproduction (Solano et al., 1998; An et al., 2010). EIN3/EIL1 regulation is attributed essentially to posttranscriptional regulation.

A major mechanism to regulate EIN3/EIL1 activity acts via controlled proteolysis by the 26S proteasome, which is mediated through recognition of EIN3/EIL1 by Skp, Cullin, F-box–containing complexes with EIN3 BINDING F-BOX PROTEINS1 and 2 (SCFEBF1/EBF2) complexes (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). Upon ethylene signaling, EBF1 and EBF2 function is prevented so that EIN3/EIL1 are stabilized for inducing downstream ethylene responses (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). In addition to protein degradation, which seems to be the major pathway regulated by ethylene signaling, differential phosphorylation through a mitogen-activated protein kinase cascade has also been reported, although it remains unclear whether or not phosphorylation depends on the same signaling pathway as proteolysis (Yoo et al., 2008; An et al., 2010). EIN3 and/or EIL1 were shown to bind to promoters of downstream target genes involved in a multitude of responses ranging from biotic stress defense (Chen et al., 2009; Boutrot et al., 2010) and chlorophyll biosynthesis (Zhong et al., 2009) to ethylene signaling (Solano et al., 1998; Konishi and Yanagisawa, 2008).

Although the physiological link between ethylene and Fe deficiency responses was an important observation, the molecular basis of this phenomenon remained elusive until recently. It was demonstrated that EIN3/EIL1 physically interacts with FIT, and contribute to full FIT downstream target gene expression (Lingam et al., 2011).

Furthermore transcriptome analyses revealed that majority of the genes were differentially regulated in *ein3 eil1* mutants vs. wild type under –Fe condition compare to +Fe condition. Surprisingly, several of the differentially expressed genes are implicated in photo-oxidative stress responses in leaves. Therefore, it was speculated that by enhancing Fe uptake through interaction with FIT and by re-organizing the photo-oxidative stress responses, EIN3/EIL1 might contribute to decreasing photo-oxidative stress that may occur under light conditions in response to Fe deficiency (Bauer and Blondet 2012).

1.7 Nitric oxide (NO)

In recent years, nitric oxide (NO), gained the attention of plant biologists due to its significant role in modulating various processes throughout the plant life. NO is known to reduce seed dormancy (Zheng et al., 2009), and induces the seed germination (Beligni and Lamattina, 2000). NO is required for the root growth and development (Pagnussat et al., 2002), and regulates gravitropism (Hu et al., 2005). NO regulates stomatal closure (Bright et al., 2005), photosynthesis (Takahashi and Yamasaki, 2002), affects the function of mitochondria (Zottini et al., 2002).

NO plays significant role in the various aspects of plant reproductive organs, for instance NO has been implicated in floral regulation, by suppressing the transition to flowering by affecting the expression of regulatory genes in flowering pathways (He et al., 2004), also involves in the re orientation of pollen tube (Prado, Porterfield and Feijo, 2004) and pollen recognition by stigma (Hiscock et al., 2007). During disease resistance, NO serves as signaling molecule (Delledonne et al., 1998), Probably, as part of its signaling mechanism, it also enhances the raised cGMP levels (Durner et al., 1998) and raises the level of cytosolic free Ca^{2+} (Durner et al., 1998; Klessig et al., 2000, Garcia-Mata et al., 2003).

NO is required for the activation of a potential mitogen-activated protein kinase (MAPK) (Clarke et al., 2000). In the same year, it has been showed that NO could induce the activation of a salicylic acid induced protein kinase (SIPK), which results the induction of defense responses in tobacco (Kumar and Klessig, 2000). In later years it has been identified that NO mediates the activation of a MAPK signaling cascade, that is activated during the adventitious rooting process induced by Indole Acetic acid (Pagnussat et al., 2004).

NO is produced in response to abiotic stress responses such as drought and salt (Neil et al., 2008), and also in biotic stress conditions that are caused by biotrophic, necrotropic pathogens and viruses. NO mediates a broad range of plant responses that comprises of defense/pathogen responsive gene regulation, and the action of hormones that participates in defense response and in the hypersensitive response (HR) development (Asai and Yoshioka, 2009; Delledonne et al., 1998 and 2005; Durner et al., 1998). NO enhances the plant adaptive responses to drought stress (Garcia-Mata and Lamattina, 2001). NO is capable of regulating the multiple plant responses caused by biotic and abiotic stresses and mitigate some of the consequences caused by oxidative stresses, and delays the senescence and fruit maturation (Crawford and Guo, 2005; Delledonne, 2005).

It has been reported that NO regulates plenty of genes for instance NO regulates the expression of genes involved in the cell cycle (Correa-Aragunde et al., 2006), genes that are responsible for the synthesis and responsive to Jasmonic acid (Orozco - Cardenas and Ryan, 2002; Jih, Chen and Jeng, 2003). The expression profiling data obtained by treating Arabidopsis plants with NO donor sodium nitroprusside (SNP) revealed that the genes involved in the synthesis and signaling of ethylene, the phenylpropanoid pathway, protein antioxidation mechanisms, photosynthesis, cellular trafficking, cell death and other basic metabolic processes are regulated by NO (Wendehenne, Durner and Klessig, 2004).

1.7.1 Affect of nitric oxide on iron uptake in plants

Recently, several reports provided evidence for the role of NO in iron homeostasis and iron metabolism. NO is identified as an early signaling candidate that drives the regulation of downstream responses of Fe deficiency signaling (Arnaud et al., 2006; Garcia et al., 2010, 2011; Chen et al., 2010; Graziano and Lamattina, 2007; Murgia et al., 2002).

In addition, NO could improve the internal Fe mobilization and availability (Graziano and Lamattina, 2002).

Fe deficiency leads to a rapid and sustained accumulation of NO in the root epidermis, chiefly in rhizodermal cells of tomato (*solanum lycopersicum*) roots which correlates with the expression of Fe deficiency induced marker genes such as *SlIRT1*, *SlFRO2*. Treatment with NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) of Fe deficient roots results the repression of Fe deficiency responsive genes *SlFRO1, SlIRT1*, and the bHLH transcription factor *AtFIT* (homolog *SlFER*). Conversely, exogenous application of NO donor S-nitrosoglutathione (GSNO) leads to the induction of the same genes (Graziano and Lamattina, 2007). Similar findings were reported for Arabidopsis (Chen et al., 2010) by showing the repression with NO scavengers/inhibitors treatment and treatment with NO donors leads to the induction of *FIT*, *FRO2*.

NO could enhance the expression of several Fe related genes. Treatment with NO donor GSNO results the high level induction of Fe deficiency related genes and ferric reductase activity at +Fe in Arabidopsis and in cucumber (Garcia et al., 2010, 2011). GSNO treatment leads to induction of genes related to Fe acquisition, transport, and homeostasis such as *AtFIT, AtFRO2, AtIRT1, AtBHLH38, AtBHLA39, AtCCCl 1,2&3, AtNAS1 &2, AtMYB72 and AtFRD3* (Garcia et al., 2010). In cucumber (*Cucumis sativus*), which is also belongs to strategy I iron uptake plants, GSNO treatment results the high level expression of Fe acquisition genes such as *CsFRO1, CsIRT1*, *CsHA1, CsHA2* (H^+ -ATPase genes) (Garcia et al., 2011).

1.8 Nitric oxide and ethylene action in Fe deficiency responses

In plants various processes in which hormones, signaling compounds, and phytochrome interact or act independently in different way to give the same response. When it comes to the iron, Fe deficiency responses are modulated by ethylene and also by nitric oxide in a similar manner (positively). Such over lapping functions led to investigate whether NO and ethylene interact or influence each other or share, act in a same signaling pathway.

Recently, the relationship between NO and ethylene has been identified. Nitric oxide and ethylene interaction has been identified. Up on O3 (Ozone) stress NO and ET amplified and cooperate to stimulate Alternative oxidase (AOX) pathway (Ederli et al., 2006). It has been reported that NO may influence ethylene biosynthesis in the maturation and senescence of plant tissue (Arasimowicz and Floryszak-Wieczorek, 2007). Ethylene production is modulated by exogenous application of NO (Zhu and Zhou, 2007). However, some reports suggested that both gases act antagonistically. In Arabidopsis S-nitrosylation of methionine adenosyltransferase (MAT1) by NO leads to the down regulation of ethylene synthesis. Inhibition of MAT1 activity by NO, leads to the reduced levels of ethylene precursor S-adenosylmethionine (SAM) (Lindermayr et al., 2006).

The role of NO and ethylene in the regulation of Fe deficiency responses in plants has been proposed by various findings (Chen et al., 2010; Graziano and Lamattina, 2007; Lucena et al., 2006; Garcia et al., 2010, 2011; Romera and Alcantara, 1994). Since NO and ET acts together and involve in regulating various plant responses, it was worth trying and interesting how these two candidates act together or regulate together Fe deficiency responses.

Most recently, it was reported that NO could increase the expression of genes involved in ethylene synthesis.

In Arabidopsis and Cucumber roots, treatment with NO donor GSNO results the induction of ethylene synthesis genes such as *AtSAM1, AtSAM2, AtACS4, AtACS6, AtACO1, AtACO2, AtMTK; CsACS2 and CsACO2*. On the other hand ethylene can enhance NO production in the roots.

Treatment with ethylene precursor ACC results the enhanced production of NO in the roots, whereas treatment with ethylene blockers such as STS and Co could alleviate NO production. Induced FCR activity caused by the ACC treatment was hindered by the NO scavenger cPTIO. Therefore, it has been proposed that both NO and ET influences the production of each other. This mutual influence might lead to the amplification of Fe deficiency responses including the induction of Fe deficiency responsive genes.

NO and ET are produced upon low Fe signal and both influence the production of the other, and low Fe signal (presumably phloem Fe) is essential for the activation of NO, ET and to be effective. This low iron situation might attribute the specificity to the responses. Hence, NO and ET that are produced in other stress conditions are unable to mediate the induction of Fe deficiency responses (Garcia et al., 2011).

It is known that posttranscriptional regulation of the transcription factors plays crucial role in various developmental stages of plants. For instance, several posttranslational modifications were well described in plants (Tootle and Rebay, 2005). Phytochrome interacting factors (PIFs) belonging to the bHLH family (similar to FIT) transcription factors can be considered as good example for such modifications, All PIFs except PIF7 are phosphorylated and subsequently ubiquitinated prior to their degradation (Shen et al., 2007, 2009; Al-Sady et al., 2006). Recently, it has been reported that IRT1 is monoubiquitinated (Barberon et al., 2011). Furthermore, it is shown that the ethylene biosynthesis protein ACC synthase 2/6 was shown to be phosphorylated by MAP kinase MPK6, that leads to enhanced ethylene signaling (Joo et al., 2008) and in addition EIN3 has also been shown to be regulated by MPKs (Yoo et al., 2008).

Involvement of MAP kinases in bHLH transcription factors are also well documented in the case of the bHLH protein SPEECHLESS (SPCL). SPLC is targeted by phosphorylation events that were transduced by MKK4/5 and MPK3 and MPK6 (Lampard et al., 2008). Besides phosphorilation and ubiquitination, another interesting and relevant posttranscriptional modification for the current study is 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., 2009). Since there is NO involvement as described above in Fe deficiency responses, which might be the same scenario in the case of FIT.

Since EIN3/EIL1 interacts with FIT (Lingam et al., 2011), this might serve as an example of integration of hormonal stimulus and signal transduction similar to that of MAP kinases in order to regulate downstream targets in upon Fe deficiency in plants.
For instance regulation *FRO2* and *IRT1* may be controlled by post-transcriptional regulation of FIT besides its transcriptional induction upon –Fe condition. Post-transcriptional regulation of FIT could be modulated by ethylene and signaling compounds such as NO. Thus, investigating the post-transcriptional regulation of FIT and the influence of ethylene and NO on FIT accumulation and abundance is essential to understand underlying mechanism of Fe sensing and uptake regulation in plants.

2. Scientific aims of the project

The objective of the present study is to unravel the posttranscriptional regulation of an iron dependent transcriptional factor FIT. Previous findings reported that FIT and its functional homolog (FER) from tomato (*Solanum lycopersicum*) are regulated by the iron deficiency status of the plant through transcriptional and posttranscriptional mechanisms (Colangelo and Guerinot 2004; Jakoby et al., 2004; Brumbarova and Bauer, 2005). To better understand the regulation of Fe acquisition in strategy I plants, investigation of FIT protein regulation is essential. However, the previous studies could not analyze the FIT protein accumulation and abundance in response to Fe nutritional status. Control of FRO2 and IRT1 activity is crucial for the plant to regulate Fe uptake into the root. Understanding the regulatory mechanisms that act upon FIT may ultimately allow us to gain insight into the signals by which plants sense their environment and internal requirement for Fe uptake.

The first goal of the current study was to generate tools to investigate endogenous FIT protein status in planta in response to Fe supply. To achieve this, a specific antiserum against FIT protein was generated with the help of in-house facilities of Saarland University in collaboration with Prof. Uli Müller, Department of Zoology. As a first step, Arabidopsis *FIT* gene has been cloned. After transformation, recombinant fusion protein was expressed in *E.coli* and purified. The purified recombinant fusion protein was then injected to animals (Mice and Rats). Later, the collected antiserum has been checked for its specificity. Finally, the obtained antiserum was used to investigate endogenous FIT protein status in plants under different Fe nutritional supply.

2. Scientific aims of the project

In addition to protein level regulation of FIT, the next level goal of this work was focused on the investigation of the factors (such as ethylene and nitric oxide) influencing accumulation, regulation and stability of FIT. It is known that ethylene and nitric oxide modulate the induction/regulation of Fe deficiency genes including *FIT*. Although the physiological link between ethylene, nitric oxide and Fe deficiency responses was an important observation, the molecular basis of this phenomenon remained elusive. To address this, corresponding mutants, overexpression lines have been analyzed. In parallel, appropriate pharmacological treatments were performed in order to decipher the involvement of ethylene and nitric oxide on FIT protein to investigate FIT stability, degradation and further effect on its downstream target genes such as *FRO2* and *IRT1*.

3.1. Materials

3.1.1. Plant material

- *Arabidopsis thaliana* ecotype Columbia (Col-0) has been used as wild type
- Arabidopsis T-DNA insertion mutant *fit-3* (described in Jakoby et al., 2004) has been used
- Seeds of *ein3-1eil1-3* mutant (*ein3eil1*) were multiplied and verified in the triple response assay (Chao et al., 1997; Binder et al., 2007)
- Non tagged FIT overexpression (FIT Ox) line (as described in Jakoby et al., 2004) was used
- HA-tagged FIT over expression line (HA-FITOx) was used as described in Meiser et al., 2011

3.1.2. Bacterial strains

- NovaBlue Singles™, Tuner (DE3)pLacI competent cells (Novagen) were used

3.1.3. Vectors and Plasmids

- pETBlue2 vector (Novagen)

3.1.4. Oligonucleotides

- Table 3.1 list of primers used in the study

	Forward primer	Reverse primer
FIT full	5'- G GAA GGA AGA GTC AAC GCT CTG-'3	5'- ACG ACC TTC GAT AGT AAA TGA CTT GAT GAA TCC AAA ACC T-'3
FIT -C	5'-A GCT TCT TTA AAC TCT ACT GGA GGG TAC-'3	5'- ACG ACC TTC GAT AGT AAA TGA CTT GAT GAA TCC AAA ACC T-'3
	pETBlueUP primer (Novagen #70604-3) 5'-TCA CGA CGT TGT AAA ACG AC-'3	pETBlueDOWN primer (Novagen#70603-3) 5'-GTT AAA TTG CTA ACG CAG TCA-'3

3.1.5. Antibodies

- FIT-C polyclonal antiserum (see section 3.2.3 for details)
- anti-mouse IgG conjugated with horseradish peroxidase (Sigma-Aldrich, USA) for the detection of anti FIT-C antibodies
- Rat IgG monoclonal anti HA antibody clone 3F10 (Roche) for the detection of HA tagged FIT protein
- Polyclonal Goat anti Rat Horseradish peroxidase secondary antibody (Sigma Aldrich) for the detection of anti HA antibodies
- Goat-anti Rat alkaline phosphatase-conjugated secondary antibody (Jackson Immuno Research, Germany) for the detection of HA antibodies on root cross sections

3.1.6. Softwares

- PlasmaDNA was used to generate the overview of the restriction sites of the recombinant plasmid (pETBlue2 with FIT-C insertion)
- ImageJ was used quantify the protein bands on western blots
- DNAstar was used for primer design and alignment

3.2 Methods

3.2.1 Plant material and growth conditions

For physiological assays seeds were surface sterilized as described in Jakoby et al., 2004.

- In the 6-day growth system, surface sterilized seeds were directly germinated on 50 µM Fe (+ Fe) or 0 µM Fe (- Fe) Hoagland agar medium and were grown at long-day conditions.

- In the 2-week growth system, plants were grown for 14 days on square plates containing Hoagland agar medium (50 µM Fe) under long-day condition (at 21°C/19°C and 16 h light, 8 h dark cycles) in plant growth chambers (CLF Plant Climatics). For Fe deficiency treatment, 14-days old plants were transferred to a fresh 0 µM Fe (- Fe) Hoagland agar plates containing 50 µM ferrozine, and grew for three days.

The following Hoagland salt concentrations have been used for the preparation of Hoagland medium.
0.1875 mM $MgSO_4 \times 7\ H_2O$, 0.125 mM KH_2PO_4, 0.3125 mM KNO_3, 0.375 mM $Ca(NO_3)_2$, 12.5 µM KCL, 12.5 µM H_3BO_3, 2.5 µM $MnSO_4 \times H_2O$, 0.5 µM $ZnSO_4 \times 7\ H_2O$,0.375 µM $CuSO_4 \times 5\ H_2O$, 0.01875 µM $(NH_4)_6Mo_7O_{24} \times 4\ H_2O$. pH has been adjusted to 6.0.

3.2.2 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 color 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 normalized 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, Methods in Molecular Biology, Issue 479).

3.2.2.1 Statistical Analysis

- Statistical evaluation was performed by *t* test using the values of biological replicates. For Figure 4.13, P values were obtained via *t* test using the GraphPad software at http://www.graphpad.com/welcome.htm.

3.2.3 FIT antiserum preparation

- The C-terminal part of FIT excluding the bHLH domain was amplified by PCR using the primer combination 5’-A GCT TCT TTA AAC TCT ACT GGA GGG TAC-‘3 and 5’-ACG ACC TTC GAT AGT AAA TGA CTT GAT GAA TCC AAA ACC T-‘3, and cloned into pETBlue-2 vector by using Perfectly Blunt® Cloning Kit, recombinant plasmid was transformed into NovaBlue Singles™ Competent Cells (Novagen, USA). After initial selection of positive colonies as per manufacturer’s instructions, colony PCR was performed for verification of positive recombinant plasmids, additional selection of positive clones has been identified by restriction digestion (see Fig. 3.3 (a) and (b) for the overview of restriction digestion sites).

After sequence verification, the recombinant plasmid was transformed into Tuner (DE3) pLacI cells and the recombinant protein induction was performed according to the manufacturer's instructions (Novagen, USA).

Insoluble FIT-C His-tagged fusion protein was isolated under denaturing conditions with 6M Guanidin-HCL and was affinity-purified by chromatography. Chromatographic column filled with TALON Metal Affinity Resin (Clontech, USA). Column preparation including resin filling and protein purification process has been done as per the instructions described in the user manual provided by the manufacturer (Clontech, USA, manual PT1320-1 (PR993342)). Purified protein was injected into mice to obtain antiserum. The obtained 4 different antiserum (namely Sh-1, Sh-2, Sh-3 and Sh-4) were tested positive for their specificity to detect bacterially expressed FIT-C peptide. Due the consistency in the detection of desired FIT-C recombinant protein, all four antiserum were pooled together in the following western blot experiments.

For use in Western blots with plant protein extracts anti-FIT-C antiserum was purified. Crude bacterial extract containing recombinant FIT-C fusion protein was loaded on a preparatory gel and blotted to a nitrocellulose membrane. After Ponceau S staining the membrane region containing the FIT-C antigen was cut off as a strip. The membrane was blocked for 1 hour at room temperature with 1% BSA dissolved in PBS-T and subsequently probed with crude mouse antiserum at 4°C overnight. Unbound fraction was collected into a new tube. The membrane was washed 3 times with PBS-T, and bound antibodies were eluted two times with elution buffer (0.1 M glycine-HCl pH 2.7, 0.5 M NaCl). The eluted antibody fractions were immediately neutralized by adding 1/10 volume of neutralization buffer (1 M Tris-HCl pH 8.0, 1.5 M NaCl, 1 mM EDTA, 0.5% NaN3) and bovine serum albumin (BSA) was added at 1 mg/ml final concentration.

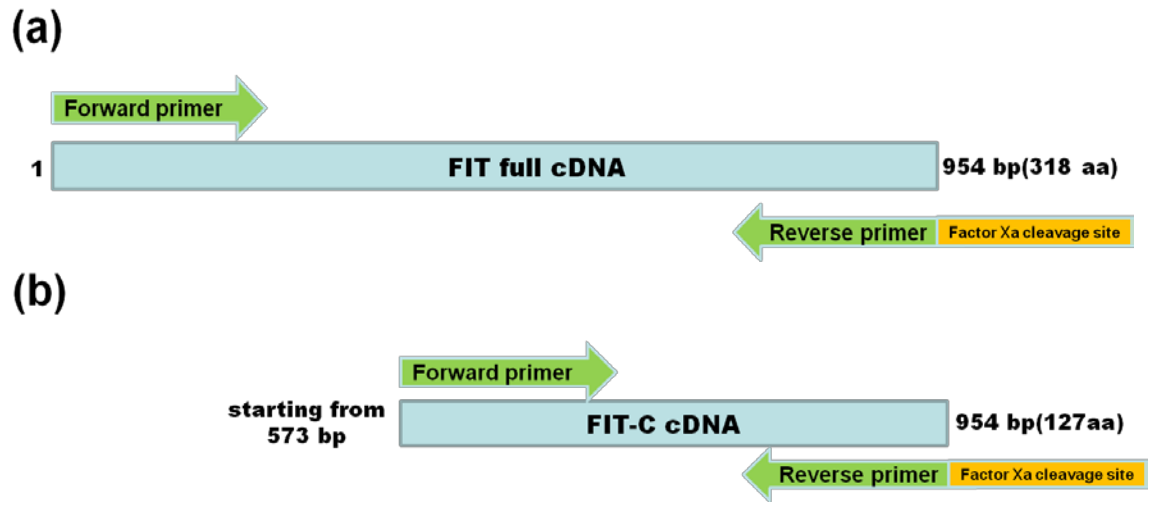


Figure 3.1 Schematic view of amplified fragments for cloning
FIT full (a) and FIT-C terminal part (b) has been amplified. Factor Xa cleavage site was added to the reverse primer. In the schematic view, fragments sizes shown without Factor Xa cleavage site sequence of the reverse primer.

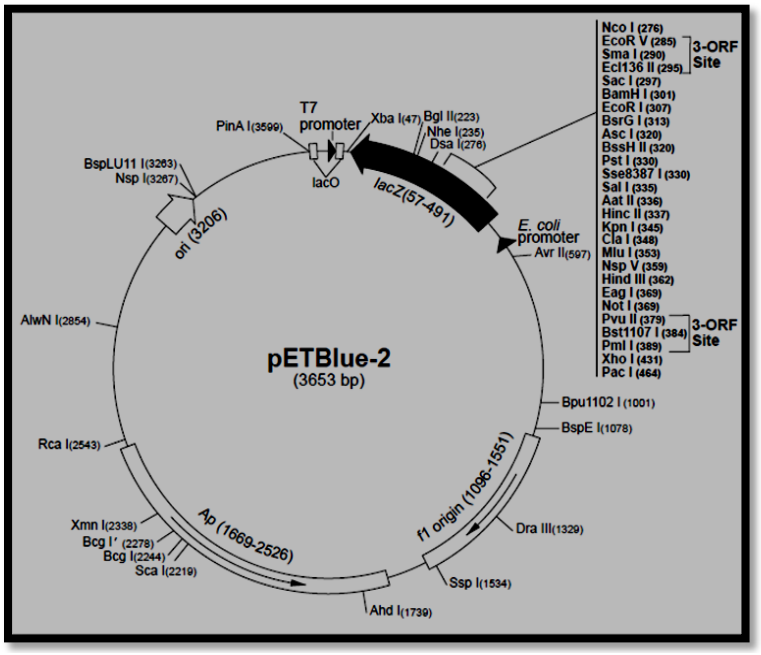
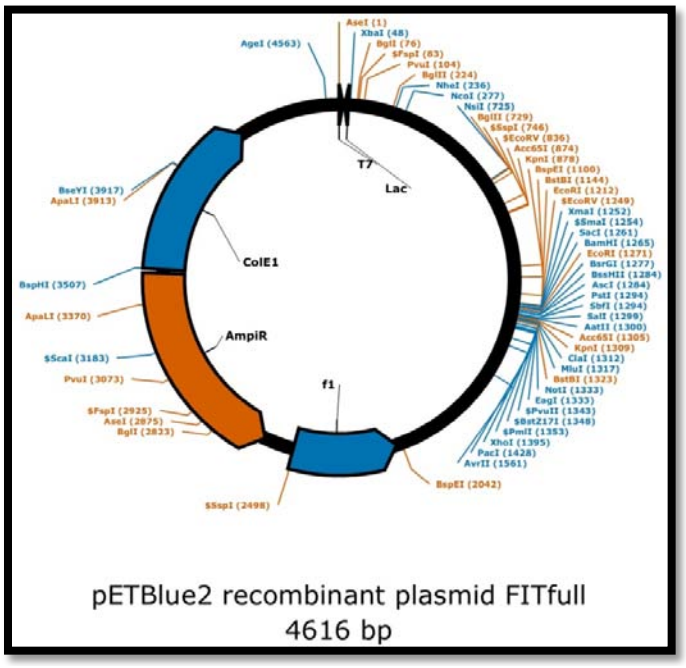


Figure 3.2 map of pETBlue2 vector
Overview of pETBlue2 vector map with multiple cloning sites.

(a)



(b)

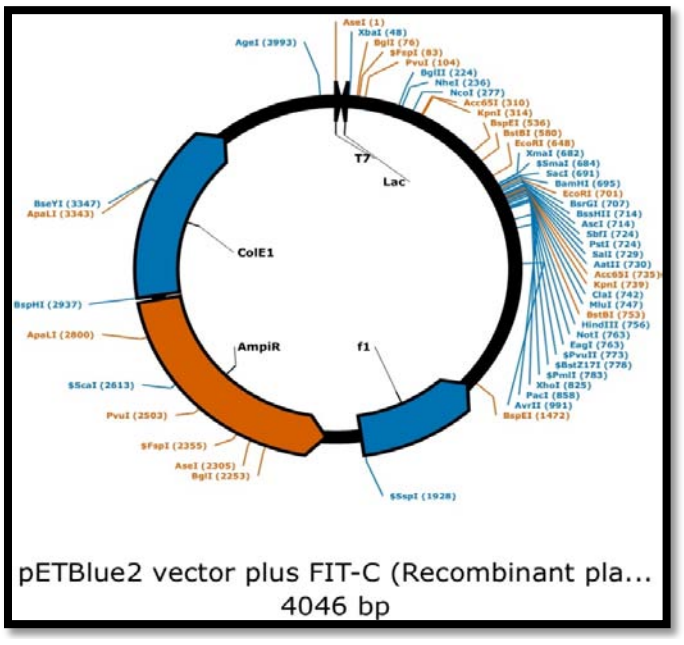


Figure 3.3 Overview of the recombinant plasmids with restriction sites
Overview of the recombinant plasmids showing restriction sites of FIT full inserted into pETBlue2 vector (a), and FIT-C inserted into pETBlue2 vector (b). Overview was generated by plasma DNA software.

3.2.4 Western Immunoblot analysis

- Total protein extracts were prepared from roots of 6-day-old plant seedlings following a described procedure (Scharf et al., 1998). Root tissue was ground in liquid nitrogen and 30 mg root powder was resuspended in an equal volume of lysis buffer (500 mM NaCl, 25 mM HEPES, 5 mM MgCl2, 1 mM Na-EDTA, 10 mM NaF, 10% (w/v) glycerole, 0.2% Nonidet P40 and one protease inhibitor cocktail tablet (Roche Diagnostics, Germany) per 50 ml of buffer. After centrifugation at 10.000 rpm for 10 min at 4°C, supernatant was transferred to a new tube and protein concentrations were determined by Bradford Assay reagent (Sigma-Aldrich, USA). 10 µg proteins were loaded per lane on a 10 % SDS-PAGE and subsequently blotted to a nitrocellulose membrane. Western blot analysis was conducted according to standard procedures.

- For detection of FIT protein, freshly purified undiluted anti-FIT-C mouse antiserum was applied. These primary antibodies were detected with anti-mouse IgG conjugated with horseradish peroxidase (1:8000 dilution, Sigma-Aldrich, USA).

- HA-FIT protein was detected by incubation with anti-HA high affinity monoclonal rat antibody (1:1000 clone 3F10, Roche, Germany) and as secondary antibody anti-rat IgG (whole molecule)-horse radish peroxidase conjugated (1:10000, Sigma-Aldrich, USA). Detection signals were developed by using an enhanced chemiluminescence detection kit (Biorad, USA) according to the manufacturer's protocol. Relative quantification of protein bands detected in Western blot experiments was calculated using the ImageJ software (Abramoff et al., 2004) and normalization to the Coumassie/Ponceau S-stained bands.

3.2.5 Pharmacological treatments

- Ethylene experiments (with ethylene inhibitors) were conducted using 6-day old seedlings. Seeds were directly germinated on 50 µM or 0 µM Fe Hoagland agar medium containing 10 µM aminoethoxyvinylglycine (AVG, Sigma-Aldrich, USA), 200 µM silver thiosulfate (STS) or 20 µM aminooxoacetic acid (AOA, Sigma-Aldrich, USA). Samples were collected on 6^{th} day and further processed for western blot analysis. For MG132 treatment, 6 day-old seedlings were treated for 4 hours in liquid Hoagland medium containing 100 µM MG132 (Calbiochem, USA) and harvested for analysis.

- Nitric oxide (NO) experiments were conducted using the 6-day growth assay. 5-day old seedlings were transferred to fresh 50 µM or 0 µM Fe Hoagland agar medium, containing as treatments 25 µM NO donor S-nitrosoglutathione (GSNO was synthesized as reported (Stamler and Loscalzo, 1992) or 1 mM cell-permeating NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl imidazoline-1-oxyl-3-oxide (cPTIO, Sigma-Aldrich), respectively. Same procedure was followed for the treatments with additional NO inhibitor such as Tungstate, L-NAME (1mM final concentration was used for the both inhibitors). 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 µM MG132 (1:1000 dilution from 42 mM stock solution diluted in DMSO) and subsequently quick frozen in liquid nitrogen for western blot analysis.

3.2.6 Immunolocalisation/Immunohistochemistry

Immunohistochemistry was carried out according to Kurata et al., 2005; and Nakajima et al., 2001 with minor alterations. Roots were fixed in 4% Paraformaldehyde solution for 1 hour with vacuum infiltration and washed three times in PBS for 10 min. Later, roots were carefully embedded in 1% agarose solution when the temperature of the solution is about 50°C. After solidification, small agarose blocks were prepared by excising the agarose surrounding the embedded roots, the roots in agarose blocks were passed through an ethanol series and further embedded in tissue embedding medium Paraplast plus (Carl-Roth GmbH, Germany). Sections (9 µm) were sliced with microtome (Reichert-Jung, Germany) and placed on poly lysine coated slides to adhere the root section on the slide surface. After de-paraffinisation with Roti-Histoclear, root sections were subjected to rehydration with ethanol series (high to low percentage of ethanol solutions). Then, the root sections were washed in PBS and treated with 20µg/ml Protinase-K (Applichem) for 15 min at room temperature. Immediately, root sections were washed in PBS-T and subsequently blocked with blocking buffer (PBS-T plus 2% BSA) for 5 h at room temperature. Later, the root sections were incubated with anti HA high affinity antibody at a 1:200 dilution for overnight at room temperature. After the incubation, slides were washed 5 times in PBS-T and incubated with Goat-anti Rat alkaline phosphatase-conjugated secondary antibody at a 1:500 dilution for 2 h at room temperature (Jackson Immuno Research, Germany). Slides were washed 3 times in PBS-T and twice in alkaline phosphatase buffer pH 9.5. The signal was developed using BCIP/NBT solutions (Carl-Roth, Germany) for 2 h at room temperature. After color development, sections were washed and passed through ethanol series and slides were dipped in Roti-Histol (Carl-Roth, Germany) prior to mount with Roti-Histokit (Carl-Roth, Germany). Images were obtained with Leica microscope (Leitz DMR B series).

4. Results

4.1 Generation of FIT antiserum

To achieve the first goal of the present study, i.e. to monitor Fe dependent expression, regulation of FIT protein in planta, it is necessary to generate antibodies that can specifically detect the FIT protein. Towards this, we have performed a series of experiments that includes cloning, transformation, heterologous expression and purification of recombinant protein, and immunizing/injecting the animals (Mouse) with purified recombinant protein to obtain the antiserum.

4.1.1 Cloning and confirmation of cloned recombinant FIT plasmid

For this purpose, we specifically amplified full length FIT (FIT full) and also a partial region of FIT from its C-terminal part (here after described as FIT-C; Fig. 4.1). The reason to select and clone the C-terminal part was to exclude the possibility of cross reactivity of the generated antibodies to other bHLH proteins (since FIT is a bHLH transcription factor protein) on western blot. Upon successful ligation and transformation, the obtained colonies were numbered and a colony PCR has been performed to check for the positive clones for the presence of recombinant plasmid. In addition, from the selected recombinant plasmids we have performed a colony PCR and also restriction digestion on the recombinant plasmid to confirm the proper orientation of the insert by ligation (Fig. 4.2 a&b, Fig. 4.3 a-d).

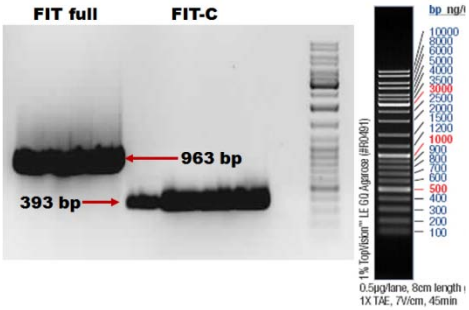


Figure 4.1 PCR amplification of FIT full and FIT-C
Agarose gel electrophoreses of PCR amplified FIT full length (963 bp) and FIT-C (393 bp) fragments. Amplified product sizes including the additional sequence of Factor Xa cleavage site of the reverse primer.

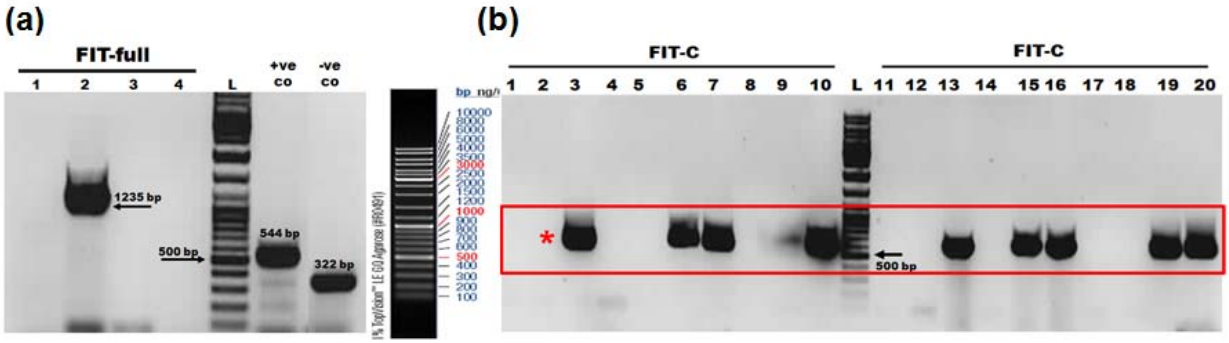


Figure 4.2 Colony PCR of FIT full and FIT-C colonies

Resulted colonies were tested for the presence of recombinant plasmid by colony PCR, colonies were numbered as 1, 2, 3…20. If the insert is in the correct orientation the expected size of the PCR product for FIT-full with the primer combination (FIT 5' and pETBlueDOWN) is 1235 bp (963 bp of FIT full plus 272 bp from the pET Blue2 vector). Only colony no. 2 of FIT full gave a PCR product at expected size, Fig. 4.2 (a). For the controls pETBlueUP and pETBlueDOWN primer combination (from the pETBlue2 vector) was used. As +ve control, vector ligated with check insert control of 212 bp insert (supplied with the kit components and used as +ve control to monitor successful ligation as well as transformation) was used. The expected band size for +ve control is 544 bp. As -ve control, empty vector (w/o PCR product) was used as template and expected PCR product is 332 bp (544-212). Fig. 4.2(b) FIT-C 5' and pETBlueDOWN primer (as 3') combination was used for FIT-C amplification, expected band size is 665bp (393 bp from FIT-C and 272 bp from the vector + factor-Xa cleavage site). Colony PCR was performed on 20 colonies. +ve colonies were highlighted in red color box and asterisks. L=ladder. Colony nos. 3, 6, 7, 10, 13,15,16,19 and 20 were positive colonies.

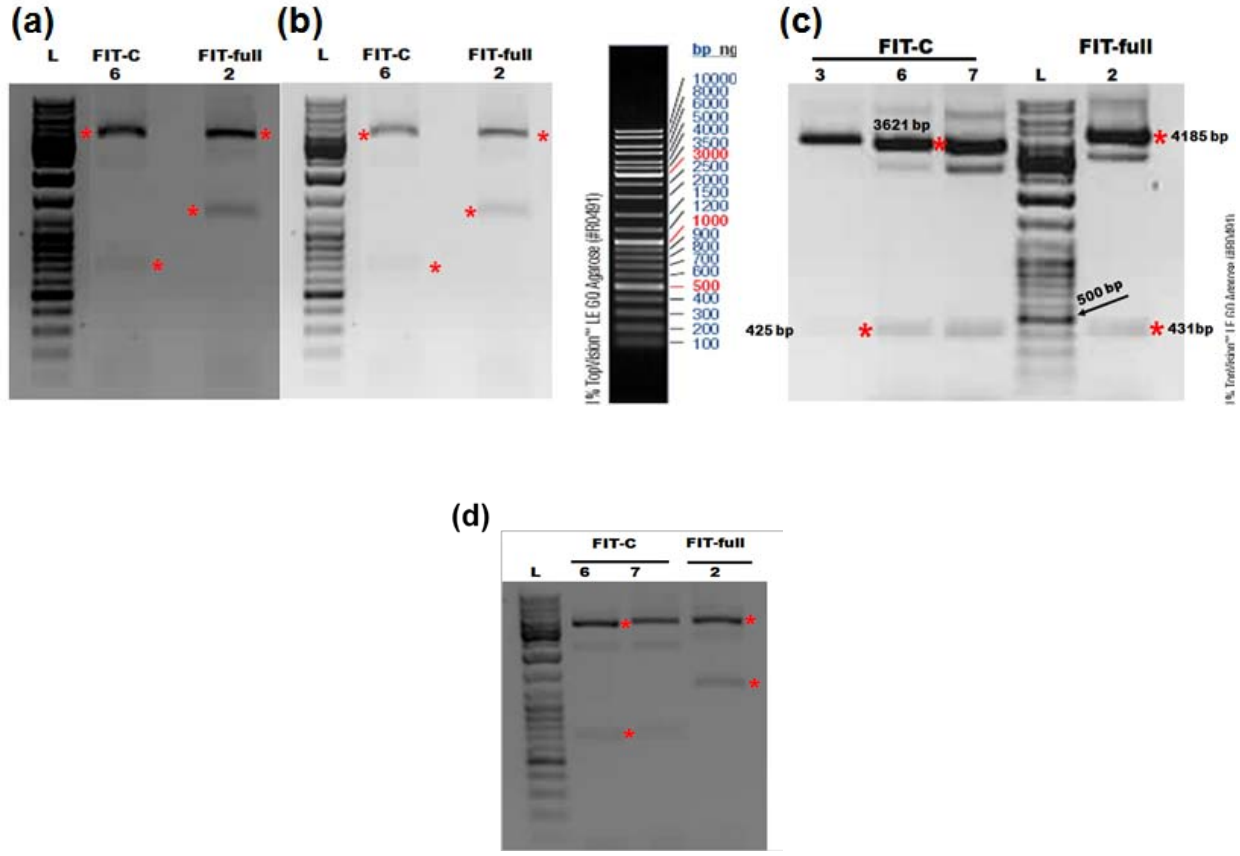


Figure 4.3 Agarose gel electrophoreses images of restriction digestion of FIT full and FIT-C recombinant plasmids

Double digestion with BamHI and XbaI, (a) over exposed gel image for the better visibility of the 647 bp band of the FIT-C, (b) less exposed gel image for the better visibility of the marker. For FIT-C, 647 and 3399 bp bands, for FIT-full 1217 and 3399 bp bands were obtained. (c) single digestion with Kpn I, FIT-C recombinant plasmids obtained from colony numbers 3, 6 and 7, for FIT-full colony no. 2 were used. For FIT-C, 425 and 3621 bp bands, and for FIT-full 431 and 4185 bp bands were obtained.(d) double digestion of FIT-C, FIT full with Xba I and Sal I, for FIT-C 681 bp, and 3365 bp, for FIT-full 1251 bp and 3365 bp bands were appeared. The resulted bands (marked with asterisks) at expected sizes indicated the correct orientation of the cloned insert. See material and methods for the overview of the restriction sites of the recombinant plasmids.

4.1.2 Expression of recombinant FIT-C fusion protein in *E.coli* and protein purification

After colony PCR, restriction digestion and sequence confirmation the selected recombinant plasmid (FIT-C 6) was transformed into the Tuner™ DE3 expression cells. Upon the successful expression of the recombinant 21 kDa FIT-C fusion protein at small scale level (Fig. 4.4a), a large scale expression and purification of FIT-C protein was performed in order to obtain sufficient FIT-C fusion protein for the immunization (Fig. 4.5).

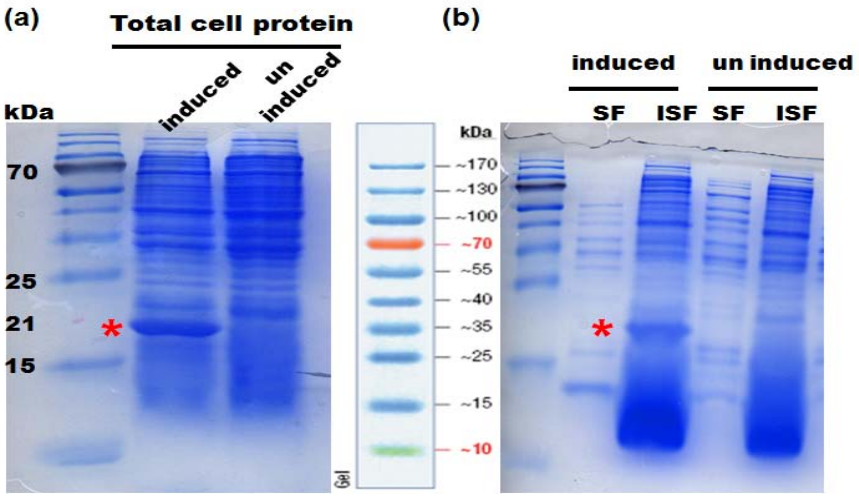


Figure 4.4 SDS-PAGE analysis of heterologously expressed recombinant FIT-C fusion protein in *E.coli*

FIT-C fusion protein expression in Tuner™ DE3 cells, Fig.(a) expression in total cell protein from induced culture, (b) expressed FIT-C protein accumulated as insoluble protein (inclusion bodies). SF means soluble fraction, ISF means insoluble fraction. Asterisks (*) indicates the ~21 kDa size FIT-C fusion protein band (14 kDa from FIT-C terminal part of 127 amino acids plus 6.4 kDa from the vector region that poses 6 His tags + 0.4 kDa from Factor Xa cleavage site).

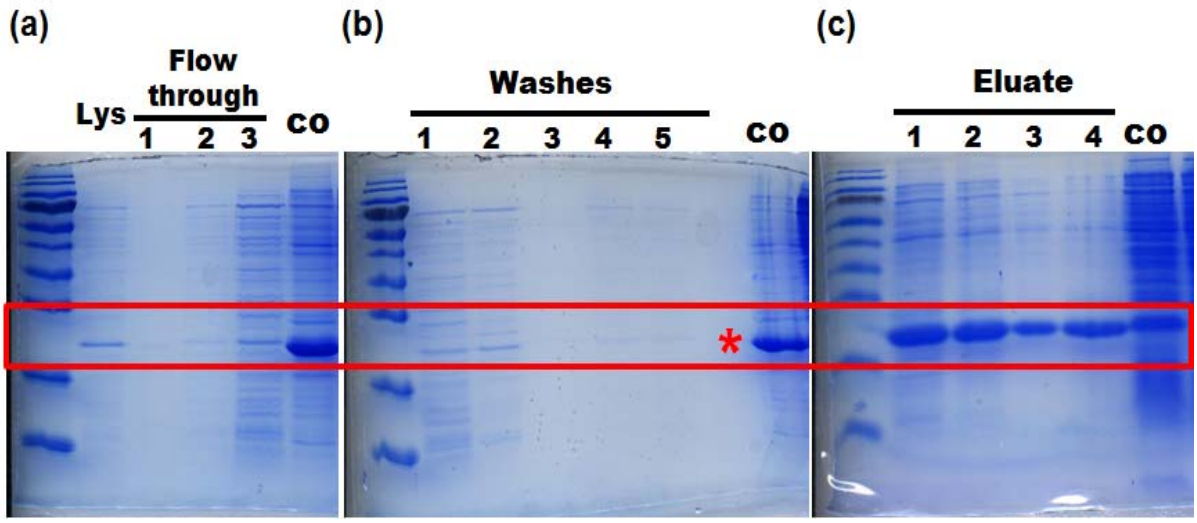


Figure 4.5 Purification of heterologously expressed recombinant FIT-C fusion protein in *E.coli*

(a) SDS-PAGE analysis of induced bacterial cell lysate (Lys), and flow through (1, 2 & 3) from the chromatographic column, (b) Washes from the TALON metal affinity resin chromatographic column (1, 2, 3, 4, & 5 washes), (c) Eluate (1, 2, 3, & 4,), as control (co) protein sample prepared from induced culture was loaded to verify the purified protein size . Asterisks (*) indicates the ~21 kDa size FIT-C fusion protein band.

4.1.3 Immunization of Mice with FIT-C fusion protein, antiserum collection and specificity test of the FIT-C antiserum

The heterologously expressed recombinant FIT-C fusion protein was affinity purified (Fig. 4.5a-c) and injected to mice. Immunization of mice and antiserum collection was kindly performed by Prof. Uli Müller and Iris Fuchs, Department of Zoology, Saarland University. The obtained antiserum was checked for its specificity on *E.coli* expressed FIT-C fusion protein (Fig. 4.6) and later used for monitoring internal / in planta FIT protein expression and accumulation/abundance.

Hence we could detect a single band on western blot that matches to the specifically expressed and desired FIT-C protein, we conclude that the generated antiserum is specific to FIT protein.

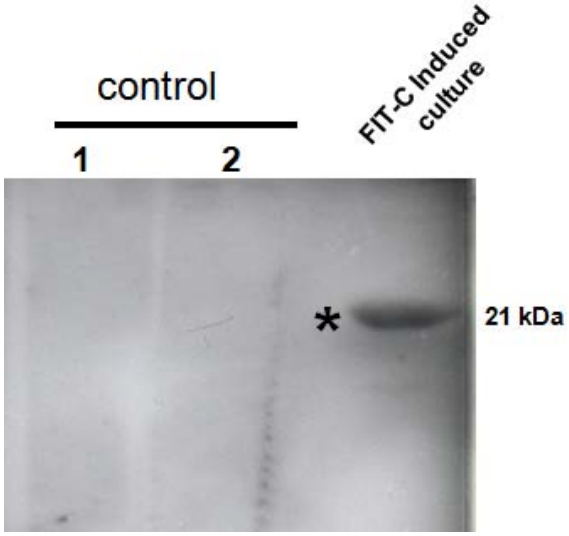


Figure 4.6 specificity of FIT-C antiserum

Westernblot image of FIT-C antiserum specificity, control 1 was loaded with uninduced bacterial culture, control 2 was loaded with FIT-C induced bacterial culture but omitted the incubation with FIT-C antiserum, and incubated with secondary antibody (to cross check for the cross reactivity of the secondary antibody). The last lane from the right hand side is loaded with FIT-C induced bacterial culture and probed/incubated with FIT-C antiserum. * indicates the position of 21 kDa FIT-C protein.

4.2 FIT protein expression, stability and regulation in planta

Previous studies reported that *FIT* is transcriptionally regulated upon iron deficiency (Jakoby et al., 2004; Colangelo and Guerinot, 2004). It was then interesting to investigate/validate the FIT protein status in response to Fe status. In order to monitor FIT protein levels in planta, a polyclonal affinity-purified antiserum directed against the C-terminal peptide of FIT has been generated for this study. This antiserum was used to monitor the status of endogenous FIT protein of wild type plants and non tagged FIT overexpression plants.

4.2.1 FIT protein accumulates under iron deficient conditions in Arabidopsis roots

To elucidate whether FIT protein expression levels were regulated by Fe, we conducted western blot analysis. Western blot results/analysis showed that in wild type (Col-0) roots, FIT was detectable under - Fe conditions but not under + Fe conditions (Fig. 4.7). Whereas in the FIT Ox plants, strong FIT protein bands were detectable under both Fe supply conditions (Fig. 4.7), indicating that FIT protein was produced at + and – Fe in FIT Ox plants. In negative control protein extracts, samples prepared from *fit* loss-of-function mutant plants, FIT protein bands were not detectable which also demonstrates the specificity of the generated antiserum (Fig. 4.7). Conclusively, these findings suggested that iron deficiency leads to FIT protein accumulation in wild type Arabidopsis roots.

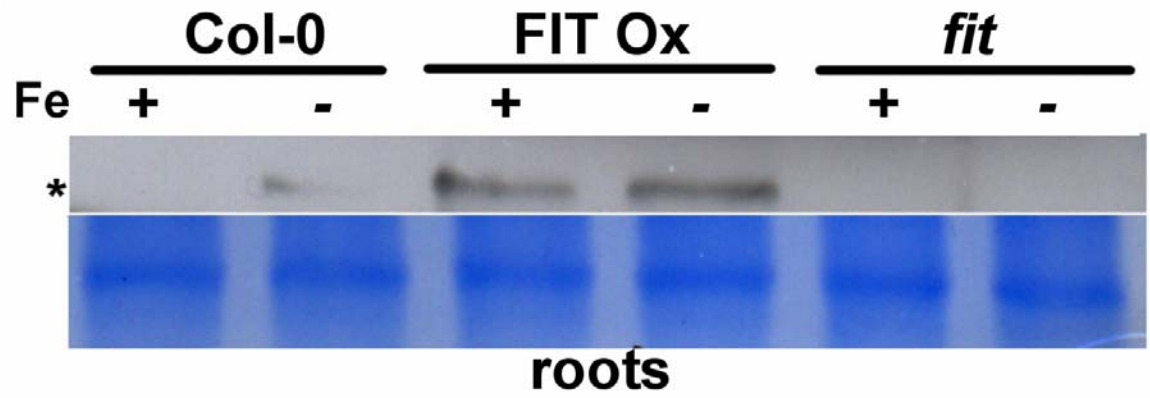


Figure 4.7 Abundance of FIT protein in wild type and in FIT overexpression plants
FIT protein was only detected at – Fe in wild type plants, while FIT was found abundant at + and – Fe in over-expression plants. FIT protein in roots of wild type Col-0, FIT Ox (positive control; Jakoby *et al.,* 2004), *fit* (negative control, note specificity of the antiserum); plants were grown in the 14-day agar growth system; FIT protein was detected by Western blot using anti-FIT-C polyclonal antiserum; asterisk indicates ~35-kDa size of FIT (upper image); Coomassie-staining represents the loading control (lower image).

4.3 Nitric Oxide (NO) as signaling component on FIT gene expression and FIT protein accumulation in Arabidopsis

Very recently, turnover control of FIT protein has been reported. Analysis of wild type and FIT over expression plants (non tagged and tagged such as GFP/FLAG/HA-FIT) revealed that FIT is subjected to turnover control (Meiser et al., 2011 [part of this dissertation]; Sivitz et al., 2011). It was then great interest to uncover which signaling components/molecules that might potentially affect FIT protein accumulation and abundance. In the present study it was also investigated that FIT stability was increased by ethylene signaling (Lingam et al., 2011). This prompted us to investigate whether NO would influence abundance and activity of FIT protein.

Several findings demonstrated that, NO positively affects Fe deficiency responses in tomato (*Solanum lycopersicum*) and Arabidopsis (Graziano et al., 2002; Graziano and Lamattina, 2007; Besson-Bard et al., 2009; Chen et al., 2010). NO and ethylene could promote/influence and regulate Fe deficiency responses in a similar fashion (Lucena et al., 2006; García et al., 2010; Wu et al., 2011).

4.3.1 Effect of NO on FIT protein accumulation and stability upon Fe deficiency

However, none of the above studies could address the regulation of FIT at protein level in Fe deficiency. With help of tools (FIT Specific antiserum) that have been developed during this study, for the first time we were able to investigate the effect of NO on FIT expression/regulation and stability during Fe deficiency. To test the effect of NO, we grew wild type and FIT overexpression (HA-FIT) plants in the 6-day growth system that is more convenient and most suitable to perform NO pharmacological treatments with the widely used NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO).

The reason behind for the selection of cPTIO is, it has been widely used and described as the most reliable common plant inhibitor for NO in the literature (Graziano and Lamattina, 2007; Chen et al., 2010). In our growth and treatment conditions, we were able to detect FIT protein in wild type control roots at –Fe (Fig. 4.8a), which was in accordance with our previous result (Fig. 4.7).

cPTIO treatment led to a strong reduction of FIT protein to 2% at –Fe compared with control roots, suggesting that inhibition of NO signaling resulted in reduced FIT protein stability and abundance (Fig. 4.8a). Interestingly, the cPTIO treated HA-FIT plant roots, also showed in a decrease of HA-FIT protein levels (to 30% at –Fe and to 50% at +Fe versus controls; Fig. 4.8b).

Wild type seedlings that grew in the presence of NO donor S-nitrosoglutathione (GSNO), did not show any difference on FIT accumulation and abundance at –Fe. Whereas, at +Fe GSNO treatment could slightly lead to FIT accumulation (Fig. 4.8a). These observations supporting that NO enhanced FIT protein accumulation, whereas NO inhibition hindered it. Besides this, we confirmed the results by analyzing HA-FIT plants under the same conditions. Treatment with cPTIO showed reduced (to 50 % at + Fe, and to 30 % at – Fe vs controls) HA-FIT protein levels (Fig. 4.8b).

However, GSNO treated seedlings did not show any difference at HA-FIT levels. This might be due to the FIT overexpression and beyond the certain threshold level GSNO may remain ineffective (Fig.4.8b).

To further validate and support the effect of NO inhibitors on FIT protein, we have tested additional NO inhibitors. In plants, the two vastly studied enzymatic sources for NO are NO synthase (NOS)-type enzyme and nitrate reductase (NR).

NOS catalyzes the conversion of L-arginine to L-citrulline and NO. N-nitro-L-arginine methyl ester hydrochloride (L-NAME), the L-arginine analogue, was used in order to block NOS in various plants (Desikan et al., 2004; Graziano et al 2007). A molybdenum-containing enzyme Nitrate reductase (NR), which catalyzes the formation of NO via nitrite reduction, as well as in generating nitrite from nitrate (Rockel et al., 2002). Tungstate, a molybdate analogue that has been previously reported to inhibit the formation of an active NR *in vivo* (Deng et al., 1989) and also reported to block NR-dependent NO production (Bright et al., 2005).

Our results showed that all three tested NO inhibitors led to reduced HA-FIT protein at –Fe, namely to 40% (tungstate), 30% (L-NAME), and 50% (cPTIO) versus the controls (Fig. 4.9). Taken together, NO inhibition certainly diminished FIT protein accumulation.

4.3.2 Influence of NO on *FIT*, *FRO2* and *IRT1* gene expression upon Fe deficiency

In addition to protein expression and regulation studies, we have also investigated whether cPTIO treatments had affected the expression of the Fe deficiency marker genes in wild type plants and HA-FIT overexpression plants from the same set of experiment. This is mainly to verify and compare our protein analysis experimental system to that of published literature in other words to cross check whether we can reproduce the findings of the previously published work in Arabidopsis (only Chen et al., 2010 used Arabidopsis). However, none of those studies analyzed FIT Ox lines for this purpose. In wild type control (w/o cPTIO) roots exposed to +Fe or –Fe, gene expression was as expected and in accordance to the results described in the previous sections of this report. *FIT* was induced 3-fold, whereas *IRT1* and *FRO2* were at least 8-fold induced by –Fe (Fig. 4.8c).

In the case of HA-FIT seedlings a similar gene expression pattern was observed, with the exception for *FIT* hence it was under constitutive promoter control compared to the wild type (Fig. 4.8d). cPTIO, Tungstate treatments resulted in a decreased expression of *FIT, FRO2,* and *IRT1* gene expression in –Fe wild type roots compared with the –Fe control (Fig. 4.8c, and 4.10a). According to the literature this expression pattern was expected (Graziano et al., 2002; Graziano and Lamattina, 2007; Besson-Bard et al., 2009; Chen et al., 2010). In contrast to cPTIO and Tungstate treatments, L-NAME has no effect on *FIT*, *FRO2* and *IRT1* gene expression (Fig. 4.10a) which is again in accordance to previous findings from *Solanum lycopersicum*. *SlFER*, *SlFRO1* and *SlIRT1* gene expression was not suppressed at -Fe upon L-NAME treatment (Graziano et al., 2007).

On the other hand, cPTIO, Tungstate and L-NAME treatments had no effect on gene expression of *FIT*, *FRO2* and *IRT1* in HA-FIT plants (Fig. 4.8d, and Fig. 4.10b). Probably, the remaining small pool of HA-FIT protein in the HA-FIT overexpression plants might be sufficient to trigger *FRO2* and *IRT1* induction at -Fe. The decrease of HA-FIT by cPTIO shows that HA-FIT protein regulation cannot be explained merely by a reduced transcriptional activation due to cPTIO but that reduced NO affected HA-FIT also at the protein level.

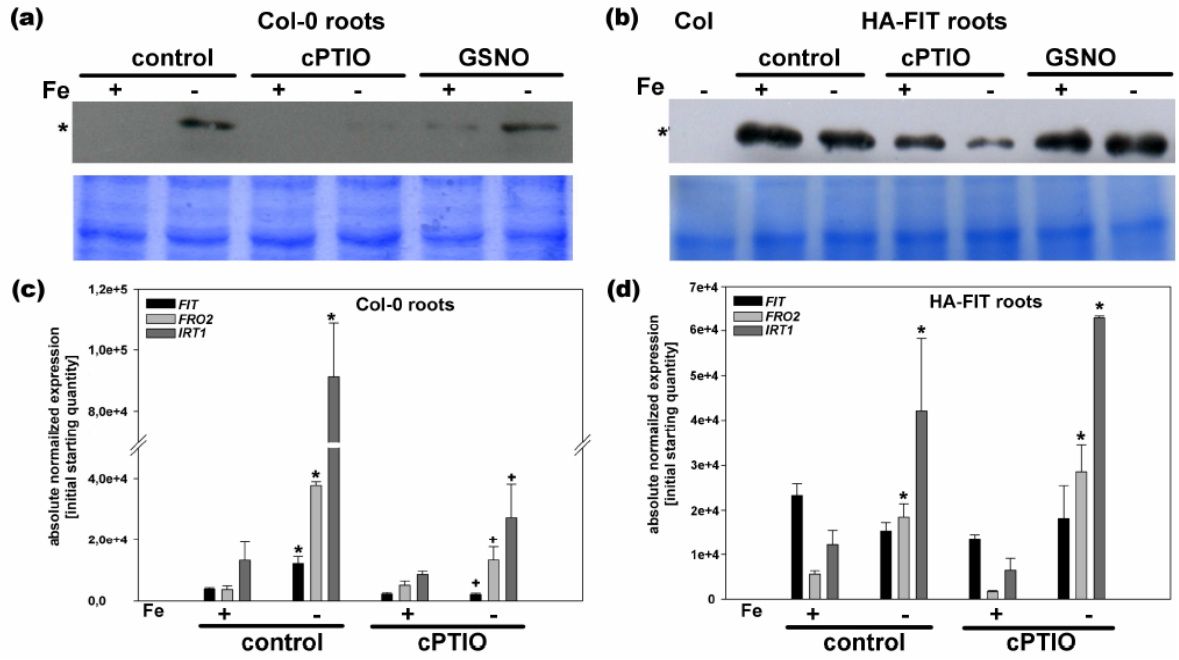


Figure 4.8 Effect of nitric oxide (NO) on FIT protein abundance and gene expression
cPTIO caused a reduction of FIT and HA-FIT protein levels, and a decrease of *FIT, FRO2* and *IRT1* gene expression in wild type, but not in HA-FIT roots. (a) FIT protein in roots of wild type, untreated (control), treated for 24 hours with 1 mM cPTIO (cPTIO) and 25 µM GSNO (GSNO); plants were grown in the 6-day agar system; FIT protein was detected by Western blot using anti-FIT-C polyclonal antiserum; * indicates the 35 kD size of FIT (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); HA-FIT protein was detected by Western blot using anti-HA monoclonal antibodies; * indicates the 42 kD HA-FIT band (upper image, Col-0 was used as negative control); Coumassie-staining represents the loading control (lower image). (c) Reverse transcription-qPCR analysis of *FIT, FRO2* and *IRT1* in wild type roots treated with or without cPTIO as described in (a); * indicates significant change versus + Fe of respective treatment ($p < 0.05$); + indicates significant change versus control at respective Fe supply ($p < 0.05$); $n = 2$. (d) Reverse transcription-qPCR analysis of *FIT, FRO2* and *IRT1* in HA-FIT 9 roots treated with or without cPTIO as described in (b); * and + as in (c).

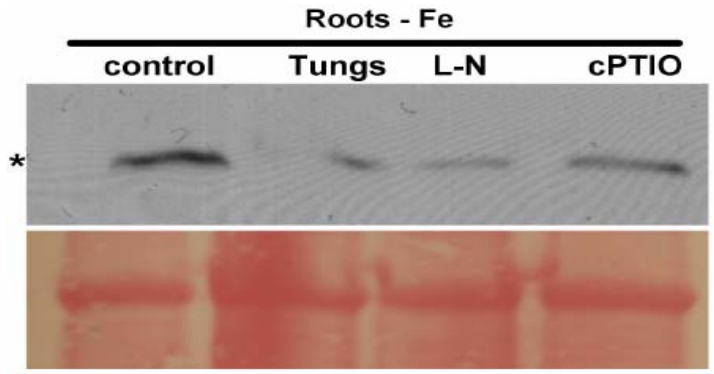


Figure 4.9 Abundance of HA-FIT protein in response to different NO inhibitors
HA-FIT protein abundance in roots of – Fe HA-FIT 9 plants, untreated (control), treated with 1 mM Tungstate (Tungs), 1 mM L-NAME (L-N) and 1 mM cPTIO (cPTIO), showing that several NO inhibitors caused reduction of HA-FIT protein amounts; plants were grown in the 6-day agar system; HA-FIT protein was detected by Western blot using anti-HA monoclonal antibodies; * indicates the 42 kD HA-FIT band (upper image); Ponceau S-staining represents the loading control (lower image).Experiment performed jointly by a co-worker Johannes Meiser, seedlings growth, NO treatments performed by Lingam, protein extraction and western blot analysis was performed by Meiser.

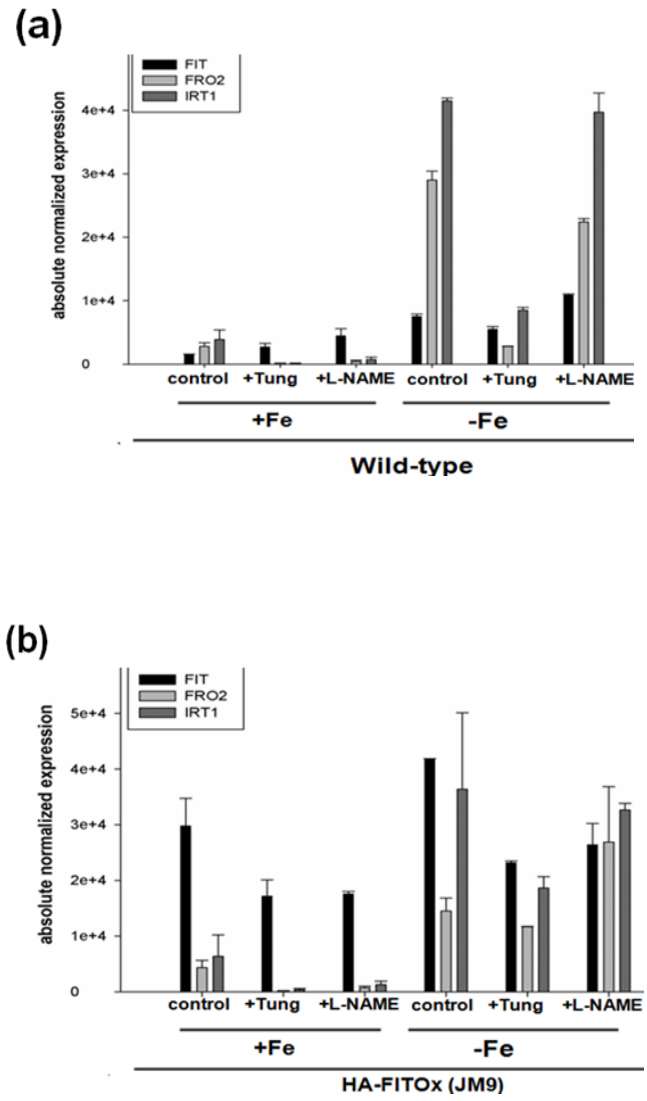


Figure 4.10 Effect of nitric oxide (NO) inhibitors on FIT gene expression
(a) Reverse transcription-qPCR analysis of *FIT, FRO2* and *IRT1* in wild type roots treated with or without Tungstate, L-NAME as described in Fig.4.9; (b) Reverse transcription-qPCR analysis of *FIT, FRO2* and *IRT1* in HA-FIT 9 roots treated with or without as described in Tungstate, L-NAME as described in Fig.4.9.

4.3.3 HA-FIT protein localization in root transverse sections in response to NO inhibition

Since NO inhibition affected FIT protein accumulation and abundance, it was then interesting to monitor whether HA-FIT protein shows any differential cellular localization pattern in response to NO inhibition and Fe supply. To address this question, we performed immunolocalization on HA-FIT Ox (JM-9) root cross sections. Seedlings were grown and cPTIO treatments were done as described in previous sections. However, there is no specific difference in signal pattern was observed at cellular level. This is probably due to the FIT over expression since it was under constitutive promoter control. Nevertheless, reduced FIT levels were observed in the roots that treated with NO scavenger cPTIO. This serves as an additional proof for the previously presented western blot results on FIT protein abundance.

Immunolocalization with internal FIT antibody on wild type roots would be more interesting to investigate the differential cellular localization of FIT protein in response to Fe and NO. Due the limited availability of the internal FIT antibody, it was not possible to investigate. Since immunolocalization procedure demands/require much more antibodies than western blot analysis. Moreover, it is not possible to recollect and reuse the antibodies after immunolocalization as in the case of western blot where the antibodies can be recollected for multiple uses.

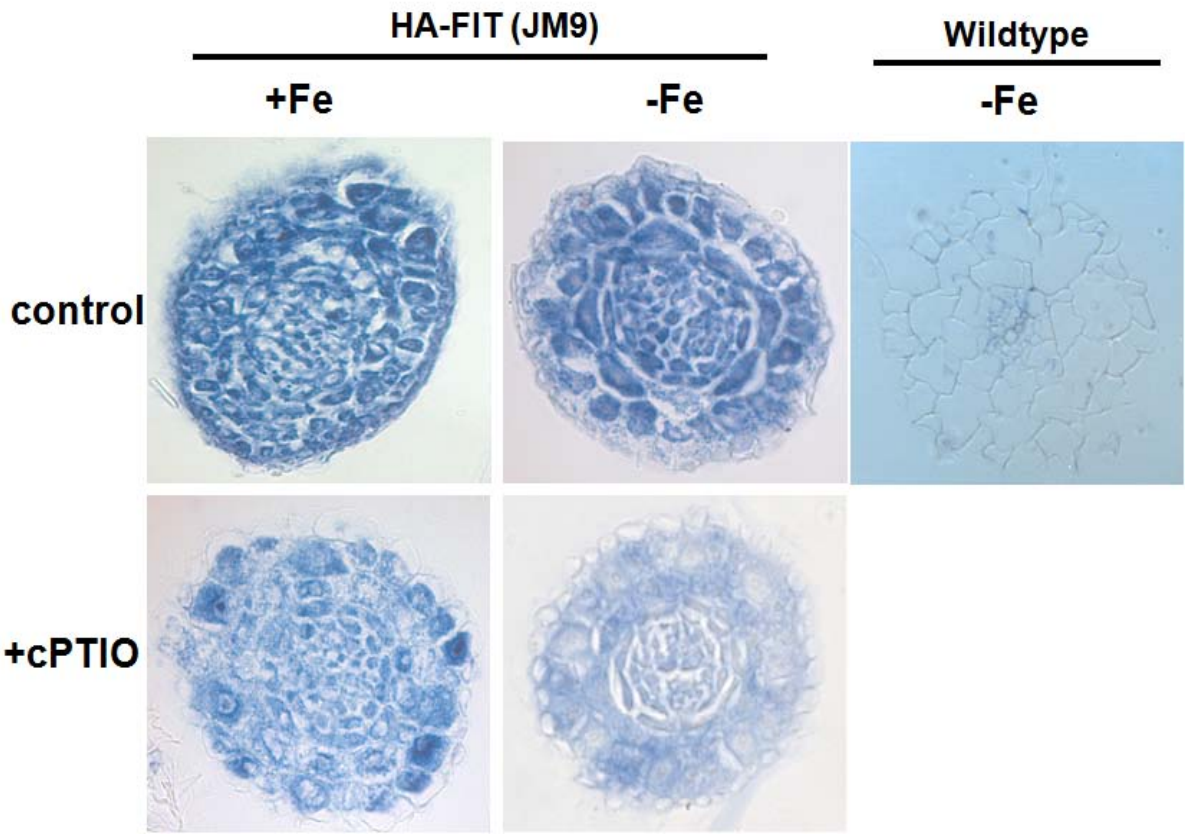


Figure 4.11 Immunolocalization of HA-FIT protein on root transverse sections
HA-FIT protein imunolocalization on Arabidopsis root cross sections using anti HA antibody, roots were embedded in paraffin and cross sectioned from meristematic zone @ 8µm thickness. Growth conditions and cPTIO treatments were done as described in Fig.4.8. Intensity of blue/violet color indicates the abundance of HA-FIT protein. Wild type (Col-0) used as negative control.

4.4 FIT Protein accumulation is counteracted by NO Inhibitors and restored by Inhibitors of proteasomal degradation

Although the above data could confirm the effect of NO inhibitors on FIT abundance, it is still remain unclear what kind of mechanisms may play a role in FIT protein degradation and stability. These observations made us curious to find out possible mechanisms. To address this, we incubated cPTIO grown HA-FIT seedlings (similarly grown as discussed in previous sections) with a widely used most common proteasome inhibitor MG132. cPTIO treatment led to reduced HA-FIT levels to 50% in –Fe and to 6% in +Fe condition compared to controls (Fig. 4.12) this is in agreement with data obtained and presented in Fig. 4.8b.

Interestingly, in MG132 treated cPTIO grown HA-FIT seedlings FIT levels were restored in both Fe conditions (Fig.4.12). Therefore, we conclude that FIT protein was susceptible to proteasome mediated degradation during the inhibition of NO signaling. Hence, treatment with proteasome inhibitors could restore the HA-FIT protein in the cPTIO grown seedlings. Taken together, we conclude that NO favors FIT stability in a similar way to ethylene through inhibition of FIT degradation via proteasome

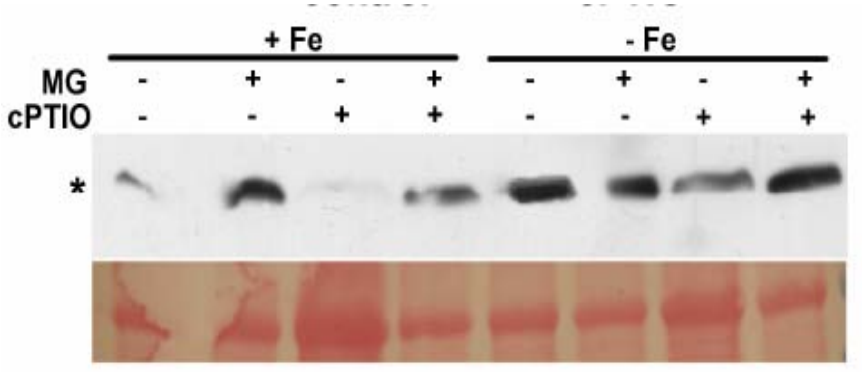


Figure 4.12 MG132 reversed the cPTIO-mediated repression of HA-FIT
HA-FIT abundance in roots of HA-FIT 9 plants grown at – or + Fe and treated as indicated for 24 h with 1 mM cPTIO and four hours with 100 µM MG132 (MG), showing that MG132 treatment reversed the cPTIO effect; plants were grown as in (a); upper and lower image as described in (b) except that Ponceau S was used as loading control (Experiment performed jointly by a co-worker Johannes Meiser, seedlings growth, NO treatments performed by Lingam, MG132 treatment sample collection was done by Lingam and Meiser, protein extraction and western blot analysis performed by Meiser).

4.5 Influence of ethylene on FIT mediated Fe deficiency responses

Multiple studies proved that plant hormone ethylene is able to promote up-regulation of Fe acquisition. Application of ethylene precursors such as 1-aminocyclopropane-1-carboxylic acid (ACC) or ethephon to plants could mimic morphological growth responses of Fe-deficient plants (Romera and Alcantara, 1994; Schmidt et al., 2000). ACC treatment enhanced molecular-physiological Fe deficiency responses like *IRT1* and *FRO2* gene expression (Lucena et al., 2006; Waters et al., 2007; García et al., 2010).

4.5.1 Analysis of FIT protein levels to ethylene inhibition

On the other hand, in contrary to the above findings, several studies reported that the application of ethylene synthesis inhibitor AVG suppresses Fe acquisition responses (Romera and Alcantara, 1994; Lucena et al., 2006; García et al., 2010). The interactions discovered between FIT and EIN3/EIL1 suggests that ethylene is involved in regulation of molecular Fe deficiency responses (work done by co-worker, published in Lingam et al., 2011). Hence, we asked the question whether ethylene regulates Fe acquisition responses via FIT protein stability.

Therefore, we first examined whether such a suppressing effect on Fe acquisition responses can also be noticeable in our 6-day growth system. Seedlings were directly germinated in the presence or absence of AVG and Fe, respectively. Gene expression analysis showed that *IRT1* and *FRO2* were induced by –Fe in the absence and presence of AVG. However, expression levels were lower in the presence of AVG than in the control (Fig. 4.13a). Expression of *FIT* was also suppressed upon AVG treatment compared to the control at – Fe. Hence, we conclude that in our seedling growth system ethylene was necessary for full level up-regulation of Fe deficiency responses.

In order to monitor the effect of AVG on FIT protein level, we have used the wild type and a FIT overexpression line (2xPro-CaMV35S:FIT, named FIT Ox; Jakoby et al., 2004). In wild type roots, FIT protein was only detectable at –Fe in the control, but not at + Fe, whereas in FIT Ox roots, FIT protein was present in + and -Fe control conditions (Fig. 4.13b). The FIT Ox line was useful for the analysis since the constitutive FIT mRNA expression allowed analysis of protein expression uncoupled from FIT gene transcription. Upon AVG treatment, FIT protein was no longer detectable in the wild type. In FIT Ox roots, FIT protein levels were reduced to 30 and 40% of control levels by AVG application under + and –Fe conditions, respectively (Fig. 4.13b).

In these plants, FIT mRNA levels were at least as high upon AVG treatment in leaves as in the controls (published in Lingam et al., 2011 as a co-work with Julia Mohrbacher). Therefore, FIT protein downregulation in AVG-treated plants was independent of transcriptional regulation of *FIT*. AVG treatment resulted in a lower gene expression level of IRT1 and FRO2 in the FIT Ox leaves (published in Lingam et al., 2011 as a co-work with Julia Mohrbacher).

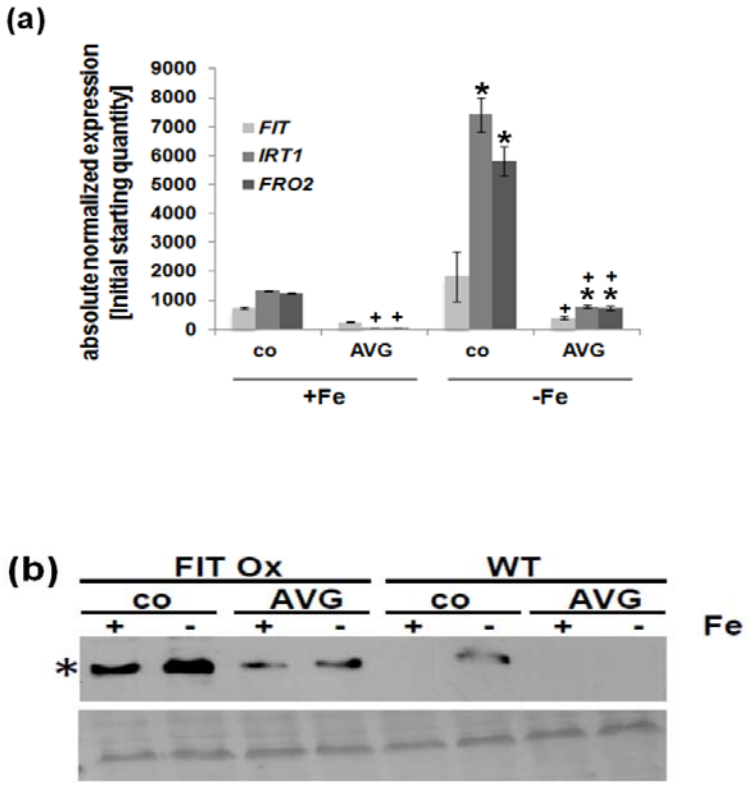


Figure 4.13 Reduced Fe deficiency gene expression and FIT abundance in response to AVG

(a) Gene and protein expression analysis in response to Fe supply (+ and –Fe) and 10 mM AVG (co means control without AVG), showing that Fe deficiency gene expression and FIT protein abundance were reduced by AVG. In (a) asterisk indicates significant change versus +Fe ($P < 0.05$); + indicates significant change versus no AVG control ($P < 0.05$). (a) qRT-PCR analysis of FIT, IRT1, and FRO2 in wild type seedlings; 6-d seedling growth assay, n = 2. SD was calculated for two biological replicates. Gene expression analysis carried out as part of co-work together with Julia Mohrbacher (published in Lingam et al., 2011). (b) FIT protein detection by immunoblot in FIT Ox and wild type (WT) seedlings with anti-FIT-C antibody (top image; asterisk indicates 35 kD position of FIT protein). Ponceau S-staining of the proteins on the same membrane is shown (bottom image).

As an additional support, and also to investigate whether HA-FIT shows differential cellular localization pattern as a result of its low abundance upon AVG treatment, we have performed immunolocalization on HA-FIT root cross sections. Although through the current approach we were unable to see the differential cellular localization of HA-FIT, we were able to reproduce the western blot data which show that reduction in HA-FIT levels upon AVG treatment (Fig 4.14).

As a control, we also tested the effect of ethylene inhibition by 20 µM AOA and 200 µM STS on FIT protein accumulation (see also Romera and Alcantara, 1994). AOA is an inhibitor of ethylene synthesis, and STS is an inhibitor of ethylene perception. We observed that AOA and STS treatment resulted in reduced FIT protein levels (Fig. 4.15a). In FIT Ox roots FIT protein reduced to 20 % and 50 % of those in control FIT Ox plants, respectively (AVG treatment caused a reduction to 60 % of the control level in this experiment).

Gene expression analysis of *FIT* has been carried out from the same batch of samples that have been treated with AOA, STS and AVG for the protein analysis by western blot. In this experiment *FIT* gene expression correlates with the FIT protein abundance in FITOx plants. In AVG treatment, *FIT* was substantially repressed as in the case of previous observations. AOA and STS treatments also suppressed *FIT* gene induction but not as strong as in the AVG treatment (Fig. 4.15b).

In conclusion, AVG did not only inhibit Fe deficiency response gene expression as reported in previous studies (Romera and Alcantara, 1994; Lucena et al., 2006; García et al., 2010) but also FIT protein accumulation. These findings suggest that ethylene regulates Fe acquisition responses also via FIT protein stability.

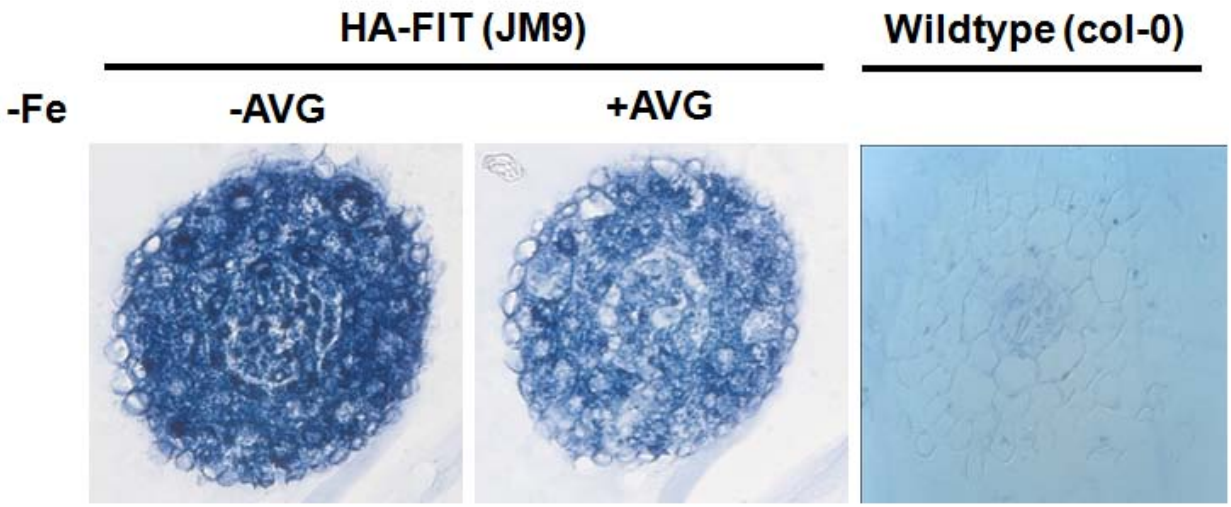


Figure 4.14 Immunolocalization of HA-FIT protein on root transverse sections
HA-FIT protein imunolocalization on Arabidopsis root cross sections using anti HA antibody, 6 day old roots were embedded in paraffin and cross sectioned from meristematic zone @ 8µm thickness. Seedling growth conditions and AVG treatments were performed similarly as described in previous sections. Intensity of blue/violet color indicates the abundance of HA-FIT protein. Wild type (Col-0) used as negative control.

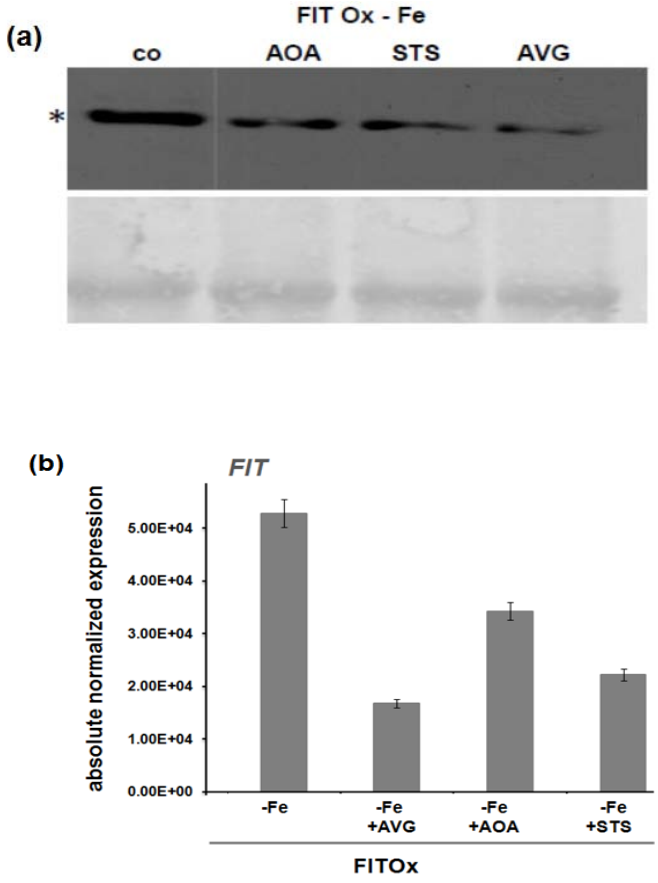


Figure 4.15 Effect of AOA and STS ethylene inhibitors on FIT protein abundance and FIT gene expression

(a) Immunoblot analysis showing that multiple ethylene inhibitors caused a reduction in FIT protein abundance; western blot performed with anti-FIT-C antibody (upper image,* indicates 35 kDa position of FIT), Ponceau S-staining of proteins (lower image). (b) Reverse transcription-qPCR analysis of *FIT* in FIT Ox; seedlings grown at – Fe in the control (co), in the presence of 20 µM AOA (AOA), 200 µM STS (STS) and 10 µM AVG (AVG)

4.5.2 Analysis of FIT protein abundance in *ein3eil1* plants

To better understand the meaning of combined action of up-regulation of Fe deficiency responses by FIT and EIN3/EIL1 on one side and protein interaction between FIT and EIN3/EIL1 on the other side (Lingam et al., 2011; Co-work with Julia Mohrbacher) we tested whether FIT protein levels may be affected by EIN3/EIL1. In wild type roots, FIT was expressed at protein level upon – Fe but not at + Fe (Fig. 4.13b see also Fig. 4.7, Fig. 4.8a and Fig. 4.16). Interestingly, we found that FIT protein levels were reduced to 8 % in the *ein3eil1* mutant compared to the WT at - Fe. However, *FIT* gene expression levels were reduced by half in *ein3eil1* at – Fe (performed by a co-worker J. Mohrbacher, Fig. 4a of Lingam et al., 2011). The reduction was only observed in wild type plants.

In FIT Ox *ein3 eil1* plants, the FIT protein level was not reduced compared with FIT Ox (in a wild type EIN3/EIL1 background), while in the same experiment, FIT protein was reduced to 30% in ein3 eil1 versus the wild type at –Fe.

These experiments indicate that EIN3/EIL1 affect FIT accumulation in the wild type. We conclude that the interaction between FIT and EIN3/EIL1 may serve to enhance FIT protein levels

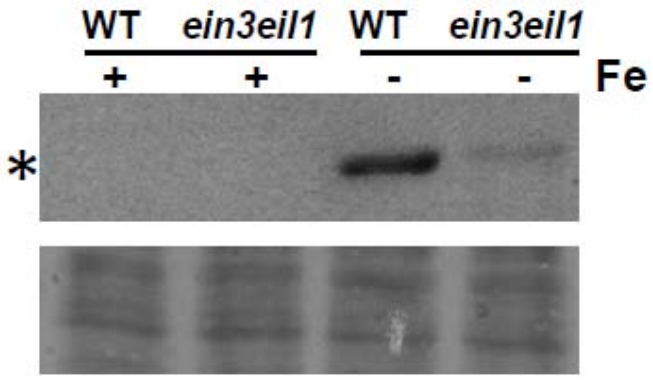


Figure 4.16 FIT protein regulation in *ein3eil1*
FIT protein abundance in seedling roots of the wild type (WT) and *ein3eil1*, exposed to + or –Fe as indicated, showing that FIT protein abundance is reduced in the *ein3 eil1* mutant.

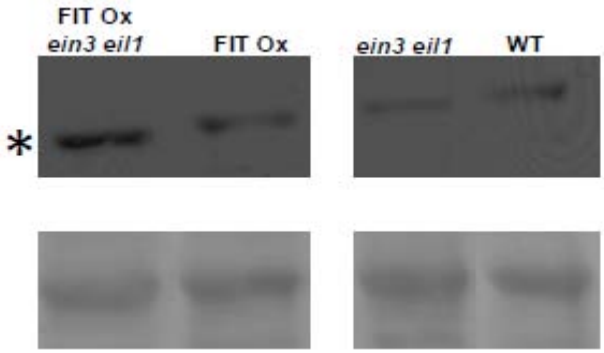


Figure 4.17 FIT protein regulation in FIT Ox *ein3eil1* plants, Immunoblot analysis of seedlings of FIT Ox *ein3eil1*, FIT Ox, *ein3eil1* and wild type, grown at - Fe, with anti-FIT-C antibody (upper image; asterisk indicates 35-kD of FIT protein), Ponceau S-staining of proteins on the same membrane (lower image). FIT protein levels were reduced to 30% in *ein3 eil1* versus the wild type, but not in FIT Ox *ein3 eil1* versus FIT Ox.

4.5.3 Treatment with MG132 restored FIT protein abundance

The downregulation of FIT levels in response to AVG and in the *ein3eil1* background suggests that perhaps FIT was destabilized and degraded by the proteasome in the absence of ethylene signaling. To check this possibility, we incubated wild type plants that grew on AVG with the proteasome inhibitor MG132. In this experiment, FIT protein was reduced to 30% upon AVG treatment at –Fe compared with the control (Fig. 4.18). Upon treatment with 100 mM MG132, FIT protein levels were restored in plants exposed to AVG (Fig. 4.18).

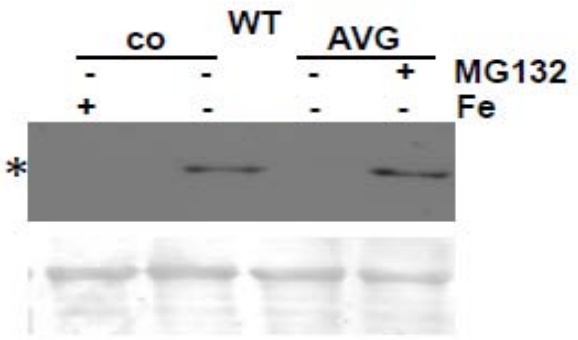


Figure 4.18 FIT protein regulation in wild type roots upon MG132 treatment
The effect of MG132 on FIT abundance in AVG-treated roots, showing that MG132 treatment restored FIT abundance upon AVG treatment. Wild type seedlings, exposed to + and –Fe, treated with 10 mM AVG or untreated (co, control) and treated for 4 h with or without 100 mM MG132 (+ or –MG132).

Furthermore, we also investigated the effect of MG132 on *ein3eil1* seedlings. In this experiment, the FIT protein level was 10% in the mutant versus wild type at –Fe. Upon treatment with 100 mM MG132, we observed a restoration of FIT protein abundance to 60% of the wild type at –Fe (Fig. 4.19). These findings suggest that in the absence of EIN3/EIL1, FIT protein was more prone to proteasome-dependent degradation than in their presence.

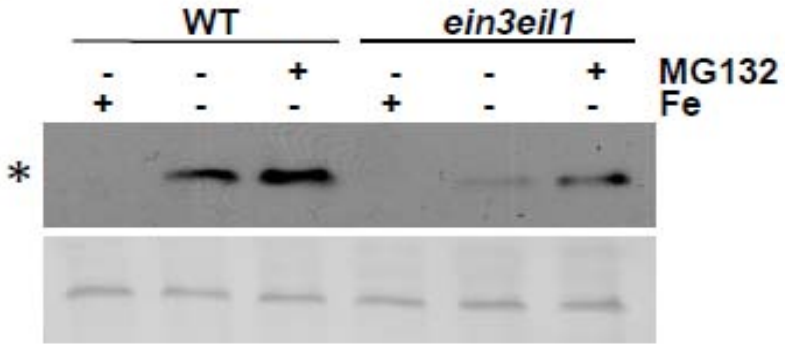


Figure 4.19 FIT protein regulation in *ein3eil1* roots upon MG132 treatment
The effect of MG132 on FIT abundance in *ein3eil1*, showing that MG132 restored FIT protein levels in *ein3eil1* mutant. Seedlings exposed to + and –Fe, treated for 4 h with or without 100 mM MG132 (+ or –MG132); immunoblot analysis with anti-FIT-C antibody (top image; asterisk indicates 35-kD position of FIT protein) and Ponceau S-staining of proteins on the same membrane (bottom image).

5. Discussion

5.1 Posttranscriptional regulation of FIT

Plants react in response to Fe starvation by upregulating the genes involved in the mobilization and subsequent uptake of Fe. In dicot plants, this regulation is controlled by a positive regulator FIT. Previous reports showed the *FIT* regulation at transcriptional level under Fe limiting condition (Colangelo and Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2005). For the first time, by developing a specific FIT polyclonal antiserum, we could detect endogenous FIT protein from the Fe starved wild type plant root extracts. Thus, we could study posttranscriptional regulation of endogenous FIT. Analysis of FIT Ox lines was useful for further analysis since the constitutive *FIT mRNA* expression allowed analysis of protein expression uncoupled from FIT gene transcription. Despite the FIT accumulation at +/-Fe in FIT Ox lines, the target genes exclusively induced upon –Fe situation supports the possible posttranslational regulation of FIT. Such possible regulatory mechanisms and the factors that can influence FIT activation, stability are discussed in the following paragraphs.

5.2 NO is required for FIT accumulation and stability

As reported previously, application of NO inhibitors such as cPTIO caused a decrease of Fe deficiency gene expression (Graziano et al., 2002; Graziano and Lamattina, 2007; Besson-Bard et al., 2009; Chen et al., 2010). Therefore, at the transcriptional level NO can induce *FIT*, *IRT1*, and *FRO2*. In addition, for the first time we demonstrated that application of cPTIO, L-NAME and Tungstate also caused a reduction of FIT protein. However, cPTIO, a scavenger of NO, caused a reduction of FIT protein levels at +Fe and –Fe in both wild type and FIT overexpression lines (HA-FIT). The observed inhibitory effect of cPTIO on FIT protein levels was not merely the result of reduced transcriptional activation of *FIT*.

Hence the reduced protein accumulation caused by cPTIO was also apparent in HA-FIT overexpression plants, where HA-FIT transcription was not regulated by nitric oxide and consequently not affected by cPTIO. Therefore, we deduce that NO promoted FIT protein levels.

Although principally low amounts of FIT protein are sufficient to trigger FIT downstream responses to the full level, this was not the case upon cPTIO treatment in the wild type. Perhaps the remaining levels of active FIT were too low in the wild type treated with cPTIO to cause downstream gene induction, the similar situation was observed in the wild type plants treated with Tungstate. However, L-NAME treatment did not show such repression on the FIT downstream responsive genes in wild type. The reason for such variations is probably due to their mode of action to inhibit NO upon Fe deficiency. Since cPTIO is a scavenger of NO, that might remove NO which is produced or synthesized by various pathways. Whereas, Tungstate and L-NAME can only target specific pathways of NO synthesis in this scenario the other or alternative source of NO synthesis may be still active and compensate the role or production of NO (L-NAME blocks NO synthase and Tunstate inhibits the formation of an active nitrate reductase). In HA-FIT overexpression plants treated with cPTIO, the levels of the 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 overexpression plants, cPTIO, L-NAME and Tungstate treatments did not affect downstream gene expression.

However, the detailed mechanism of NO involvement in activating and stabilizing FIT is not clear, one could speculate a couple of possibilities. One of such possibilities could be the nitrosylation of Cys residues (Tada et al., 2008; Lindermayr and Durner, 2009). The presence of 3 cystein residues in FIT amino acid sequence hints such possibility. Moreover, majority of these 3 cystein residues (2 out of 3) are present in the C-terminal part of FIT (work done by co-worker Johannes Meiser, Johannes Meiser 2011) which could be the potential target for regulation.

Interestingly, all these three are conserved to *Sl*FER (*Solanum lycopersicum*). In such scenario of nitrosylation of FIT in presence of NO could answer the differential physiological responses between the controls and cPTIO treated plants. In addition to *FIT*, NO (GSNO treatment) could induce other Fe dependant genes such as *BHLH038* and *BHLH039* (García et al., 2010). Similarly, bHLH038/039 might also regulate at transcriptional and posttranscriptional level in response to NO just like FIT. It was reported that FIT interacts with bHLH038/039. Probably, the posttranslational modifications (nytrosylation) of these bHLHs in response to NO might facilitate such interaction (Yuan et al., 2008). In addition, the presence of conserved cystein residues in bHLH 038/039/100 (Vorwieger et al., 2007) is thought-provoking the possibility of nitrosylation of cysteins in response to NO.

5.3 NO reduced the proteasomal degradation of FIT

Another novelty of the present study is that, MG132, which acts to inhibit the proteasome, 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 the proteasomal degradation of FIT.

Sivitz et al. (2011) proposed that the activity of FIT was related to its constant turnover and that ubiquitination and proteasomal degradation of FIT, stimulated by –Fe, might be needed to maintain a turnover of FIT for its transcriptional activity at its target binding sites. On the other hand, we proposed that the differential FIT activity was due to the activation of FIT from a large inactive pool to a small active pool, both of which might be targeted by the proteasome (Lingam et al., 2011). Here, we showed that the activity of FIT was not compromised by CHX treatment (Meiser et al., 2011).

Obviously, low amounts of FIT protein were sufficient to trigger IRT1 and FRO2 induction, so we assume that these low amounts contained sufficient active FIT that the synthesis of “fresh” FIT (Sivitz et al., 2011) was not immediately needed.

By comparing the amounts of protein at +Fe and –Fe upon CHX and cPTIO treatment and the untreated controls (Meiser et al., 2011), we suggest that a large pool of FIT that was targeted by the proteasome must have been inactive FIT. Since the proteasome did not appear to select between active and inactive FIT, the proteasomal degradation may not play an important role for increasing the pool of active FIT. This leads to the question of what other mechanism could activate FIT. One possibility is that the active and inactive states differ by specific covalent modifications. If the transfer from the inactive state to the active state has a bottleneck, this could be achieved through limitation of the enzymes that may confer or remove covalent modifications to “activate” FIT (Meiser et al., 2011).

In this study, we could show a novel degradation mechanism of root Fe uptake regulating transcription factor FIT and we are able to propose the reasons and mechanisms for the turnover and activation control of FIT. To our knowledge, this is a new link between FIT activity and NO that could be concluded from our work.

5.4 EIN3/EIL1 affect FIT abundance

We present evidence that FIT abundance can be modulated in plants and that this modulation can be achieved by hormonal cues. We showed in two independent experimental assays that ethylene affected the levels of FIT. We demonstrated that the presence of the ethylene inhibitor AVG resulted in lower FIT amounts in plant seedlings than in the control. Pharmacological treatment using ethylene inhibitors is considered suboptimal, since the available substances have side effects.

However, in combination with mutant analysis of the signaling pathway (*ein3eil1*), the pharmacological results gained significant support. We presented evidence that in the absence of EIN3/EIL1, namely, in *ein3eil1* mutants, FIT was present at lower levels than in the wild type control. FIT levels were not found to be proportional to the amounts of measured FIT transcripts. In the wild type situation, FIT was detectable at –Fe but not at +Fe. On the other hand, the difference in FIT expression between + and –Fe was only two to threefold in the wild type. In the *ein3 eil1* background, FIT levels were decreased in a stronger manner (reduction to 8, 10, and 30% of wild type levels in different experiments) than *FIT mRNA* levels (reduction to 30 to 50%).

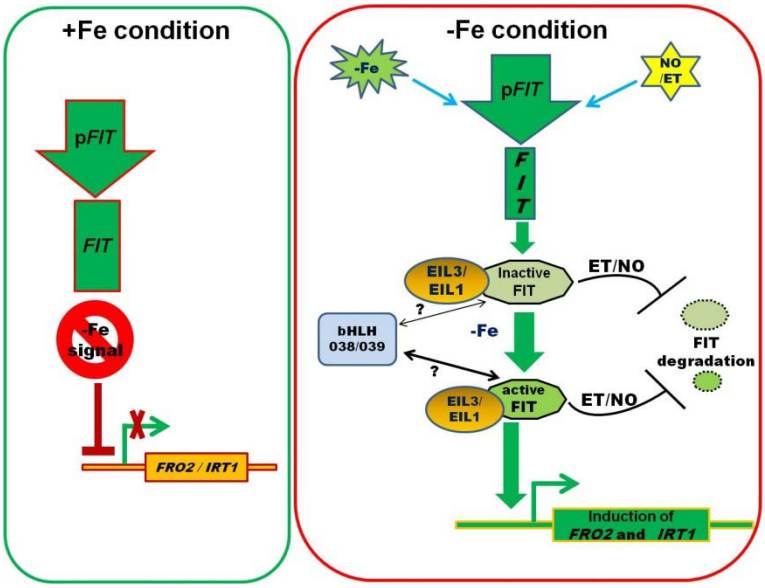
Two conclusions can be drawn from our results, the level of FIT is a target of regulation in plant cells, and ethylene signaling positively affects FIT levels. We thus propose in our model (Figure 5.1) that FIT is not stable in plant cells. One explanation is that ethylene signaling via EIN3/EIL1 results in the production of an unknown factor that is needed for maintaining a high level of FIT. Another explanation is that EIN3/EIL1 themselves are the factors needed for maintaining the high level of FIT. Support for this latter explanation comes from the observed protein interaction between FIT and EIN3/EIL1 (Lingam et al., 2011).

In a continuation study, to understand the meaning /benefit of the identified interaction between FIT and EIN3/EIL1 and the importance of EIN3/EIL1 in stabilizing FIT by interacting with FIT, a microarray analysis of *ein3/eil1* and wild type under +/-Fe have been performed. These analysis suggested that majority of the genes were differentially regulated in *ein3 eil1* mutants vs. wild type upon Fe deficiency than in +Fe condition. Therefore it was concluded that by enhancing Fe uptake through interaction with FIT and by re-organizing the photo oxidative stress responses, EIN3/EIL1 might contribute to decreasing photo-oxidative stress that may occur under light conditions in response to Fe deficiency (Lingam et al.,2011, Bauer and Blondet 2011).

In the model (Figure 5.1), we propose that the physical protein interaction may thus serve to modulate the stability of FIT. Through the increase in FIT stability, EIN3/EIL1 can then indirectly, in a nonsynergistic manner with FIT, contribute to full expression of FIT downstream target genes. The question remained as to how EIN3/EIL1 interaction may affect FIT protein stability. An answer was suggested from experiments with the proteasomal inhibitor MG132. Application of this inhibitor could alleviate the downregulation of FIT abundance upon AVG treatment and in the *ein3 eil1* background. It is therefore a likely possibility that FIT is targeted by the proteasome and that the interaction with EIN3/EIL1 upon ethylene signaling may counteract this effect, hence resulting in a stronger FIT protein abundance and action. Since it was shown that BHLH038 and BHLH039 can interact with FIT (Yuan et al., 2008), it will be interesting to investigate the posttranscriptional regulation of these bHLH factors as well and perhaps their capacity to interact with EIN3/EIL1.

Thus, FIT might play pivotal role in the FIT based Fe mobilization network in plants by coordinating the various signals that contribute for the successful Fe mobilization and uptake as per the Fe demand of the plant. The sophisticated fine tuning FIT activity may act as safeguard to by preventing over accumulation of Fe and subsequent Fe toxicity.

a) Wild type



b) FITox

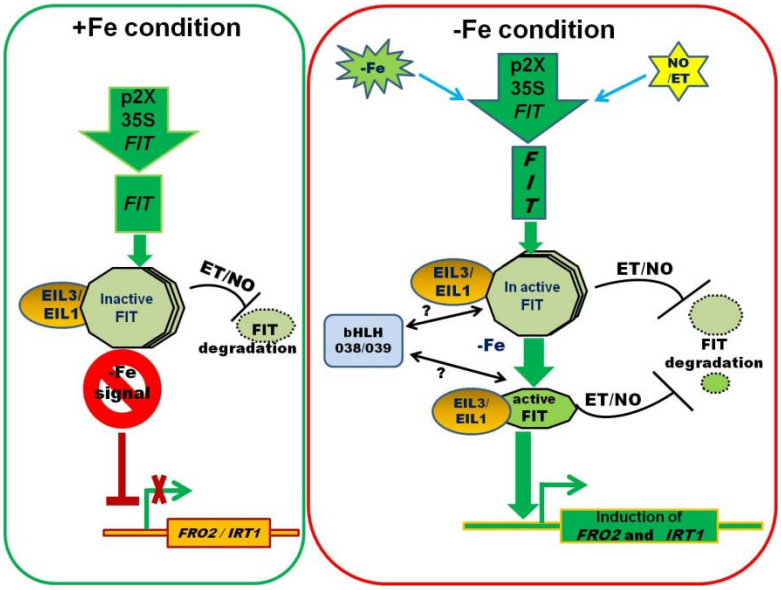


Figure 5.1 model explaining a summary of events that activate, control and stabilize FIT

The collective summary of our findings were depicted in +/-Fe, in wild type (a) and FIT overexpression (b) scenarios (tagged and non tagged FITOx plants).
Upon –Fe FIT is produced in wild type plants, where as FIT is produced independent of Fe supply in FIT Ox plants. The activity of FIT is tightly regulated. In response to –Fe FIT is activated and leads to subsequent induction of downstream targets such as *FRO2* and *IRT1* expression. Based on the relative *IRT1* and *FRO2* expression levels and the levels of FIT protein, we propose that only a small pool of FIT protein is active, while a large pool remains inactive. Active and inactive FIT undergoes rapid constant turnover in plant cells. FIT protein can dimerize with bHLH038 and bHLH039 (Yuan et al., 2008) and might drive the downstream responses of *IRT1* and *FRO2*. Fe deficiency leads to ethylene production (Romera et al., 1999; Li and Li, 2004; Zuchi et al., 2009). EIN3/ EIL1 activated in the ethylene signaling pathway physically interact with FIT, NO and ethylene enhances FIT accumulation by counteracting proteasomal degradation of FIT. According to our model, EIN3/EIL1 does not primarily participate in conjunction with FIT to induce *IRT1* and *FRO2*. We favor the hypothesis that EIN3/EIL1 function to amplify Fe acquisition through stabilization of FIT.

Interestingly, the NO effect on gene expression and FIT protein regulation paralleled that of ethylene (Graziano et al., 2002; Lucena et al., 2006; Graziano and Lamattina, 2007; Besson-Bard et al., 2009; Chen et al., 2010; García et al., 2010; Lingam et al., 2011; Wu et al., 2011). Ethylene, like NO, is required for full-level upregulation 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 promote or influence each other (García et al., 2010, 2011; Romera et al., 2011).

References

Abramoff, M.D., Magelhaes, P.J., and Ram, S.J. (2004). Image Processing with ImageJ. Biophot. Intl. **11:** 36-42.

Al-Sady, B., Ni, W., Kircher, S., Schäfer, E., and Quail, P. H. (2006). Photoactivated phytochrome induces rapid PIF3 phosphorylation prior to proteasome-mediated egradation. Molecular cell **23:** 439-446.

An, F., Zhao, Q., Ji, Y., Li, W., Jiang, Z., Yu, X., Zhang, C., Han, Y., He, W., Liu, Y., Zhang, S., Ecker, J.R., and Guo, H. (2010). Ethylene-induced stabilization of ETHYLENE INSENSITIVE3 and EIN3-LIKE1 is mediated by proteasomal degradation of EIN3 binding F-box 1 and 2 that requires EIN2 in Arabidopsis. Plant Cell **22:** 2384-2401.

Andaluz, S., López-Millán, A.F., De las Rivas, J., Aro, E.M., Abadía, J., and Abadía, A. (2006). Proteomic profiles of thylakoid membranes and changes in response to iron deficiency. Photosynth Res. **89:** 141-155.

Arasimowicz, M. and Floryszak-Wieczorek, J. (2007). Nitric oxide as a bioactive signalling molecule in plant stress responses. Plant Science **172:** 876-887.

Arnaud, N., Murgia, I., Boucherez, J., Briat, JF., Cellier, F., and Gaymard, F. (2006). An iron-induced nitric oxide burst precedes ubiquitin-dependent protein degradation for Arabidopsis *AtFer1* ferritin gene expression. J. Biol. Chem. **281:** 23579-22388.

Asai, S., and Yoshioka, H. (2009). Nitric oxide as a partner of reactive oxygen species participates in disease resistance to necrotrophic pathogen Botrytis cinerea in Nicotiana benthamiana. Molecular Plant-Microbe Interactions **22:** 619-629.

Ashley, M., Grant, M., and Grabov, A. (2006). Plant responses to potassium deficiencies: a role for potassium transport proteins. J. Exp. Bot. **57:** 425-436.

Barberon, M., Zelazny, E., Robert, S., Conéjéro, G., Curie, C, Friml, J., and Vert, G. (2011). Monoubiquitin-dependent endocytosis of the IRON-REGULATED TRANSPORTER 1 (IRT1) transporter controls iron uptake in plants. Proc. Natl. Acad. Sci. USA **1:** E450-E458.

Bauer, P., Ling, H.Q., and Guerinot, M.L. (2007). FIT, the FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR in Arabidopsis. Plant Physiol. Biochem. **45:** 260-261.

Bauer, P., and Blondet, E. (2011). Transcriptome analysis of *ein3eil1* mutants in response to iron deficiency. Plant signaling & behavior **6:** 1669-1671.

Beligni, M.V and Lamattina, L. (2000). Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. Planta **210:** 215-221.

References

Besson-Bard, A., Gravot, A., Richaud, P., Auroy, P., Duc, C., Gaymard, F., Taconnat, L., Renou, J.P, Pugin, A., and Wendehenne, D. (2009). Nitric oxide contributes to cadmium toxicity in Arabidopsis by promoting cadmium accumulation in roots and by up-regulating genes related to iron uptake. Plant Physiol. **149:** 1302-1315.

Binder, B.M., Walker, J.M., Gagne, J.M., Emborg, T.J., Hemmann, G., Bleecker, A.B., and Vierstra, R.D. (2007). The Arabidopsis EIN3 binding F-Box proteins EBF1 and EBF2 have distinct but overlapping roles in ethylene signaling. Plant Cell **19:** 509-523.

Boutrot, F., Segonzac, C., Chang, K.N., Qiao, H., Ecker, J.R., Zipfel, C., and Rathjen, J.P. (2010). Direct transcriptional control of the Arabidopsis immune receptor FLS2 by the ethylene-dependent transcription factors EIN3 and EIL1. Proc. Natl. Acad. Sci. USA **107:** 14502-14507.

Briat, J.F., Ravet, K., Arnaud, N., Duc, C., Boucherez, J., Touraine, B., Cellier, F., and Gaymard, F. (2010). New insights into ferritin synthesis and function highlight a link between iron homeostasis and oxidative stress in plants. Ann. Bot. **105:** 811-822.

Bright, J., Desikan, R., Hancock, J.T., Weir, I.S., and Neill, S.J. (2005). ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H_2O_2 synthesis. Plant J. **45:**113-122.

Brumbarova, T., and Bauer, P. (2005). Iron-mediated control of the basic helix-loop-helix protein FER, a regulator of iron uptake in tomato. Plant Physiol. **137:** 1018-1026.

Brumbarova, T., Matros, A., Mock, H.P., and Bauer, P. (2008). A proteomic study showing differential regulation of stress, redox regulation and peroxidase proteins by iron supply and the transcription factor FER. Plant J. **54:** 321-334.

Buckhout, T.J., Yang, T.J.W., and Schmidt, W. (2009). Early iron-deficiency-induced transcriptional changes in Arabidopsis roots as revealed by microarray analyses. BMC genomics **10:** 147.

Carretero-Paulet, L., Galstyan, A., Roig-Villanova, I., Martínez-García, J.F., Bilbao-Castro, J.R., and Robertson, D.L. (2010). Genome-wide classification and evolutionary analysis of the bHLH family of transcription factors in Arabidopsis, poplar, rice, moss, and algae. Plant Physiol. **153:** 1398-1412.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J.R. (1997). Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell **89:** 1133-1144.

References

Chen, H., Xue, L., Chintamanani, S., Germain, H., Lin, H., Cui, H., Cai, R., Zuo, J., Tang, X., Li, X., Guo, H., and Zhou, J.M. (2009). ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE3-LIKE1 repress *SALICYLIC ACID INDUCTION DEFICIENT2* expression to negatively regulate plant innate immunity in Arabidopsis. Plant Cell **21:** 2527-2540.

Chen, W.W., Yang, J.L., Qin, C., Jin, C.W., Mo, J.H., Ye, T., and Zheng, S.J. (2010). Nitric Oxide Acts Downstream of Auxin to Trigger Root Ferric-Chelate Reductase Activity in Response to Iron Deficiency in *Arabidopsis thaliana*. Plant Physiol. **154:** 810-819.

Cholodny, N. (1931). Zur Physiologic des pflanzlichen Wuchshor- mons. Planta **14:** 207-216.

Ciereszko, I., and L. A., Kleczkowski. (2002). Effects of phosphate deficiency and sugars on expression of rab18 in Arabidopsis: hexokinase-dependent and okadaic acid-sensitive transduction of the sugar signal. Biochimica et Biophysica Acta **1579:** 43-49.

Clarke, A., Desikan, R., Hurst, R.D., Hancock, J.T., and Neill, S.J. (2008). NO way back: nitric oxide and programmed cell death in Arabidopsis thaliana suspension cultures. Plant J. **24:** 667-677.

Colangelo, E.P and Guerinot, M.L. (2004). The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. Plant Cell **16:** 3400-3412.

Connolly, E.L. (2002). Expression of the IRT1 Metal Transporter Is Controlled by Metals at the Levels of Transcript and Protein Accumulation. Plant Cell **14**: 1347-1357.

Connolly, E.L., Campbell, NH., Grotz, N., Prichard, C.L., and Guerinot, M.L. (2003). Overexpression of the FRO2 Ferric Chelate Reductase Confers Tolerance to Growth on Low Iron and Uncovers Posttranscriptional Control. Plant Physiol. **133**: 1102–1110.

Correa-Aragunde, N., Graziano, M., Chevalier, C., and Lamattina, L. (2006). Nitric oxide modulates the expression of cell cycle regulatory genes during lateral root formation in tomato. J. Exp. Bot. **57:** 581-588.

Crawford, N. M., and F. Q. Guo. (2005). New insights into nitric oxide metabolism and regulatory functions. Trends Plant Sci. **10:** 195-200.

Crowe, M.L., Serizet, C., Thareau, V., Aubourg, S., Rouze, P., Hilson, P., Beynon, J., Weisbeek, P., van Hummelen, P., Reymond, P., and al., e. (2003). CATMA: a complete Arabidopsis GST database. Nucl. Acids Res. **31:** 156–158.

References

Curie, C., Alonso, J.M., Le Jean, M., Ecker, J.R., and Briat, J.F. (2000). Involvement of NRAMP1 from Arabidopsis thaliana in iron transport. Biochemical journal **347**: 749-55.

de Benoist, B., McLean, E., Egli, I., and Cogswell, M. (2008). Worldwide prevalence of anaemia 1993-2005. ISBN: 9789241596657.

Delledonne, M., Xia, Y., Dixon, R. A., and Lamb, C. (1998). Nitric oxide functions as a signal in plant disease resistance. Nature **394:** 585-588.

Delledonne, M. (2005). NO news is good news for plants. Curr. Opin. Plant Biol. **8:** 390–396.

Deng, M., Moureaux, T., and Caboche, M. (1989). Tungstate, a molybdate analog inactivating nitrate reductase, deregulates the expression of the nitrate reductase structural gene. Plant Physiol. **91:** 304-309.

Desikan, R., Cheung, M. K., Bright, J., Henson, D., Hancock, J.T., and Neill, S.J. (2004). ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. J. Exp. Bot. **55:** 205-212.

Dinneny, J.R., Long, T. a., Wang, J.Y., Jung, J.W., Mace, D., Pointer, S., Barron, C., Brady, S.M., Schiefelbein, J., and Benfey, P.N. (2008). Cell identity mediates the response of Arabidopsis roots to abiotic stress. Science **320:** 942-945.

Divi, U.K., and Krishna, P. (2009). Brassinosteroid: a biotechnological target for enhancing crop yield and stress tolerance. New Biotechnology **26:**131-136.

Durner, J., Wendehenne, D., and Klessig, D.F. (1998). Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. Proc. Natl. Acad. Sci. USA **95:** 10328-10333.

Ederli, L., Morettini, R., Borgogni, A., Wasternack, C., Miersch, O., Reale, L., Ferranti, F., Tosti, N., and Pasqualini, S. (2006). Interaction between nitric oxide and ethylene in the induction of alternative oxidase in ozone-treated tobacco plants. Plant Physiol. **142:** 595-608.

Eide, D., Broderius, M., Fett, J., and Guerinot, M.L. (1996). A novel iron-regulated metal transporter from plants identified by functional expression in yeast. Proc. Natl. Acad. Sci. USA **93:** 5624-5628.

Franco-Zorrilla, J.M., González, E., Bustos, R., Linhares, F., Leyva, A., and Paz-Ares, J. (2004). The transcriptional control of plant responses to phosphate limitation. J. Exp. Bot. **55:** 285-293.

Gagne, J.M., Smalle, J., Gingerich, D.J., Walker, J.M., Yoo, S.D., Yanagisawa, S., and Vierstra, R.D. (2004). Arabidopsis EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. Proc. Natl. Acad. Sci. USA **101:** 6803-6808.

References

Gagnot, S., Tamby, J.P., Martin-Magniette, M.L., Bitton, F., Taconnat, L., Balzergue, S., Aubourg, S., Renou, J.P., Lecharny, A., and Brunaud, V. (2007). CATdb: a public access to Arabidopsis transcriptome data from the URGV-CATMA platform. Nucl. Acids Res. **36:** D986-D990.

García-Mata, C. and L. Lamattina. (2001). Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Physiol. **126:** 1196-1204.

Garcia-Mata, C., Gay, R., Sokolovski, S., Hills, A., Lamattina, L., and Blatt, M. R. (2003). Nitric oxide regulates K+ and Cl-channels in guard cells through a subset of abscisic acid-evoked signalling pathways. Proc. Natl. Acad. Sci. USA **100:** 11116-11121.

García, M.J., Lucena, C., Romera, F.J., Alcántara, E., and Pérez-Vicente, R. (2010). Ethylene and nitric oxide involvement in the up-regulation of key genes related to iron acquisition and homeostasis in Arabidopsis. J. Exp. Bot. **61:** 3885-3899.

García, M.J., Suárez, V., Romera, F.J., Alcántara. E., and Pérez-Vicente, R. (2011). A new model involving ethylene, nitric oxide and Fe to explain the regulation of Fe-acquisition genes in Strategy I plants. Plant Physiol. Biochem. **49:** 537-544.

Giehl, R.F.H., Lima, J.E., and von Wirén, N. (2012). Localized iron supply triggers lateral root elongation in Arabidopsis by altering the AUX1-mediated auxin distribution. Plant Cell **24:** 33-49.

Gitan, R.S., and D.J., Eide. (2000). Zinc-regulated ubiquitin conjugation signals endocytosis of the yeast ZRT1 zinc transporter. Biochemical Journal **346**: 329.

Graziano, M., Beligni, M.V., and Lamattina, L. (2002). Nitric oxide improves internal iron availability in plants. Plant Physiol. **130:** 1852-1859.

Graziano, M., and Lamattina, L. (2007). Nitric oxide accumulation is required for molecular and physiological responses to iron deficiency in tomato roots. Plant J. **52:** 949-960.

Grotz, N., and Guerinot, M.L. (2006). Molecular aspects of Cu, Fe and Zn homeostasis in plants. Biochimica et biophysica acta **1763:** 595-608.

Guo, H., and Ecker, J.R. (2003). Plant responses to ethylene gas are mediated by SCF(EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. Cell **115:** 667-677.

Guzman, P., and Ecker, J.R. (1990). Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell **2:** 513-523.

He, Y., Doyle, M.R., and Amasino, R.M. (2004). PAF1-complex-mediated histone methylation of FLOWERING LOCUS C chromatin is required for the vernalization-responsive, winter-annual habit in Arabidopsis. Genes Dev. **18:** 2774-2784.

References

Heim, M.A., Jakoby, M., Werber, M., Martin, C., Weisshaar, B., and Bailey, P.C. (2003). The basic helix-loop-helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. Mol Biol Evol. **20:** 735-747.

Henriques, R., Jásik, J., Klein, M., Martinoia, E., Feller, U., Schell, J., Pais, M.S., and Koncz, C. (2002). Knock-out of Arabidopsis metal transporter gene IRT1 results in iron deficiency accompanied by cell differentiation defects. Plant Mol. Biol. **50:** 587-597.

Hilson, P., Allemeersch, J., Altmann, T., Aubourg, S., Avon, A., Beynon, J., Bhalerao, R.P., Bitton, F., Caboche, M., Cannoot, B., and al., e. (2004). Versatile gene-specific sequence tags for Arabidopsis functional genomics: transcript profiling and reverse genetics applications. Genome Res. **14:** 2176–2189.

Hiscock, S., Bright, J., McInnis, S.M., Desikan, R., and Hancock, J.T. (2007). Signaling on the stigma: potential new roles for ROS and NO in plant cell signaling. Plant signaling & behavior **2:** 23-24.

Hsiao, P.W., and Chang, C. (1999). Isolation and characterization of ARA160 as the first androgen receptor N-terminal-associated coactivator in human prostate cells. J. Biol. Chem. **274:** 22373-22379.

Hu, X., Neill, S.J., Tang, Z., and Cai, W. (2005). Nitric oxide mediates gravitropic bending in soybean roots. Plant Physiol. **137:** 663-670.

Ivanov, R., Brumbarova. T., and Bauer P. (2011) Fitting into the harsh reality: regulation of iron-deficiency responses in dicotyledonous plants. Mol. Plant **5:** 27–42.

Jackson, W.T. (1960). Effect of indoleacetic acid on rate of elongation of root hairs of *Agrostis alba L*. Physiologia Plantarum **13:** 36-45.

Jakoby, M., Wang, H.Y., Reidt, W., Weisshaar, B., and Bauer, P. (2004). *FRU* (*BHLH029*) is required for induction of iron mobilization genes in *Arabidopsis thaliana*. FEBS Lett. **577:** 528-534.

Jiang, C., Gao, X., Liao, L., Harberd, N.P., and Fu, X. (2007). Phosphate starvation root architecture and anthocyanin accumulation responses are modulated by the gibberellin-DELLA signaling pathway in Arabidopsis. Plant Physiol. **145:** 1460-1470.

Jih, P.J., Chen, Y.C., and Jeng, S.T. (2003). Involvement of hydrogen peroxide and nitric oxide in expression of the ipomoelin gene from sweet potato. Plant Physiol. **132:** 381-389.

References

Joo, S., Liu, Y., Lueth, A., and Zhang, S. (2008). MAPK phosphorylation-induced stabilization of ACS6 protein is mediated by the non-catalytic C-terminal domain, which also contains the cis-determinant for rapid degradation by the 26S proteasome pathway. Plant J. **54:** 129-140.

Kerkeb, L., Mukherjee. I., Chatterjee. I., Lahner, B., Salt. D.E., and Connolly, E.L. (2008). Iron-induced turnover of the Arabidopsis IRON-REGULATED TRANSPORTER1 metal transporter requires lysine residues. Plant Physiol. **146:** 1964-1973.

Kim, S.A., and Guerinot, M.L. (2007). Mining iron: Iron uptake and transport in plants. FEBS Lett. **581:** 2273-2280.

Klatte, M., and Bauer, P. (2009). Accurate real-time reverse transcription quantitative PCR. Meth. Mol. Biol. **479:** 61-77.

Klatte, M., Schuler, M., Wirtz, M., Fink-Straube, C., Hell, R., and Bauer, P. (2009). The analysis of Arabidopsis nicotianamine synthase mutants reveals functions for nicotianamine in seed iron loading and iron deficiency responses. Plant Physiol. **150:** 257-271.

Klessig, D.F., Durner, J., Noad, R., Navarre, D.A., Wendehenne, D., Kumar, D., Zhou, J.M., Shah, J., Zhang, S., and Kachroo, P. (2000). Nitric oxide and salicylic acid signaling in plant defense. Proc. Natl. Acad. Sci. USA **97:** 8849-8855.

Kobayashi, T., and Nishizawa, N.K. (2012). Iron uptake, translocation, and regulation in higher plants, Annu. Rev. Plant Biol. **63:** 1–22.

Konishi, M., and Yanagisawa, S. (2008). Ethylene signaling in Arabidopsis involves feedback regulation via the elaborate control of EBF2 expression by EIN3. Plant J. **55:** 821-831.

Konishi, M., and Yanagisawa, S. (2009). Ethylene signaling in Arabidopsis involves feedback regulation via the elaborate control of *EBF2* expression by EIN3. Plant J. **55:** 821-831.

Kopriva, S. (2006). Regulation of sulfate assimilation in Arabidopsis and beyond. Ann. Bot. **97:** 479-495.

Kumar, D., and Klessig, D. F. (2000). Differential induction of tobacco MAP kinases by the defense signals nitric oxide, salicylic acid, ethylene, and jasmonic acid. Molecular Plant-Microbe Interactions **13:** 347-351.

Kurata, T., Ishida, T., Kawabata-Awai, C., Noguchi, M., Hattori, S., Sano, R., Nagasaka, R., Tominaga, R., Koshino-Kimura, Y., and Kato, T. (2005). Cell-to-cell movement of the CAPRICE protein in Arabidopsis root epidermal cell differentiation. Development **132:** 5387-5398.

Laganowsky, A., Gómez, S.M., Whitelegge, J.P., and Nishio, J.N. (2009). Hydroponics on a chip: Analysis of the Fe deficient Arabidopsis thylakoid membrane proteome. J. Proteomics **72:** 397-415.

Lampard, G.R, Macalister, C.A., and Bergmann, D.C. (2008). Arabidopsis stomatal initiation is controlled by MAPK-mediated regulation of the bHLH SPEECHLESS. Science **322:** 1113-1116.

Landsberg, E.C. (1984). Regulation of iron-stress-response by whole-plant activity. J. Plant. Nutr. **7:** 609-621

Landsberg, E.C. (1986). Function of rhizodermal transfer cells in the Fe-stress response mechanism of *Capsicum annuum L*. Plant Physiol. **82:** 511-517.

Landsberg, E. C. (1996). Hormonal regulation of iron-stress response in sunflower roots: a morphological and cytological investigation. Protoplasma **194:** 69-80.

Lei, M., Zhu, C., Liu, Y., Karthikeyan, A.S., Bressan, R.A., Raghothama, K.G., and Liu, D. (2011). Ethylene signalling is involved in regulation of phosphate starvation-induced gene expression and production of acid phosphatases and anthocyanin in Arabidopsis. New Phytologist **189:**1084-1095.

Li, L., Cheng, X., and Ling, H.Q. (2004). Isolation and characterization of Fe(III)-chelate reductase gene *LeFRO1* in tomato. Plant Mol. Biol. **54:** 125-136.

Li, X., and Li, C. (2004). Is ethylene involved in regulation of root ferric reductase activity of dicotyledonous species under iron deficiency. Plant Soil **261:** 147-153.

Lin, Z., Zhong, S., and Grierson, D. (2009). Recent advances in ethylene research. J. Exp. Bot. **60:** 3311-3336.

Lincoln, C., Britton, J.H., and Estelle, M. (1990). Growth and development of the axr1 mutants of Arabidopsis. Plant Cell. **2:** 1071-1080.

Lindermayr, C., Saalbach, G., Bahnweg, G., and Durner, J. (2006). Differential inhibition of Arabidopsis methionine adenosyltransferases by protein S-nitrosylation. J. Biol. Chem. **281:** 4285-4391.

Lindermayr, C., and Durner, J. (2009). S-Nitrosylation in plants: pattern and function. J. Proteomics **73:** 1-9.

Lingam, S., Mohrbacher, J., Brumbarova, T., Potuschak, T., Fink-Straube, C., Blondet, E., Genschik, P., and Bauer, P. (2011). Interaction between the bHLH transcription factor FIT with ETHYLENE INSENSITIVE3/ETHYLENE INSENSITIVE3-LIKE1 reveals molecular linkage between iron acquisition regulation and ethylene signaling in Arabidopsis. Plant Cell **23:** 1815–1829.

Ling, H.Q., Bauer, P., Bereczky, Z., Keller, B., and Ganal, M. (2002). The tomato *fer* gene encoding a bHLH protein controls iron- uptake responses in roots. Proc. Natl. Acad. Sci. USA **99:** 13938-13943.

Long, T. a, Tsukagoshi, H., Busch, W., Lahner, B., Salt, D. E., and Benfey, P. N. (2010). The bHLH Transcription Factor POPEYE Regulates Response to Iron Deficiency in Arabidopsis Roots. Plant cell **22:** 2219-2236.

Lucena, C., Waters, B.M., Romera, F.J., García, M.J., Morales, M., Alcántara, E., and Pérez-Vicente, R. (2006). Ethylene could influence ferric reductase, iron transporter, and H^+-ATPase gene expression by affecting FER (or FER-like) gene activity. J. Exp. Bot. **57:** 4145-4154.

Lurin, C., Andres, C., Aubourg, S., Bellaoui, M., Bitton, F., Bruyere, C., Caboche, M., Debast, C., Gualberto, J., Hoffmann, B., and al., e. (2004). Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell **16:** 2089–2103.

Ma, Z., Baskin, T.I., Brown, K.M., and Lynch, J.P. (2003). Regulation of root elongation under phosphorus stress involves changes in ethylene responsiveness. Plant Physiol. **131:** 1381-1390.

Marschner, H., and Römheld, V. (1994). Strategies of plants for acquisition of iron. Plant Soil **165:** 261–274.

Marschner, H. (1995). Mineral Nutrition of Plants. Academic Press, Boston.

Maruyama-Nakashita, A., Nakamura, Y., Yamaya, T., and Takahashi, H. (2004). A novel regulatory pathway of sulfate uptake in Arabidopsis roots: implication of CRE1/WOL/AHK4-mediated cytokinin-dependent regulation. Plant J. **38:** 779-789.

Meiser, J. Lingam, S., and Bauer, P. (2011). Posttranslational regulation of the iron deficiency basic helix-loop-helix transcription factor FIT is affected by iron and nitric oxide, Plant Physiol. **157:** 2154–2166.

Molassiotis, A., Therios, I., Dimassi, K., Diamantidis, G., and Chatzissavvidis, C. (2005). Induction of Fe (III)-chelate reductase activity by ethylene and salicylic acid in iron-deficient peach rootstock explants. J. Plant. Nutr. **28:** 669-682.

Murgia, I., Delledonne, M., and Soave, C. (2002). Nitric oxide mediates iron-induced ferritin accumulation in Arabidopsis. Plant J. **30:** 521-528.

Noguchi, T., Fujioka, S., Choe, S., Takatsuto, S., Yoshida, S., Yuan, H., Feldmann, K.A., and Tax, F.E. (1999). Brassinosteroid-insensitive dwarf mutants of Arabidopsis accumulate brassinosteroids. Plant Physiol. **121:** 743–752.

Orozco-Cárdenas, M.L. and Ryan, C.A. (2002). Nitric oxide negatively modulates wound signaling in tomato plants. Plant Physiol. **130:** 487-493.

Pagnussat, G.C., Simontacchi, M., Puntarulo, S., and Lamattina, L. (2002). Nitric oxide is required for root organogenesis. Plant Physiol. **129:** 954-956.

Pagnussat, G.C., Lanteri, M.L., Lombardo, M.C., and Lamattina, L. (2004). Nitric oxide mediates the indole acetic acid induction activation of a mitogen-activated protein kinase cascade involved in adventitious root development. Plant Physiol. **135:** 537-544.

Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C., and Genschik, P. (2003). EIN3-dependent regulation of plant ethylene hormone signaling by two arabidopsis F box proteins: EBF1 and EBF2. Cell **115:** 679-689.

Prado, A.M., Porterfield, D.M., and Feijó, J.A. (2004). Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. Development **131:** 2707-2714.

Robinson, N.J, Procter, C.M, Connolly, E.L, and Guerinot, M.L. (1999). A ferric-chelate reductase for iron uptake from soils. Nature **397:** 694-697.

Rockel, P., Strube, F., Rockel, A., Wildt, J., and Kaiser, W.M. (2002). Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. J. Exp. Bot. **53:** 103-110.

Rogers, E.E., and Guerinot, M.L. (2002). FRD3, a member of the multidrug and toxin efflux family, controls iron deficiency responses in Arabidopsis. Plant Cell **14:** 1787-1799.

Römheld, V., and Marschner, H. (1986) Different strategies in higher plants in mobilization and uptake of iron. J. Plant. Nutr. **9:** 695-713.

Romera, F.J., and Alcantara, E. (1994). Iron-Deficiency Stress Responses in Cucumber (*Cucumis sativus L*.) Roots A Possible Role for Ethylene? Plant Physiol. **105:** 1133-1138.

Romera, F.J., Alcantara, E., and de la Guardia, M.D. (1999). Ethylene production by Fe-deficient roots and its involvement in the regulation of Fe-deficiency stress responses by Strategy I plants. Ann. Bot. **83,** 51-55.

Rubio, V., Bustos, R., Irigoyen, M.L., Cardona-López, X., Rojas-Triana, M., and Paz-Ares, J. (2009). Plant hormones and nutrient signaling. Plant Mol. Biol. **69:** 361-373.

Sakakibara, H., Takei, K., and Hirose, N. (2006). Interactions between nitrogen and cytokinin in the regulation of metabolism and development. Trends Plant Sci. **11:** 440-448.

Santi, S., Cesco, S., Varanini, Z., and Pinton, R. (2005). Two plasma membrane H^+-ATPase genes are differentially expressed in irondeficient cucumber plants. Plant Physiol. Biochem. **43:** 287–292.

Santi, S., and Schmidt, W. (2009). Dissecting iron deficiency-induced proton extrusion in Arabidopsis roots. New phytologist **183:** 1072-1084.

Sasse, J. M. (2003). Physiological actions of brassinosteroids: an update. Journal of Plant Growth Regulation **22:** 276-288.

Scharf, K.D., Heider, H., Höhfeld, I., Lyck, R., Schmidt, E., and Nover, L. (1998). The tomato Hsf system: HsfA2 needs interaction with HsfA1 for efficient nuclear import and may be localized in cytoplasmic heat stress granules. Mol. Cell Biol. **18:** 2240-2251.

Schikora, A., and Schmidt, W. (2001). Acclimative changes in root epidermal cell fate in response to Fe and P deficiency: a specific role for auxin? Protoplasma **218:** 67-75.

Schmidt, W., Tittel, J., and Schikora, A. (2000). Role of Hormones in the Induction of Iron Deficiency Responses in Arabidopsis Roots. Plant Physiol. **122:** 1109–1118.

Schmidt, W. (2003). Iron solutions: acquisition strategies and signaling pathways in plants. Trends Plant Sci. **8:** 188–193.

Schuler, M., Keller, A., Backes, C., Philippar, K., Lenhof, H.P., and Bauer, P. (2011). Transcriptome analysis by GeneTrail revealed regulation of functional categories in response to alterations of iron homeostasis in Arabidopsis thaliana. BMC plant biology **11:** 87.

Séguéla, M., Briat, J.F., Vert, G., and Curie, C. (2008). Cytokinins negatively regulate the root iron uptake machinery in Arabidopsis through a growth-dependent pathway. Plant J. **55:** 289-300.

Shen, Y., Khanna, R., Carle, C.M., and Quail, P.H. (2007). Phytochrome induces rapid PIF5 phosphorylation and degradation in response to red-light activation. Plant Physiol. **145:** 1043-1051.

Shen, Y., Zhou, Z., Feng, S., Li, J., Tan-Wilson, A., Qu, L.J., Wang, H., and Deng, X.W. (2009). Phytochrome A mediates rapid red light-induced phosphorylation of Arabidopsis FAR-RED ELONGATED HYPOCOTYL1 in a low fluence response. Plant Cell **21:** 494-506.

Shin, H., Shin, H.S., Chen, R., and Harrison, M.J. (2006). Loss of At4 function impacts phosphate distribution between the roots and the shoots during phosphate starvation. Plant J. **45:** 712-726.

Shin, R., and Schachtman, D.P. (2004). Hydrogen peroxide mediates plant root cell response to nutrient deprivation. Proc. Natl. Acad. Sci. USA **101:** 8827-8832.

Signora, L., De Smet, I., Foyer, C.H., and Zhang, H. (2002). ABA plays a central role in mediating the regulatory effects of nitrate on root branching in Arabidopsis. Plant J. **28:** 655-662.

Sivitz, A., Grinvalds, C., Barberon, M., Curie, C., and Vert, G. (2011) Proteasome-mediated turnover of the transcriptional activator FIT is required for plant iron-deficiency responses. Plant J. **66:**1044-1052.

References

Smalle, J., Haegman, M., Kurepa, J., Van Montagu, M., and Straeten, D.V. (1997). Ethylene can stimulate Arabidopsis hypocotyl elongation in the light. Proc. Natl. Acad. Sci. USA **94:** 2756-2761.

Solano, R., Stepanova, A., Chao, Q., and Ecker, J.R. (1998). Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev. **12:** 3703-3714.

Stamler, J.S., and Loscalzo, J. (1992). Capillary zone electrophoretic detection of biological thiols and their S-nitrosated derivatives. Analytical chemistry **64:**779-85.

Tada, Y., Spoel, S.H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., Zuo, J., and Dong, X. (2008). Plant immunity requires conformational changes of NPR1 via S-nitrosylation and thioredoxins. Science **321:** 952-956.

Takahashi, S., and Yamasaki, H. (2002). Reversible inhibition of photophosphorylation in chloroplasts by nitric oxide. FEBS Lett. **512:**145-148.

Tian, Q.Y., Sun, P., and Zhang, W.H. (2009). Ethylene is involved in nitrate-dependent root growth and branching in Arabidopsis thaliana. New Phytologist **184:** 918-931.

Ticconi, C.A., and Abel, S. (2004). Short on phosphate: plant surveillance and countermeasures. Trends Plant Sci. **9:** 548-555.

Tootle, T.L., and Rebay, I. (2005). Post-translational modifications influence transcription factor activity: a view from the ETS superfamily. BioEssays **27:** 285-298.

Triantaphylidès, C., and Havaux, M. (2009). Singlet oxygen in plants: production, detoxification and signaling. Trends Plant Sci. **4:** 219-228.

Varotto, C., Maiwald, D., Pesaresi, P., Jahns, P., Salamini, F., and Leister, D. (2002). The metal ion transporter IRT1 is necessary for iron homeostasis and efficient photosynthesis in Arabidopsis thaliana. Plant J. **31:** 589-599.

Vert, G., Grotz, N., Dedaldechamp, F., Gaymard, F., Guerinot, M.L., Briat, J.F., and Curie, C. (2002). IRT1, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. Plant Cell **14:** 1223-1233.

Von Wirén, N., Klair, S., Bansal, S., Briat, J.F., Khodr, H., Shioiri, T., Leigh, R.A., and Hider, R.C. (1999). Nicotianamine chelates both FeIII and FeII. Implications for metal transport in plants. Plant Physiol. **119:**1107-1114.

Vorwieger, A., Gryczka, C., Czihal, A., Douchkov, D., Tiedemann, J., Mock, H.P., Jakoby, M., Weisshaar, B., Saalbach, I., and Bäumlein, H. (2007). Iron assimilation and transcription factor controlled synthesis of riboflavin in plants. Planta **226:** 147-158.

References

Walker, E.L., and Connolly, E.L. (2008). Time to pump iron: iron-deficiency-signaling mechanisms of higher plants. Curr. Opin. Plant Biol. **11:** 530-535.

Wang, H.Y., Klatte, M., Jakoby, M., Baumlein, H., Weisshaar, B., and Bauer, P. (2007). Iron deficiency-mediated stress regulation of four subgroup Ib *BHLH* genes in *Arabidopsis thaliana*. Planta **226:** 897-908.

Wang, X., Kong, H., and Ma, H. (2009). F-box proteins regulate ethylene signaling and more. Genes Dev. **23:** 391-396.

Wang, B., Li, Y., and Zhang, W.H. (2012). Brassinosteroids are involved in response of cucumber (*Cucumis sativus*) to iron deficiency. Ann. Bot. **110:** 681-688.

Waters, B.M., Lucena, C., Romera, F.J., Jester, G.G., Wynn, A.N., Rojas, C.L., Alcántara, E., and Pérez-Vicente, R. (2007). Ethylene involvement in the regulation of the H^+-ATPase *CsHA1* gene and of the new isolated ferric reductase *CsFRO1* and iron transporter *CsIRT1* genes in cucumber plants. Plant Physiol. Biochem. **45:** 293-301.

Wendehenne, D., Durner, J., and Klessig, D.F. (2004). Nitric oxide: a new player in plant signalling and defence responses. Curr. Opin. Plant Biol. **7:** 449-455.

Wu, J., Wang, C., Zheng, L., Wang, L., Chen, Y., Whelan, J., and Shou, H. (2011). Ethylene is involved in the regulation of iron homeostasis by regulating the expression of iron-acquisition-related genes in *Oryza sativa*. J. Exp. Bot. **62:** 667-674.

Yendrek, C.R., Lee, Y.C., Morris, V., Liang, Y., Pislariu, C.I., Burkart, G., Meckfessel, M.H., Salehin, M., Kessler, H., and Wessler, H. (2010). A putative transporter is essential for integrating nutrient and hormone signaling with lateral root growth and nodule development in *Medicago truncatula*. Plant J. **62:** 100-112.

Yang, Y.H., Dudoit, S., Luu, P., Lin, D.M., Peng, V., Ngai, J., and Speed, T.P. (2002). Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucl. Acids Res. **30:** e15.

Yi, Y., and Guerinot, M.L. (1996). Genetic evidence that induction of root Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency. Plant J. **10:** 835-844.

Yoo, S.D., Y.H., C., G., T., Xiong, Y., and Sheen, J. (2008). Dual control of nuclear EIN3 by bifurcate MAPK cascades in C_2H_4 signalling. Nature **451:** 789-795.

Yuan, Y., Wu, H., Wang, N., Li, J., Zhao, W., Du, J., Wang, D., and Ling, H.Q. (2008). FIT interacts with AtbHLH38 and AtbHLH39 in regulating iron uptake gene expression for iron homeostasis in Arabidopsis. Cell Res. **18:** 385-397.

References

Yuan, Y.X., Zhang, J., Wang, D.W., and Ling, H.Q. (2005). AtbHLH29 of Arabidopsis thaliana is a functional ortholog of tomato FER involved in controlling iron acquisition in strategy I plants. Cell Res. **15:** 613-621.

Zheng, C., Jiang, D., Liu, F., Dai, T., Liu, W., Jing, Q., and Cao, W. (2009). Exogenous nitric oxide improves seed germination in wheat against mitochondrial oxidative damage induced by high salinity. Environmental and Experimental Botany **67:** 222-227.

Zhong, S., Zhao, M., Shi, T., Shi, H., An, F., Zhao, Q., and Guo, H. (2009). EIN3/EIL1 cooperate with PIF1 to prevent photo-oxidation and to promote greening of Arabidopsis seedlings. Proc. Natl. Acad. Sci. USA **106:** 21431-22146.

Zhu, S., and Zhou, J. (2007). Effect of nitric oxide on ethylene production in strawberry fruit during storage. Food Chemistry **100:**1517-1522.

Zhu, Z., and Guo, H. (2008). Genetic basis of ethylene perception and signal transduction in Arabidopsis. Journal of integrative plant biology **50:** 808-815.

Zottini, M., Formentin, E., Scattolin, M., Carimi, F., Lo Schiavo, F., and Terzi, M. (2002). Nitric oxide affects plant mitochondrial functionality in vivo. FEBS Lett. **515:** 75-78.

Zuchi, S., Cesco, S., Varanini, Z., Pinton, R., and Astolfi, S. (2009). Sulphur deprivation limits Fe-deficiency responses in tomato plants. Planta **230:** 85-94.

Acknowledgements

The time has finally come to an end and I would like to acknowledge all the people who have helped, without their support this wouldn't have been possible.

First and foremost I want to thank my supervisor **Professor Dr. Petra Bauer,** who accepted me to do PhD studies in her lab. I thank you so much for believing in me and supporting me all these years. I am indebted to her more than she knows.

A special thanks to **Professor Dr. Uli Mueller,** Department of Zoology, Saarland university, for his kind help in producing anti-FIT antiserum.

I would like to convey my deep thanks to my colleagues and friends **Julia Mohrbacher** and **Johannes Meiser** for their valuable contribution for the successful completion of the projects. Thank you for your help and nice discussions. It was great fun and very comfortable to work with you guys. It was indeed a great time to work with friendly team members **Tzvetina** and **Rumen** (for helpful comments, discussions, outdoor trips and parties)**, Felix, Mara** (for nice friendship)**, Cham, Marco, Hans-Jörg Mai, Angelika Anna** (you are so kind to me), and **Christine Zehren.** Thanks to **Iris Fuchs** and **Angelika,** Department of Zoology for the help and support for producing FIT antibodies, for Immunostaining procedure.

Thank you so much **Monika Schäfer** (**Moni**) for your kind help and cooperation particularly in the very beginning of my stay in Saar. It would have been very difficult to manage several issues without your help.

Thanks to my Indian friends at Uni-Saarbrueken, **Aravind, Harish, Kiran, Ram, Sathish, Senthil, Shyam, Srikanth** and other friends particularly **Kostya** and **Mark.** Guys, thank you for everything. We had so much fun and nice time together.

I am indebted to **my parents Naga Mallikharjuna Rao & Satya vani** and brother **Gopi Nath** for their support all my life. I thank you for always believing in whatever I did. I simply cannot thank you my family. Thanks to my grandfather **Bhaskar Rao** and babay **Subba Rao** for their support for my education. I hope to make you happy and proud. A special thanks to my new family members **vadina**, **mother-in-law**, **Shankar anna** and **Pandu**. I thank all of you for your love and support.

Finally, I acknowledge my beloved wife **Anu (Chinnu)**, my better half, who has entered and enriched my life. Chinnu, your love and patience make all the difference. I thank you for your caring, trust and support.

Thanks to all my teachers in **India** and **Sweden** (particularly Dr. Marcus Grebe, Prof. Rishi Bhalerao) it is all your teachings and the inspiration that you have created during my education made me what I am today.

Curriculum Vitae

Personal Information

Name: Brahmasivasenkar Lingam

Date of Birth: 05.08.1978

Place of Birth: Tiruvuru, Andhra Pradesh, India

Marital status: Married

Nationality: Indian

Academic Profile

2006 – 2011: **PhD** student, thesis title **"Regulation of Fe DEFICIENCY INDUCED TRANSCRIPTION FACTOR (FIT) protein abundance in response to ethylene and nitric oxide"**
Supervisor: **Prof. Dr. Petra Bauer**, Dept. of Biosciences-Botany, Saarland University, Germany.

08.2004 – 05.2005: **M.Sc,** in Molecular Biology, Umeå University, Sweden
Thesis title **"Molecular characterization of *ELD 1* of Arabidopsis"**
Umeå Plant Science Centre, Umeå, Sweden.
Supervisor: **Prof. Rishikesh Bhalerao**

09.1999 – 05.2001: **M.Sc**, in Biotechnology, Andhra University, India

09.1995 – 06.1998: **B.Sc**, (Botany, Zoology & Chemistry), Andhra University, India

Student internships/Work experience

01.09.2005 - 01.11.2005: **Project student**, Umeå Plant Science Centre, Umeå, Sweden.
Supervisor: **Dr.Markus Müller**

01.06.2005 - 01.08.2005: **Project student**, Umeå Plant Science Centre, Umeå, Sweden.
Supervisor: **Dr. Markus Grebe**

01.11.2001–01.07.2004: **Senior Research Fellow**, A.N.G. R. Agricultural University, India
"Processing of pork broiler & eggs", National Agri. Tech. Project

Dec 2000: **Student Research Trainee**, Pasteur Institute of India, Coonoor, India**.** Trained in the production of different vaccines such as rabies, diphtheria, tetanus, and pertussis

Technical skills:

- PCR, recombinant plasmids construction for cloning purposes
- Transformation techniques
- Nucleic acids extraction
- Gel Electrophoresis
- Real-time qRT-PCR
- Expression and Purification of Recombinant Proteins in *E.coli*
- Affinity Chromatography for purifying recombinant proteins
- Western blotting
- Fractionation of membrane proteins by density(sucrose) gradient ultracentrifugation
- GUS reporter gene assay(Flourimetric-quantification)
- Confocal and Fluorescence Microscopy
- Arabidopsis seedling/tissue embedding(paraffin), sections preparation
- Immunolocalization on Arabidopsis root sections
- Whole mount technique of Arabidopsis seedlings
- Reporter gene/GUS Histological studies
- well versed with culturing, handling, maintenance and various other routinely used techniques which are important for *Arabidopsis thaliana* laboratories
- Stable(Arabidopsis) and transient (in Tobacco leaves) plant transformation

Computer skills: MS-Office, DNAStar, PrimerExpress, Genevestigator & WinRhizo

Language skills: English (Proficient) German & Hindi (Basic) & **Telugu** (Mother tongue)

Conferences attended

09. 2006: 3rd Tri-National Arabidopsis Meeting, Tübingen, Germany, Poster presentation

09. 2007: 4th Tri-National Arabidopsis Meeting, Vienna, Austria, Poster presentation

Fellowships/Awards

09. 2007- 06. 2010: **DAAD** (German Academic Exchange Service) Teaching & Research assistantship, through International office, Saarland University

List of Publications:

1. **Lingam, S.,** Mohrbacher, J., Brumbarova, T., Potuschak. T., Fink-Straube, C., Blondet, E., Genschik, P., and Bauer, P. (2011). Interaction between the bHLH transcription factor FIT with ETHYLENE INSENSITIVE3/ETHYLENE INSENSITIVE3-LIKE1 reveals molecular linkage between iron acquisition regulation and ethylene signaling in Arabidopsis. Plant Cell **23:** 1815–1829.

2. Meiser, J., **Lingam, S.,** and Bauer, P. (2011). Posttranslational regulation of the iron deficiency basic helix-loop-helix transcription factor FIT is affected by iron and nitric oxide. Plant Physiol. **157:** 2154–2166.