THESIS

IRON ECONOMY IN ARABIDOPSIS THALIANA ROSETTES

Submitted by

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ABSTRACT

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Iron is important for plant growth and lack of iron negatively affects crop productivity. When we understand more about how plants prioritize their iron use we can use this knowledge to benefit the people of the world. Plant metabolism can be altered to allocate iron in such a way creating larger and/or healthier edible parts.

In the first chapter of this thesis I briefly discuss the biological roles of iron and its function in plants with an emphasis on photosynthesis.

In the second chapter I investigated the potential prioritization of iron-dependent proteins in *Arabidopsis thaliana* plants that were grown hydroponically and exposed to one week of iron deprivation followed by one week of iron resupply. Through the one week of iron depletion the treated plants became visually chlorotic and after one week of iron recovery the treated plants appeared to have fully recovered from the chlorosis. At the end of the recovery week treated plants had significantly less biomass than the control plants yet suffered no indirect effects. To investigate if the decrease in biomass was caused by defects in photosynthetic electron transport chlorophyll fluorescence was measured as well as the photooxidation-reduction values of photosystem I (PSI). Indeed photosynthesis was affected and it was found that there was a decrease in electrons flowing to PSI. With the use of immunoblots it was discovered that the cytochrome b_6f complex proteins were strongly affected by iron depletion followed by the subunits of PSI. Furthermore, I found that the abundance of sulfur metabolism proteins decreased in reaction to decreased iron nutrition.

In the third chapter I discuss the implications of my findings for plant science and society and I give recommendations for follow up work on this project.

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Chapter I

General Introduction

Iron, Fe, is a versatile and useful element for living systems. Fe is typically found in two oxidation states, either the ferric (Fe^{+3}) or ferrous (Fe^{+2}) form. In the cell iron is redox active and switches between the ferric and ferrous forms by accepting or donating electrons (Marschner, 2012). Iron's redox properties have made it an ideal element for living organisms to incorporate into enzymes or proteins with a redox function. Thus, iron containing proteins are essential for electron transport, oxygen transport, protection against reactive oxygen species (ROS), nutrient metabolism as well as DNA synthesis and repair.

Different types of iron cofactors have evolved to exploit the beneficial properties of iron. There are three main types of iron cofactors, heme, non-heme and iron sulfur (Fe-S) clusters (for a list of Fe-S proteins see Balk and Pilon, 2011). Heme is composed of a porphyrin ring with Fe in the center. Oxygen transport in animals is a well-known function of the heme protein hemoglobin; other heme proteins typically function in electron transport (Smith *et al*., 2010). The structure of heme and chlorophyll is very similar as both contain a porphyrin ring. In fact, in green organisms they share the same biosynthetic pathway (Marks, 1966); which branch off at the metal insertion step, Fe in heme, Mg in chlorophyll (Cornah *et al.,* 2003). Non-heme Fe proteins contain one or more iron atoms but lack the heme porphyrin ring. Non-heme Fe proteins include purple acid phosphatase (Beck *et al.,* 1986) and copper response defect1, (CRD1) also known as CHL27, a protein involved in chlorophyll synthesis (Moseley *et al*., 2000; Tottey *et al.,* 2003). The formation of iron sulfur clusters requires sulfur to be removed from cysteine and then to be combined with iron on scaffold proteins (Balk and Pilon, 2011). In the chloroplast, CpNifS (NFS2) and SufE1 form a cysteine desulfurase that provides the S for Fe-S cluster formation (Pilon-Smits *et al*., 2002; Ye *et al*., 2006). There are several candidates that may serve as scaffold proteins but the direct source of Fe that is used for Fe-S cluster formation has not been identified (For

reviews see Balk and Schaedler, 2014;Balk and Pilon, 2011). The SufA (IscA) protein might act as a carrier for Fe-S cluster transfer to targets such as ferredoxin (Abdel-Ghany *et al*., 2005).

The diverse roles that iron plays in living systems make it indispensable to plants (Marschner, 2012). Despite the abundance of iron in the earth's crust, the second most abundant metal after aluminum (Misra, 2000), there are regions where iron is not bioavailable to plants. This is most common in calcareous soils where Fe is oxidized and insoluble at a neutral or basic pH (Guerinot and Yi, 1994). Plants use several strategies to extract iron from the soil. The most common strategies are Strategy I, utilized by most plants including *Arabidopsis thaliana*, and Strategy II, utilized by the Graminacea (for a review see Kobayashi and Nishizawa, 2012). In Strategy I plants, root membrane-bound ferric reductase oxidase (FRO2) reduces Fe^{3+} to the more soluble Fe^{2+} (Robinson *et al*., 1999; Connolly *et al*., 2003). The cation transporter, iron-regulated transporter 1 (IRT1) is upregulated during iron deficiency and imports Fe^{2+} into the root symplast (Vert *et al*., 2002). Then, $Fe^{2+}$is transported from the root cell symplast into the xylem by the ferroportin transporter (FPN). In the root xylem Fe^{2+}must be bound to a chelator, either citrate or nicotianamine (NA), for transport to the shoot (Brown and Chaney, 1971; von Wiren, 1999). In *A. thaliana* an exporter for citrate is required to allow Fe to be mobile in the apoplast (Roschzttardtz *et al*., 2011). For a review on root to shoot transport see Curie *et al.*2009. Iron may also move from the xylem into the phloem through a member of the oligopeptide-transporter family of Yellow Stripe-Like transporters (YSL) (Curie *et al*., 2001; Koike *et al*., 2004). Iron is then transported through the phloem to its sink destination bound to NA or citrate (Koike *et al*., 2004; Roberts *et al*., 2004; Grotz and Guerinot, 2006).

Major cellular iron sinks are in the vacuoles, mitochondria and chloroplasts. In *A. thaliana*, iron storage in seeds occurs in the vacuoles (Lanquar *et al*., 2005). The vascular iron transporter1(VIT1) imports iron into vacuoles (Kim *et al*., 2006) and the natural resistance-associated macrophage protein family member (NRAMP) exports iron from the vacuoles (Lanquar *et al*., 2005). In the mitochondria Fe is a cofactor in enzymes found in the electron transport chain

of the tricarboxylic acid (TCA) cycle (Balk and Pilon, 2011). Iron transporters in the plant mitochondria have not been confirmed to date. Chloroplasts are reported to contain up to 80% of the leaf cellular iron (Shikanai *et al.,* 2003) and Fe plays a vital role in photosynthetic electron transport chain. Without a supply of Fe, these Fe sinks may not function properly.

Some of the effects of iron deprivation in plants can be easily seen and measured. The observed effects include Fe-chlorosis of the leaves, decreased biomass production and shorter roots (Marschner, 2012). Chlorophyll can be seen because it is green in color; it is an essential component of the photosynthetic electron transport chain as it functions in light harvesting (Gratani *et al*., 1998). Although chlorophyll does not contain iron, the biosynthesis of chlorophyll relies on iron availability (Pushnik and Miller, 1989; Bang *et al*., 2008). When iron is not available, chlorophyll content in the leaves is decreased (Nishio *et al*., 1984b). In an attempt to uptake more iron, plant roots are structurally modified. Roots no longer elongate and root hair formation increases (Marschner, 2012). Root hairs are small enough to go into the crevices of soil particles and extract Fe that has been made more soluble by the excretion of protons (Marschner, 2012). When plants do not obtain sufficient iron, overall plant growth declines.

There is an abundance of iron containing proteins in the chloroplasts. These proteins function not only in electron transport but also in chlorophyll synthesis and ROS scavenging (Willows, 2006). Iron containing proteins in the electron transport system include photosystem II (PSII), cytochrome b_6f (cytb_6f), photosystem I (PSI) and ferredoxin. There are roughly 20 Fe atoms per full electron transport system. PSII contains 2 Fe, cytb_6f has 4 Fe, PSI is composed of 12 Fe and ferredoxin has 2 Fe atoms (Raven *et al.,* 1999) (Figure 1). Photosynthesis cannot function properly without iron.

Farmers, whether they are part of a large corporation or a subsistence farmer, rely on plant productivity. All people who eat plants or who eat the animals that eat plants rely on plant productivity. Iron deficiency leads to decreased biomass and a smaller harvest (Marschner,

2012). For plants iron is not easily extracted from the soil and mobilized into the vegetative tissues that are the edible parts of harvested crops.

According to the World Health Organization (WHO) there are 7 billion people in the world relying on plant productivity, it is evident that plant nutrition needs to be fully understood to feed everyone. Of these 7 million people, 2 billion of them suffer from iron deficiency anemia that representing over 30% of the population (http://www.who.int/nutrition/topics/ida/en/). Iron deficiency is a form of hidden hunger causing increased rates of diseases and decreased physical energy affecting people's ability to work which consequently lowers the total economic output of entire countries (Oppenheimer, 2011; http://www.who.int/nutrition/topics/ida/en/). Iron deficiency is more often a problem for people that eat very little or no meat. When we better understand how iron functions in plants that knowledge can be used to improve iron nutrition levels not only of plants but of people who rely on plants for their iron.

The study of iron-dependent proteins in iron-deprived plants has yet to be accomplished. I have identified priorities among some Fe-dependent proteins through the use of a wide array of techniques including elemental composition analysis via inductively coupled plasma optical emission spectroscopy (ICP-OES), immunoblots, chlorophyll fluorescence analysis and measurements of PSI activity. From these experiments, I conclude that plants do have an economy system for iron.

Chapter II

A spatial-temporal analysis of the effects of short-term iron depletion and resupply on Arabidopsis rosettes

Summary

Iron serves several important biological roles in plants. I aimed to identify which iron-dependent proteins involved in specific biological processes were most affected by iron depletion. Four week-old *Arabidopsis thaliana* plants were subjected to one week of iron depletion followed by one week of iron resupply in hydroponics. I analyzed the effects of this treatment on plant growth, mineral composition, physiology and the accumulation of specific iron proteins. I found that iron depletion affected plant growth and photosynthesis especially in the newly developing leaves and that these effects were reversible by iron resupply. Specific iron proteins were affected by iron depletion these included components of the photosynthetic electron transport system as well as enzymes functioning in sulfur assimilation and iron sulfur (Fe-S) cluster assembly. There was no clear correlation between the type of iron cofactor and a protein's tendency to be retained during iron deficiency. This study indicates that iron depletion specifically affects a subset of the iron proteome.

Introduction

Iron, Fe, is an essential micronutrient for plants. Plants need iron cofactors because of iron's oxidation-reduction properties, which are important in electron transfer and redox reactions. Indeed, iron cofactors are found in several components of both the chloroplastic and mitochondrial electron transfer chains. In addition, Fe is a cofactor for enzymes that function in reductive sulfate and nitrate assimilation in plastids. Furthermore, Fe cofactors play roles in catalysis in a diverse array of reactions. For instance, Fe is a cofactor in chloroplastic iron superoxidase dismutase enzymes, and it is an essential part of cofactors for enzymes that function in the biosynthesis of chlorophyll, nicotinamide adenine dinucleotide phosphate (NADPH), as well as in DNA and RNA

metabolism. In order to fulfill its many functions, Fe is used in proteins in many different molecular arrangements, but three major classes of Fe cofactors have been distinguished; heme, non-heme and FeS clusters (Balk and Schaedler, 2014).

It is common for plants to experience iron deprivation due to a lack of iron in the soil or low bioavailability of iron because it is oxidized and insoluble at neutral pH (Guerinot and Yi, 1994). Plants have evolved two different strategies to acquire iron from the soil called, Strategy I and Strategy II (for a review see Kobayashi and Nishizawa, 2012). All higher plants except the Graminacea utilize Strategy I. In iron limiting environments these plants express H^+-ATPases in their roots that release protons which by acidifying the rhizosphere make Fe^{3+} more water soluble (Santi *et al*., 2005). Then the root membrane-bound ferric reductase oxidase (FRO2) reduces Fe^{3+} to Fe^{2+} (Robinson *et al*.,1999; Connolly *et al*., 2003), which can then be taken up into the root symplast by the cation transporter called iron-regulated transporter 1 (IRT1) (Vert *et al*., 2002). When iron availability is low in the rhizosphere, IRT1 and FRO2 are up regulated in the roots to facilitate the uptake of iron (Vert *et al*., 2002). Strategy II is employed by the Graminacea. When iron is limited, Graminacea secrete Mugineic acids (MAs) by a member of the phytosiderophore transporter family which is called OsTOM1 in rice and HvTOM1 in barley (Nozoye *et al*., 2011). In the rhizosphere, these phytosiderophores chelate Fe^{3+}forming a stable soluble complex (von Wiren *et al*., 1995). Fe^{3+} bound to MA complexes are taken up into the root symplast by transporters of the Yellow Stripe-Like family (YS1 and YSL) (Curie *et al*., 2001 and Inoue *et al*., 2009 respectively).

Once in the root, free iron is transported from the root cell symplast into the xylem by the ferroportin transporter (FPN). There are two orthologs in *A. thaliana,* FPN1 and FPN2, they are expressed and localized in roots, AtFPN2 is expressed during iron deficiency (Morrissey *et al.,* 2009). Iron's reactive nature leads to the formation of detrimental reactive oxygen species (ROS) (Halliwell and Guttheridge, 1984). Therefore in the root, Fe must be bound to a chelator to be safely transported to its destination. Inside the xylem Fe has been found chelated to citrate and

nicotianamine (NA) (Brown and Chaney, 1971; von Wiren, 1999). For a review on root to shoot transport see Curie *et al.,* 2009. Iron can move from the xylem into the symplast of vegetative cells in leaves or into the phloem with help from a member of YSL, a subfamily of the oligopeptide-transporters (OPT) (Curie *et al*., 2001; Koike *et al.,* 2004). Iron may be transported through the phloem safely bound to NA or citrate thus avoiding the toxic effects of free iron (Roberts *et al.,* 2004; Koike *et al.,* 2004; Grotz and Guerinot, 2006). Phloem transport is important because xylem takes longer to mature; it is phloem that feeds Fe to newly developing tissues.

Major cellular iron sinks include vacuoles, mitochondria and chloroplasts. Vacuoles serve as an important Fe storage organelle for seeds (Lanquar *et al.,* 2005). Iron enters the vacuole through the vascular iron transporter 1 (VIT1) (Kim *et al.,* 2006) where it is stored for later use. Later, Fe can be released from vacuoles by means of transporters of the natural resistance-associated macrophage protein family member (NRAMP) (Lanquar *et al.,* 2005). Mitochondria are a significant Fe sink because Fe is used as a cofactor in the electron transport chain and in tricarboxylic acid (TCA) cycle enzymes (Balk and Pilon, 2011). Iron transporters into the plant mitochondria have not been confirmed to date. Chloroplasts represent the most important Fe sink quantitatively in green tissues. Indeed, each photosynthetic electron transport chain contains roughly 20 iron atoms (Raven *et al.,* 1999). It has been reported that roughly 80% of the leaf cellular iron is contained in chloroplasts of *A. thaliana* (Shikanai *et al.,* 2003) and 90% in sugar beet (Terry and Abadia, 1986). Thus, Fe starvation should especially affect chloroplast functions. A ferric reductase at the chloroplast surface is required for optimal Fe delivery to chloroplasts (Jeong *et al.,* 2009). Permease in chloroplasts 1 (PIC1) has been identified as a possible transporter of Fe in the chloroplast inner membrane (Duy *et al.,* 2007).

When plants are deprived of iron they exhibit characteristic symptoms (Marschner, 2012). Iron deprived plants have decreased biomass and shorter roots (Chatterjee *et al*., 2006; Abbott, 1968). Iron is required for the synthesis of chlorophyll, the green light harvesting pigment crucial to photosynthesis (Nishio *et al.,* 1985b). Therefore, a readily noticeable symptom of iron depletion

is Fe- chlorosis caused by a decrease in chlorophyll. Efficiency rates of photosynthesis also decrease and it has been noted that photosystem I (PSI) was more affected than photosystem II (PSII) (Nishio *et al*., 1985a) and that cytochrome b_6f (cytb_6f) was less affected than PSI (Spiller, S. and Terry, N., 1980). In *Chlamydomonas* PSI has been identified as the first target in the photosystems to be modified during Fe deficiency (Moseley *et al.,* 2002). The proteins that make up light harvesting complexes of PSII and PSI were reported to decrease in pear and spinach, possibly as a protective mechanism to prevent damage to the photosystems (Morales *et al.,* 1994; Timperio *et al.,* 2007).

Plants commonly grow in iron limiting conditions and therefore it is important to understand iron's utilization within plants. Iron is important an important component of fertilizers (Marschner, 2012). Plant iron nutrition directly and indirectly affects human nutrition because Fe availability affects crop productivity and the Fe content of edible plant parts. Vegetable matter is the source of Fe for a large section of the human population which rely on vegetarian diets. After gaining a better understanding of how plants utilize iron, this knowledge can be put to use benefiting both plant and human nutrition. Studies investigating the effects of iron depletion on plants have mainly focused on the regulation of Fe uptake and distribution. Much less attention has been devoted to the possible prioritization of Fe use among iron-dependent proteins. I am interested in determining if plants regulate Fe use by having a prioritization system for proteins to maintain during Fe depletion. In these experiments, I used a model plant *A. thaliana* ecotype Columbia grown hydroponically to control Fe supply. I examined the effects of a two week time course consisting of one week of Fe depletion followed by one week of iron resupply. Through the time course I analyzed physiological and molecular changes. Protein prioritization was analyzed from whole shoot samples taken at several time intervals during the iron depletion and recovery weeks. My hypothesis was that protein conservation would be linked to a type of iron containing protein or to a specific biological function.

Materials and methods

Materials – Murashige and Skoog (MS) salts were obtained from Caisson Labs (North Logan, UT). Agar (Phyto grade) was from Research Products international (Mt. Prospect, IL). Fe-EDTA (electrophoresis grade) for use in liquid plant growth media was from Fisher Scientific (Fair Lawn, NJ). Dithiothreitol, β-mercaptoethanol and nitrocellulose (0.2 μm pore size) were purchased from Bio-Rad (Hercules, CA). Goat anti-rabbit antibody conjugated to alkaline phosphatase was from Sigma-Aldrich (St. Louis, MO). Other analytical grade reagents for plant growth media, buffers, electrophoresis and protein blotting were obtained from Fisher Scientific.

Seed sterilization, Plant growth and Sampling – Arabidopsis thaliana ecotype Columbia (Col-0) was used. For surface sterilization, the seeds were treated as follows: 4 min in 70% ethanol, 4 min in 95% ethanol, 4 min in 70% ethanol. Seeds were air dried before being sown in square Petri dishes containing ½ strength MS media (Murashige and Skoog, 1962) with 1% (w/v) sucrose and 0.6% (w/v) agar. The plates were incubated at 4^{o}Cfor two days in the dark for stratification then placed vertically into a growth chamber at a temperature of 23^{o}C and a light intensity of 250 μmol photons m^{-2} s^{-1} and an 8-h light /16-h dark cycle; relative humidity was 74%. After one week, the seedlings were transplanted to be grown hydroponically within the same growth chamber. Individual seedlings were held in place by a foam plug in the top 2 cm of a cut 1.5 ml microcentrifuge tube that was inserted into a hole drilled into the lid of the container. Each container held thirty plants and contained 5 L of 1/5 strength modified Hoagland's solution (Hoagland and Arnon, 1950) containing 10 μM Fe, EDTA and 907.23 μM KH_2PO_4.The hydroponics solution was shielded from light and changed once every 7 days. Iron depletion was induced three weeks after seedlings were transplanted by lowering Fe-EDTA to 10 nM. Iron depletion lasted for seven days after which Fe-EDTA was restored to 10 μM for another week (recovery). Control plants were maintained at 10 μM Fe-EDTA for the entire period. Samples were taken right before the initiation of Fe depletion (T0) and again two (T2), four (T4) seven (T7, right before the start of recovery), nine (T9), eleven (T11) and fourteen (T14) days after the start

of Fe depletion (Figure 2).All plant tissue was collected at the beginning of the light period. Samples were flash frozen in liquid nitrogen and stored at -80^{o}C until used. Frozen tissue was finely ground in liquid nitrogen using a mortar and pestle, weight was determined while frozen.

Chlorophyll concentration - Chlorophyll was extracted from frozen tissue as indicated in (Porra *et al*., 1989). Chlorophyll a and b concentrations were analyzed by measuring absorbances at 662 nm and 645 nm in a Beckman DU 530 spectrophotometer.

Leaf area – Leaf area was measured with a conveyor belt system attached to the LI-3050C in RealTime Capture mode (Li-Cor, Inc., Lincoln, NE). Individual leaves of the entire rosette were placed on the belt that passed by the scanning head. Measurements were made using electronic rectangular approximation with 1mm^2 resolution.

Protein extraction, Immunoblots and Quantification - Proteins were extracted from entire frozen shoots. One part of ground tissue was mixed with in 1.5 volumes of protein extraction buffer (40 mM K_2HPO_4, 9.9 mM KH_2PO_4, ascorbate 2.5 mg/ml, Triton X-100 0.05% (v/v) and 7.15 mM β-mercaptoethanol). The mixtures were incubated on ice for 10 min with occasional vortexing and then centrifuged at 13,000 rpm for 15 min at 4^{o}C.The supernatant was collected and an aliquot was mixed with 1/3 volume of SDS sample buffer (250 mM Tris/HCl pH 6.8, glycerol 20% (v/v), SDS 4% (w/v), 80 mM dithiothreitol, bromophenol-blue 0.1% (w/v)and immediately heated for 5 min. The remainder of the supernatant was used to measure the protein concentration according to Bradford (1976) using bovine serum albumin as a standard. Proteins were separated by SDS-PAGE (Laemmli, 1970) before electro-blotting onto nitrocellulose. Proteins were immuno-detected as described (Ravet *et al.,* 2011). Antibodies specific for aconitase (ACO1), alternative oxidase 1 and 2 (AOX 1-2), L-ascorbate peroxidase (APX), catalase (CAT), copper response defect 1 (CRD1), cytochrome *b-559*α-subunit of PSII (PsbE), cytochrome b_6f complex proteins (Cytb_6, Rieske and Cyt*f*), cysteine desulfurase (CpNifS), cytosolic ascorbate peroxidase (cAPX), cytosolic fructose-1,6-bisphosphatase (cFBPase), glutamine oxoglutarate

aminotransferase (GOGAT), iron SOD (FeSOD1), light harvesting complex of PSI (LHCA2), nitrate reductase (NR), plastocyanin (PC), PSI core subunit proteins A, B and C (PsaA, PsaB and, PsaC), and rubisco large sub-unit (RbcL) were purchased from Agrisera, (Vannas, Sweden) and used in the recommended dilutions. Antiserum for adenosine 5'-phosphosulfate reductase(APR2) was a generous gift from Dr. S. Kopriva (J. Innes Center, Norwich, UK) and used at a 1:5000 dilution. Antibodies specific for the following proteins have been described before: CpNifS (1:5000) (Pilon-Smits *et al*., 2002), Cu/Zn SOD (CSD2)(1:1000) (Cohu *et al*., 2009), ferredoxin (Fdx) (1:1000) and sulfite reductase (SiR) (1:2000) (Yonekura-Sakakibara *et al*., 2000), ferritin (FER) (1:5000) (Ravet K. *et al*. 2009b), manganese SOD (MnSOD) (1:1000) (Kliebenstein *et al*., 1998), SufA (1:1000) (Abdel-Ghany *et al.,* 2005), SufE (1:5000) (Ye H *et al*., 2006). A goat anti-rabbit secondary antibody coupled to alkaline phosphatase was used for band detection in a solution containing 342.45 μM nitro blue tetrazoluim chloride, and 40.25 μM 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt.

The band intensities of immunoblots were quantified using ImageJ software (1.43u, publically available at: http://rsbweb.nih.gov/ij/. Dilution series of 150%, 100%, 75%, 50% and 25% were made from the T0 control samples and used to create calibration curves with R^2 values above 0.90. Data shown represents averages from at least three biological replicates. cFBPase was used as loading control and as reference protein for abundance ratios from control and treated groups.

Chlorophyll Fluorescence and Determination of P700 redox changes - Plants were collected in the morning, dark adapted and held at room temperature for 1-3 hours prior to testing. For chlorophyll fluorescence and P700 testing a single leaf from the fifth to the sixth node after the first true leaves was tested. Chlorophyll fluorescence was measured with a Hansatech Fluorescence Monitoring System (Cohu and Pilon, 2007). The following actinic light intensities were used 43, 207, 350 and 535 μmol m^{-2} s^{-1}. Whole rosette images were captured using a PSI

FluorCam 701 MF system with software version 7.1 (Photon System Instruments, Brno, Czech Republic). Fluorescence imaging was performed on plants after 7 days of iron depletion (T7) and again after seven days of iron re-supply (T14) at a light intensity of 150 μmol m^{-2} s^{-1}. Parameters F_V/F_M and Φ PSII were calculated according to Maxwell and Johnson (2000). Reduction states of P700 were analyzed at room temperature, 22ºC, using a Walz Dual PAM 100 running software version 1.18 (Heinz Walz, Effeltrich, Germany). Actinic light settings ranged from 17 – 1000 μmol m^{-2} s^{-1} with a 60 sec. wait between pulses. (Klughammer and Schreiber, 2008). All parameters for Y(I), Y(ND) and Y(NA) were calculated according to (Klughammer and Schreiber, 2008).

Gas Exchange Measurements – To estimate mitochondrial respiration levels measurements were taken in the dark four to six hours after the initiation of the plant's normal night at 20ºC. Analysis for each plant began ten minutes after the plant was sealed in an air tight chamber and the CO_2levels in the chamber stabilized. The CO_2 evolution rate (Rd), stomatal conductance (g_s) and intercellular CO_2 concentrations (ci) were obtained using a LI-6400 portable photosynthesis system (Li-Cor, Inc., Lincoln, NE) adapted with a custom built full rosette chamber (Christman *et al*., 2008). CO_2 was evacuated from the chamber prior to reintroduction of CO_2 into the chamber and maintained at ambient [CO_2], 400 μmol mol^{-1}. A green headlamp was used to prevent the initiation of photosynthesis during manipulations. Five plants from each treatment were tested and measurements were logged ten consecutive times per plant. All parameters for Rd, g_s and ci were calculated according to (licor.com). The values for each plant were averaged, from these averages the mean for the five plants was calculated along with the standard deviation.

Oxygen consumption was measured with a Hansatech LD2/3 O_2 electrode system maintained at 26ºC with light intensity of 370 μmol m^{-2} s^{-1} as described by (Van Hoewyk *et al*., 2007). Five leaves from the fifth to the sixth node up after the first true leaves were excised and places in the chamber for analysis.

Isolation of Chloroplasts - Chloroplasts were isolated as described by (Tapken *et al*., 2012) using fresh leaves from plants at 25 days after the start of hydroponics (T4). Final chloroplast yield was 6.84 μg plant $^{-1}$ for control plants and 6.18 μg plant $^{-1}$ for treated plants.

Elemental analysis - Fresh shoots were harvested and dried at 50^{o}C for one week. 20-50 mg of dried shoot material was digested in 1 mL of nitric acid for 2 hrs at 60^{o}C followed by 6 hrs at 130^{o}C. Digested material was diluted to 10 mL with double distilled water and analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES) as described (Ravet *et al.,* 2011).

Statistical analysis - JMP IN software (version 3.2.6; SAS Institute) was used for statistical analysis. Figures and data are representative of at least three biological replicates. A student's t test was performed to calculate significant differences ($P < 0.05$). The total sample size is given where appropriate.

Results

To investigate how iron deficiency affects iron-dependent proteins in shoots of *A. thaliana* (Col-0), seedlings were grown in hydroponics in controlled conditions optimized for vegetative growth. Three weeks after the seedlings were transplanted into hydroponics iron depletion was induced by lowering Fe-EDTA from 10 μM to 10 nM. Depletion lasted for seven days and then Fe was restored to 10 μM for seven more days (recovery). Control plants were maintained at 10 μM Fe-EDTA for the entire duration. Iron depletion levels were determined from preliminary experiments. The criteria for the depletion procedure was as follows: the plants had to endure seven days of iron depletion and become visibly Fe-chlorotic then after seven days of iron resupply the plants were to have visually recovered from the Fe-chlorosis and have no visible signs of permanent damage or bolting. Samples were taken right before the initiation of Fe depletion (T0) and again two (T2), four (T4) and seven days later (T7, right before the start of

recovery), then nine (T9), eleven (T11) and fourteen (T14) days after the start of Fe depletion through iron resupply (Figure 2).

Characterization of iron starvation - I analyzed the growth as well as chlorophyll and iron content of plants grown in iron depleted conditions for characteristic symptoms of iron deficiency. After only four days of iron depletion, plants showed visual signs of chlorosis in the developing leaves (Figure 3A). After seven days of iron depletion, only the oldest leaves appeared green in color whereas the rest of the rosette was chlorotic (Figure 3A). Typical of Fe depletion, affected plants had decreased shoot growth. The decrease in shoot size was most evident at T14, seven days after Fe was resupplied. The shoot weight and leaf area of the treated plants were significantly less than that of the control plants (Figure 3B and Figure 4 respectively). This reflects a developmental delay in shoot growth caused by iron depletion. Shoot size and biomass of the treated plants never caught up to that of the control plants over the duration of the experiment. It is well established in the literature that root length decreases when plants experience Fe deprivation (Abbott, 1968). Indeed the roots of the treated plants were significantly shorter than those of control plants. This decrease in root length was evident after only four days of iron depletion and remained so through one week of Fe resupply (Figure 3C). It should be noted that the root systems were largely developed at T0 and therefore the effect of low Fe treatment was small for the roots compared to the shoots (Figure 3).

Since the treated plants were chlorotic, I measured chlorophyll content. Treated plants had significantly less chlorophyll after four days of iron depletion than the control plants (Figure 3D). During the recovery phase, chlorophyll levels of the treated plants increased significantly from T11 to T14. After seven days of iron resupply chlorophyll levels in the treated plants had fully recovered, indicating that the plants were not damaged by the week of iron depletion.

Shoot mineral composition is altered by iron deficiency - Probing for trace metals using inductively coupled plasma optical emission spectroscopy (ICP-OES) I observed that iron depletion affects the accumulation of several different elements. As expected the treated plants

contained significantly less iron than untreated plants. This decrease was already significant after two days of iron depletion (Figure 5A). The decrease of shoot Fe at T2 indicated that the treatment effect was fast and that the reduced Fe content should be an important limitation for optimal shoot development. The iron content began to increase at T9, two days after iron resupply, and the levels from control and treated plants equaled out by T11. Then two days later, at T14, the treated plants contained significantly more iron than the control plants. This over-accumulation of Fe may be caused by the over expression of IRT1 induced by the experienced iron deficiency (Vert *et al*., 2002). Manganese content decreased significantly from T4 and remained low through T14 (Figure 5C). A fast and significant increase in K was seen at T7 and continued through T14 (Figure 5G). Sulfur was also significantly affected by iron deletion. The increase in S was evident at T7 where treated plants contained more S than control plants (Figure 5H). There was a significant decrease of Zn content at T2 that was not seen at any other time point (Figure 5B). Copper content appeared to be slightly affected by Fe depletion. There was as a trend of increasing Cu concentrations in treated plants that was evident from T7 - T14; however standard deviations were high and there were no statistically significant differences between the samples (Figure 5D). Calcium content was unaffected by iron deprivation (Figure 5E). Another element that was relatively unaffected by Fe depletion is Mg, only at T11 was there a difference between the samples when the treated plants contained significantly more Mg than the control plants (Figure 5F). When hydroponically grown *A. thaliana* were supplied all nutrients sufficient for growth expect for Fe the accumulation of other nutrients was affected.

Iron deficiency affects photosynthesis – In order to determine how iron depletion affects photosynthesis, I analyzed chlorophyll florescence and the reduction/oxidation states of PSI.

Iron cannot be remobilized during iron deficiency and therefore it is expected that the young leaves will be more affected than mature leaves. To analyze the spatial effects of iron deficiency on the photosynthetic performance in the rosettes, I used FluorCam imaging. Chlorophyll distribution can be seen visually. The untreated plants were uniformly green

throughout the rosette while treated plants were chlorotic in the center of the rosette, where the younger leaves are while the outermost and older leaves remained green (Figure 6A left).

To visualize how much of the light energy absorbed by chlorophyll molecules associated with PSII was used to drive electron transport, the parameter $\Phi_{PSII,}$ the Quantum yield of PSII, can be analyzed (Maxwell and Johnson, 2000). As indicated by the false color scale, the center of the treated plants had very low Φ_{PSII} values (Figure 6A right). The low Φ_{PSII} values could indicate a decrease in the efficiency of electrons flowing through the electron transport chain. Seven days after iron was resupplied, it appears that the treated plants had recovered because they had a more uniform distribution of Φ_{PSII} (Figure 6B left) and the values in control and treated plants were almost identical for all the leaves (Figure 6B right). This indicates that the photosystems of *A. thaliana* did not suffer permanent damage since plants were able to fully recover photosynthetic electron transport after one week of iron resupply.

For a more quantitative analysis of chlorophyll fluorescence, I used a Hansatech FMS system. One chlorophyll fluorescence parameter is $F_V/F_{M,}$ it provides a measurement of the maximum quantum yield for PSII in dark adapted plants (Maxwell and Johnson, 2000). F_V/F_M is also considered a measurement of the overall intactness and performance of PSII reaction centers. Optimal F_V/F_M values are near 0.83 with a decrease suggesting stress-induced photoinhibition (Bjorkman and Demming, 1987; Maxwell and Johnson, 2000). As soon as shoot iron concentrations in the treated plants decreased at T2 of Fe depletion the F_V/F_M also decreased. Plants that experienced iron deprivation had F_V/F_M values that were only slightly yet significantly lower than control plants (Figure 6C). The slight decrease in F_V/F_M may indicate that the majority of PSII centers are functional. This decline in F_V/F_M is evident after only two days of Fe depletion indicating a rapid response to lowered Fe levels. F_V/ F_M values remained low until seven days after Fe was resupplied. However at T14, F_V/F_M levels were the same for control and

treated plants, suggesting that the PSII reaction centers were fully recovered and that no secondary effects had been introduced into the system.

To obtain a measurement of how much of the light energy absorbed by chlorophyll molecules associated with PSII was used to drive electron transport I measured the parameter Φ_{PSII} at various light intensities. The results from a light intensity of 350 μmol m^{-2} s^{-1} are shown because this is slightly higher than what the plants were grown at. After four days without sufficient iron plants had a significant and large reduction in Φ_{PSII} when compared to control plants (Figure 6D). These plants showed full recovery of Φ_{PSII} at T14 corresponding to Figure 6A and B. This observation indicates that although electron transport rates were diminished by iron depletion the treated plants were not permanently damaged as was evident in the full recovery of Φ_{PSII} seven days after iron was resupplied.

In a previous study PSII had been reported to be less affected by iron deprivation than PSI in sugar beet (Nishio *et al*., 1985a; Terry and Abadia, 1986). To gain a better understanding of how PSI is affected I measured oxidation-reduction rates of PSI using a Walz Dual PAM system. I analyzed Y(I), which indicates the amount of reduced P700 that can be oxidized and is not limited by the acceptor side or any downstream processes; Y(ND), which indicates the amount of P700 that remains oxidized due to donor side limitations; and Y(NA), which measures limitations on the acceptor side of PSI (Klughammer and Schreiber, 2008). Data for T0 are not shown because the leaves at this time point were too small to fit in the leafclip. Multiple light intensities were tested but data shown were from 354 μmol photons m^{-2} s^{-1} because this is close to what the plants were accustomed to. At T2 the lack of iron caused a noticeable decrease in Y(I) and by T7 the Y(I) of treated plants had significantly decreased from the control plants. Once decreased, Y(I) remained low until after four days of Fe resupply when this parameter increased and came up to levels similar to the untreated plants (Figure 7A).As the Y(I) of treated plants decreased the Y(ND) increased. The Y(ND) of the treated plants was significantly higher than

the control plants at T7 and remained high until T14 when the levels between the control and treated plants equaled out (Figure 7B). Although the Y(NA) of treated plants was lower than that of the control plants, there was no significant difference between the two groups (Figure 7C). In the treated plants, the fraction of PSI that is not oxidizable was only slightly larger than in the control plants, Y(NA) was relatively unaffected. Because Y(I) of the treated plants was low I conclude that electron flow through PSI was hampered by the low Fe treatment. The correspondingly high Y(ND) and stable Y(NA) may indicate that the treated plants were not receiving electrons from cytb_6f via plastocyanin (PC).

Iron-dependent proteins decrease during iron deficiency – To analyze if specific iron-dependent proteins are affected by Fe depletion, I analyzed immunoblots of whole shoot samples collected throughout the iron depletion and resupply time course. I probed for iron containing proteins involved in various metabolic functions. Cytosolic fructose-1,6-bisphosphatase (cFBPase) is a non-Fe protein that was used as a control because its abundance was not affected by the iron treatment. The accumulation of cytochrome *b-559*α a PS II subunit, PsbE, a heme protein, was relatively unchanged. Of the three PSI complex core Fe-S cluster proteins PsaC abundance appears to have decreased the most followed by PsaB, the PsaA subunit appears to be unchanged. The cytochrome b_6f complex contains three subunits; cytb_6, cyt*f* and Rieske. At day 4 of iron depletion there was a noticeable decrease in the cyt*f,* a heme protein, and by day 7 it was obvious that the abundance of the Rieske, a Fe-S protein, and cytb_6, a heme protein, had also declined (Figure 8). All three of these iron containing proteins decreased with the Rieske subunit having the most robust decrease followed by cyt*f* and then cytb_6 (Figure 8). I found that proteins of the cytochrome b_6f complex were strongly reduced in abundance when compared to those in PSII and PSI (Figure 8). PC and Rubisco are two key proteins in photosynthesis the abundance of both of these remained unchanged.

Six proteins that function in protecting the plant against ROS were tested; L-ascorbate peroxidase (APX), catalase (CAT) and cytosolic ascorbate peroxidase (cAPX) are all heme

proteins, manganese superoxide dismutase (MnSOD) and Cu/Zn superoxide dismutase (CSD2) are both are non-Fe proteins, and ferritin (Fer) an Fe binding protein. Of these, only APX showed a reduction in abundance (Figure 8). The other ROS scavenging enzymes appeared unaffected, presumably their continued activity is required as a protective mechanism. Ferritin, an iron sequestering protein, became visible at T11 when iron content in the rosette was increasing (Figure 8). At T14 iron concentrations in the rosette were significantly higher than the control plants and the abundance of ferritin was even higher than at T11, this is in agreement with the observed accumulation of ferritin in plants with elevated Fe (Lobreaux *et al.,* 1992). This increase is most likely correlated with the proposed function of ferritin, acting as a regulator of iron homeostasis specifically in preventing Fe toxicity and Fe induced oxidative stress (Ravet *et al*., 2009a).

Three chloroplastic Fe-S assembly system proteins were tested; SufA a Fe-S cluster protein, SufE is a S protein and CpNifS is a P protein. Only the abundance of SufA, a potential iron sulfur cluster transfer protein, was affected, its abundance was strongly reduced from T4 to T11 (Figure 8). SufA may function as a Fe-S cluster assembly and transport protein (Ollagnier-de Choudens *et al.,* 2003; Abdel-Ghany*et al.,* 2005). The lower SufA accumulation could indicate that when plants experience iron depletion the biogenesis of Fe-S clusters decreases causing a decrease in demand for Fe-S cluster transport.

The abundance of two Fe proteins that function in nitrogen assimilation, ferredoxin-dependent glutamine oxoglutarate aminotransferase (GOGAT) a Fe-S cluster protein and nitrate reductase (NR) a heme protein, and one non-Fe protein for carbon metabolism, cFBPase, were tested. None of these appeared to change during iron depletion which also justified to me the use of cFBPase as a loading control (Figure 8). When plants experience iron depletion continued plant growth is severely decreased, but it seems that these enzymes may be important for basic maintenance.

Sulfur metabolism appeared to be affected by iron depletion. The abundance of two proteins involved in sulfur metabolism, adenosine 5'-phosphosulfate reductase (APR2) a Fe-S cluster protein and sulfite reductase (SiR) a heme and Fe-S cluster protein, were tested. Both APR2 and SiR decreased in abundance on low Fe. APR2 is involved in sulfate reduction (Takahashi *et al*., 1997; Smith *et al*., 2000). It should be noted that APR2 decreases with developmental age seen at T9 in the control plants but in treated plants the decrease is seen at T2 (Figure 8). SiR functions in sulfite reduction (Takahashi *et al*., 1996; Khan *et al*., 2010). A decrease in SiR abundance was evident at T4 and it appears to have fully recovered by T14 (Figure 8). During iron depletion the demand for sulfur may decrease causing a decrease in the need for sulfur metabolism proteins.

The abundance of two mitochondrial Fe-S cluster proteins were tested; alternative oxidase 1 and 2 (AOX1-2) and aconitase (ACO1). AOX1-2 was unchanged while ACO1 decreased (Figure 8). ACO1 is involved in the tricarboxylic acid (TCA) cycle catalyzing the isomerization of citrate to isocitrate. The abundance appears to be only slightly decreased indicating that mitochondrial functions may be decreased but are still largely functional.

Through the use of immunoblots performed throughout the plants exposure to iron deprivation, I have attempted to characterize the abundance of iron-dependent proteins in plants growing in limited Fe. These time course immunoblots indicate that the first proteins to be diminished by iron deprivation are APX and APR2. These proteins have different biological functions and harbor different types of iron cofactors. It appears that *A. thaliana* does not readily cutback on one biological function or type of iron binding protein when iron is scarce. It is only after prolonged iron depletion, seven days, that it becomes evident that photosynthesis and sulfur metabolism are reduced.

Many of the proteins tested did not appear to change in abundance. Most notably proteins involved in carbon and nitrogen metabolism were unaffected as well as most of the proteins that protect against ROS indicating that there may be a priority given to preserve proteins involved in

carbon and nitrogen metabolism as well as ROS protection. But these carbon and nitrogen metabolism and ROS protecting proteins are composed of different types of iron-dependent proteins indicating that it is unlikely that a specific type of iron binding protein is maintained.

I did a quantitative analysis of immunoblots probing for several strongly affected proteins from shoots collected after seven days of iron depletion. This analysis demonstrated that all the cytb_6f complex proteins, cytb_6, Rieske and cyt$f$, had decreased significantly due to iron deprivation as well as one of the PSI proteins, PsaB (Figure 9 and 10). The Rieske protein decreased the most, 79%, followed by cyt$f$ and then cytb_6 which decreased 64% and 25% respectively. While the abundance of PsaB decreased by 62% the abundance of the other two PSI subunits PsaA and PsaC changed but not significantly. In partial agreement with the FMS data, the PSII PsbE subunit showed no significant change during seven days of iron depletion. This analysis clearly demonstrates that the cytb_6f complex proteins are among the first targets of iron depletion followed by one of PSI proteins, PsaB (Figure 9 and 10).

Not only did Fe depletion affect proteins involved in photosynthesis but it also affected the abundance of proteins involved in other metabolic functions. APX, an ROS protecting protein, decreased by 70% followed closely by both of the sulfur metabolism proteins APR2 and SiR (65% and 75% respectively). SufA, the Fe-S cluster assembly protein decreased by 46% and the mitochondrial ACO1 protein decreased by 59%. Copper response defect 1, CRD1, a protein involved in chlorophyll synthesis and GOGAT were decreased by 39% and 26% respectively (Figure 9 and 10). Proteins involved in sulfur metabolism decreased in abundance along with proteins involved in other metabolic processes, this may function to slow down the plant's metabolism rate when iron is limiting.

To investigate how chloroplastic proteins were affected by iron deprivation the proteins were extracted from the chloroplasts of plants after four days of iron treatment, T4. At the T7 time point the chloroplast yield was too low to work with. These proteins were separated by gel electrophoresis and stained with Coomassie Brilliant Blue (Figure 11). There were no major

differences in protein bands between the control and treated chloroplasts. This observation indicates that the majority of the chloroplast proteins in the rosettes were not affected by iron depletion. Therefore, I conclude that the observed changes in the iron proteome were specific.

Many other proteins tested showed no significant changes after seven days of iron depletion these include; PC a copper containing protein involved in transferring electrons from cytb_6f to PSI, MnSOD, the mitochondrial, AOX 1-2 and the nucleotide binding protein 35(NBP35) that mediates Fe-S cluster assembly (Figure 9 and 10). This observation is in agreement with the lack of observable changes in (Figure 11).

Mitochondrial respiration appeared unaffected by iron depletion -To see if iron depletion affects mitochondrial respiration I tested dark respiration rates on plants 28 days after the start of hydroponics after being exposed to seven days of iron depletion (T7) and again 35 days later seven days of iron resupply (T14). Surprisingly, there was no significant difference between the intracellular CO_2concentration, Ci; stomatal conductance, g_s; or respiration rates, Rd, between control and treated plants nor was there any difference in O_2 consumption (Table1). Respiration and stomatal conductance increased slightly but not significantly in treated plants at T7. I conclude that mitochondrial respiration was not significantly affected by iron depletion.

Discussion

How plants adapt to iron depletion has been investigated for many years providing a wealth of knowledge about how iron is taken up into the roots, transported through the plant to its designated sink. What is unknown is whether or not plants have an iron economy system similar to what has been proposed for copper (Ravet *et al*., 2011).

I followed the abundance of iron-dependent proteins through one week of iron deprivation and then through one week of iron recovery. I also tracked how this treatment affected biomass, photosynthesis, mitochondrial functions and other biological functions. Through the use of immunoblots I observed the decrease of several photosynthesis related proteins well as proteins

involved in various other biological functions such as carbon, nitrogen and sulfur metabolism after the iron depletion treatment. I noticed photosynthesis declined during the treatment and that mitochondrial respiration was relatively unaffected. Shoot weight and root length and chlorophyll content were negatively affected by iron depletion.

After only four days several observations were made of the iron depleted plants. I observed a significant decrease of shoot iron content as well as chlorophyll content. The roots of the treated plants were significantly shorter that those of the control plants. From measuring chlorophyll fluorescence, there was a slight yet significant decrease in the photosynthesis parameter F_V/ F_M and a more pronounced decrease in Φ_{PSII} as well. Confirming that PSII was relatively unaffected by Fe depletion as found by (Nishio *et al*., 1985a).From measuring oxidation-reduction rates of P700 I found a trend of decreasing Y(I) corresponding to increasing Y(ND), while Y(NA) for the treated plants was lower than that of the control plants it remained relatively steady and unaffected. After analyzing the effects iron deprivation had on PSI, I noticed that PSI was somewhat functional and what appeared to be a defect upstream of PSI. This is not in agreement with (Terry, 1980; Nishio and Terry, 1983; Nishio *et al.,* 1985a; Moseley *et al*., 2002), as it suggests that there is something upstream of PSI that is more vulnerable to Fe depletion than PSI itself. I decided to investigate immunoblots by probing for photosynthesis related proteins. Indeed after four days of treatment there was a noticeable decrease in the abundance of Rieske and cyt*f* subunits of the cytochrome b_6f complex.

Products of photosynthesis give plants energy to grow. Because electron transport rates were decreased by the iron treatment it was to be expected that the shoot weight of the treated plants decreased when compared to the control plants. In essence, the treated plants put very little energy into making new leaves while the control plants continued to increase vegetative growth. It has long been understood that lack of iron nutrition has a negative effect on photosynthesis in various plant species (Marschner, 2012). It was reported that PSI was the main target of iron deficiency in the chloroplast followed bycytb_6f and that PSII is not very affected

(Terry, 1980; Nishio and Terry, 1983; Perez *et al*., 1995; Jordan *et al*., 2001; Jeong, 2008). Indeed, PSI is the largest iron sink followed by the cytb_6f complex in the electron transport chain as described by (Raven *et al.,* 1999). As expected, I observed a decreased abundance of PSI subunits, especially PsaB. The assembly process of PSI in *Chlamydomonas* is controlled by epistasy of synthesis (CES) and requires the insertion of PsaB before PsaA is translated (Wostrikoff *et al*., 2004).However, from my observations of immunoblots the subunits of cytb_6f showed a very large decrease caused by iron depletion. The lack of cyt$b_6f$function is also supported by the decrease in electrons flowing through the electron transport chain four days after initiation of iron depletion. In testing the parameter Y(ND) it showed a strong defect upstream of PSI. Whereas both PSI and cytb_6f are dramatically affected by iron deficiency the electron carrier between these two complexes plastocyanin, PC, a copper protein was not affected, indicating that it is not co-regulated.

Because the flow of electrons in photosynthesis had decreased, I decided to look into mitochondrial respiration. In testing several parameters; CO_2evolution, Ci, $g_{s,}$ Rd, and O_2consumption I did not find any significant differences between control and treated plants. Only two proteins in the mitochondria were tested for by immunoblots. One decreased after seven days of treatment, ACO1, and the other was unaffected, AOX1-2. It was observed that in sugar beets exposed to iron deprivation mitochondrial functions increase and carbon is relocated from the roots to the shoots (Lopez-Millan *et al*., 2000a; Lopez-Millian *et al.,* 2000b). This does not appear to be the case with Arabidopsis and is in agreement with Terry (1980), and Pascal and Douce (1993).

Other proteins decreased in abundance after four days of treatment. The accumulation of SufA appears to be severely affected by iron deprivation while two core Fe-S assembly proteins, SufE and CpNifS, do not appear to be affected. If it is SufA that donates Fe-S clusters to build Fe-S proteins than it seems that it is not required to maintain all Fe-S proteins as several Fe-S enzymes were not affected in my treatments. Perhaps there is regulation occurring at SufA

to decrease the assembly of certain new Fe-S clusters. The turnover rates for Fe-S clusters were not analyzed and perhaps seven days with limited iron is not long enough to see a true priority of these proteins.

For all of the proteins that changed in abundance during the iron treatment neither protein stability nor mRNA levels for gene abundance was looked at. These two techniques would give better insight as to how the proteins are being regulated and it would give a clearer picture of what metabolic functions are being regulated.

Is there a priority of iron-dependent proteins in plants during deprivation? While the abundance of many proteins did not change during the treatment it appears that there are some priorities. Some proteins were lost, including many of the proteins involved in photosynthetic electron transport as well as some involved in S metabolism.

Chapter III

Summarizing discussion

Evaluation of experimental approach

It has been proposed that plants have a copper economy system; the abundance of specific proteins decreased allowing essential proteins to use the available copper (Burkhead *et al,* 2009). Direct evidence for the copper economy model was obtained from studies of poplar (Ravet e*t al*., 2011). To test the hypothesis that plants use an iron economy system during iron (Fe) deprivation, I used *Arabidopsis thaliana* plants, which were grown hydroponically and exposed to Fe depletion. I limited my analysis to the green vegetative tissues, the rosettes because they contain more iron utilizing proteins than the roots (for a review on iron dependent proteins in roots see Li *et al*., 2008; Donnini *et al*., 2010 and Lan *et al*,. 2011). When the plants were four weeks old they were exposed to Fe deprivation for one week followed by one week of Fe recovery. The plants were sampled and tested throughout the Fe starvation process and recovery to analyze physiological and molecular changes. The treatment did not seem to produce secondary effects because all the parameters tested that were changed by Fe deficiency recovered within seven days of Fe resupply.

Summary of the most important findings

When plants are exposed to iron deprivation they lose chlorophyll. This loss of chlorophyll decreases photosynthesis rates but, there is more going on inside the plant. The lack of iron causes proteins involved in photosynthesis to decrease. When I tested the abundance of proteins involved in photosynthesis I observed that the first proteins to decrease are in the cytochrome b_6f complex (cytb_6f). This was supported by testing the redox rates of photosystem (PSI), there is

something upstream of PSI preventing the flow of electrons. This is important to understand because the bioavailability of iron and how plants use it affects the food that we grow and rely on.

Interpretation of my findings

Based on my observations, I propose that plants do have a prioritization system of proteins to discard and retain during iron starvation. Iron starvation affects several biological functions, most noticeably photosynthesis and S metabolism. In less than one week, even a few days, the treated plants showed specific symptoms. Symptoms of deficiency include decreased chlorophyll content, lower photosynthesis rates, dramatically reduced shoot weight, slightly shorter root length, and the reduced accumulation of certain Fe proteins that have diverse biological functions. With the exception of the photosynthesis components, PSI and cytb_6f, and iron-sulfur (Fe-S) cluster proteins involved in S metabolism it was common that within one biological function only one or two proteins would decrease during Fe depletion.

Within the chloroplast, I observed that PSI is largely functional but that there is something upstream inhibiting electron flow during Fe deprivation. To narrow down the cause of decreased electron transport rates, immunoblots were analyzed probing for photosynthesis related proteins. It was evident after four days of treatment that a decrease in the abundance of the Rieske and cyt*f* subunits of the cytochrome b_6f complex had occurred. After seven days of treatment the abundance of cytb_6, the other cytb_6f subunit, and PsaB, a PSI subunit, had decreased dramatically while the abundance of PsbE, a PSII subunit, did not change. Thus, from photosynthetic data and immunoblots it appears that the cytochrome b_6f is more affected by iron depletion followed by PSI whereas photosystem II (PSII) is much less affected.

With photosynthesis rates decreased due to a lack of iron, I investigated mitochondrial functions to find out if they were affected in the same way. From testing the plants for changes in respiration rates after seven days of iron depletion I found no statistically significant changes in mitochondrial respiration. In order to further investigate mitochondrial functions, immunoblots for mitochondrial proteins were analyzed which showed that the abundance of aconitase (ACO1)

decreased while alternative oxidase 1 and 2 (AOX1-2) did not appear to change. To fully understand how the mitochondria are affected by Fe depletion more analysis are required. It would be especially useful to analyze Fe-dependent mitochondrial respiratory chain components.

During Fe deprivation plants do seem to have priorities for iron-dependent proteins. Throughout the one week of iron depletion the abundance of many proteins remained the same while others decreased. Those that appeared to be unaffected, function in carbon and nitrogen metabolism. The abundance of four proteins that function in the protection against reactive oxygen species was also analyzed; I found that only one decreased by Fe deprivation. This observation may indicate that these could be necessary for anti-oxidant functions in the plant and are therefore maintained. Some proteins were lost, including many of the proteins involved in photosynthetic electron transport as well as those involved in S metabolism, indicating that these functions may not be essential to the plant's survival under a short term Fe starvation regime.

Future experiments and outlook

How is the response to iron deficiency in the chloroplast regulated? My data give support for an iron economy model but at this point I cannot distinguish between the effects of iron depletion on protein stability vs. an effect on mRNA translation. It would be very useful to measure the abundance of specific mRNA levels for the genes that show strong effects at the protein level. This could be achieved via qRT-PCR. A potential problem that will have to be taken into account is that mRNA from chloroplastic genes do not have a poly-A tail which is often used in the selection of mRNA for reverse transcription. Protein stability is more challenging to analyze but Tapken *et al*., 2012 used cycloheximide to block 80S ribosomal protein synthesis. However this experiment was done using a liquid culture system which is difficult to combine with my hydroponics. Therefore, I propose that the best approach to address the mechanism of regulation is by transcript analysis. If we would find a specific effect of iron depletion on certain transcripts encoding iron proteins then the implication would be that there are iron sensors.

I noted that there were effects on the abundance of subunits of the electron transport chain but we do not know yet if these changes affect the assembly of complexes in the thylakoids. Jeong et al., 2008 used blue native page to analyze super complex assembly in plants that lacked the FRO7 protein which functions in chloroplast iron uptake. I propose to use a similar approach that would require chloroplast isolations over the time course of the treatment.

I observed effects of iron depletion on the elemental composition of the shoots. Specifically, sulfur was increased and there was a trend for copper to do the same suggesting that there is a link between iron depletion and sulfur and copper uptake. The high S content might be explained by up-regulation of sulfate uptake in the absence of the sulfur assimilation enzyme APR, which was found to be strongly reduced by the iron depletion. Lack of APR would result in a defect in accumulation of reduced sulfur compounds, cysteine and glutathione which are suspected regulators of sulfur assimilation. In the future it would be interesting to see if the regulation of S and Cu assimilation is directly linked to the regulation of the abundance of specific iron proteins found to be down regulated in this thesis.

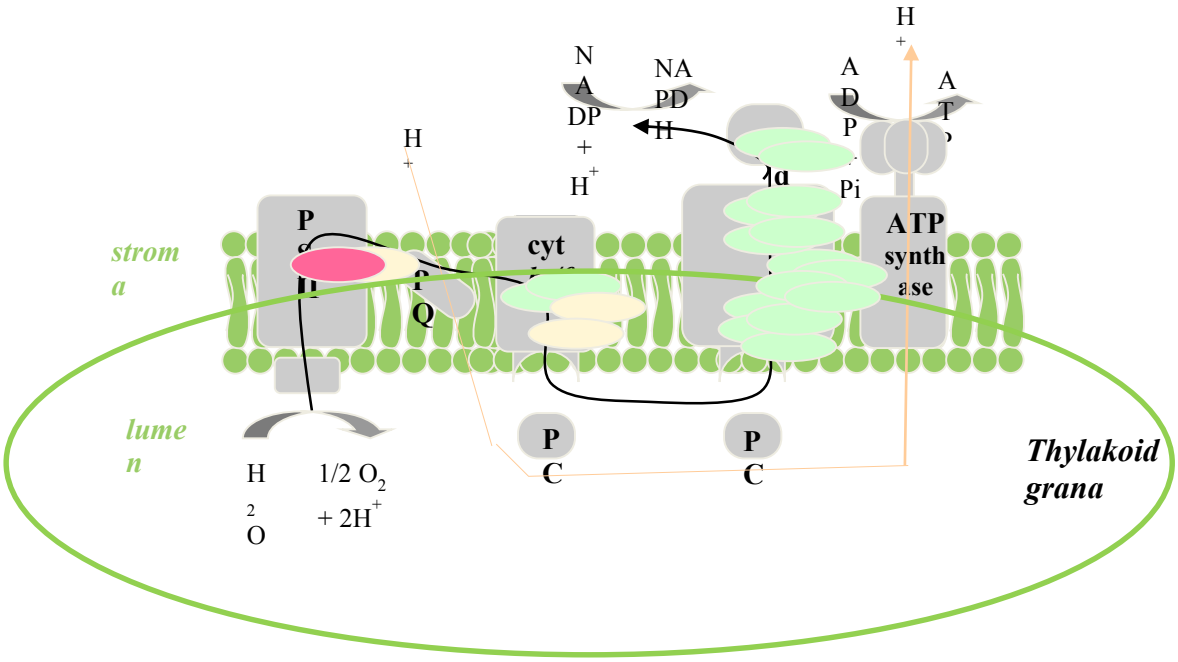
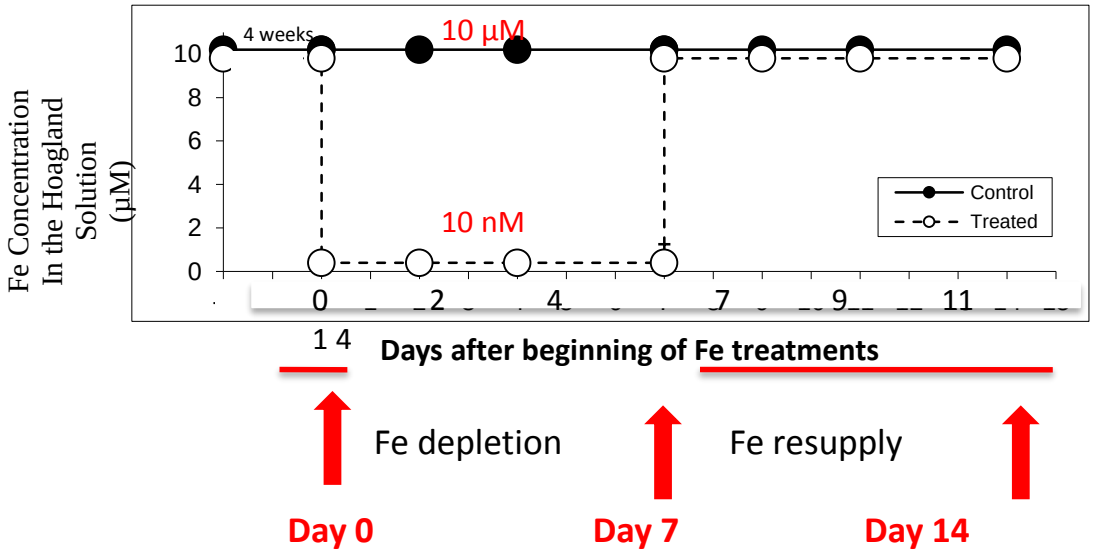


Figure 1. Electron transport chain. Schematic diagram of Fe containing proteins in the thylakoids showing the number of iron atoms in each. Figure drawn by Dr. Karl Ravet and used with permission.



Sampling and phenotypic analyses were carried out on days 0, 2, 4, 7, 9, 11 and 14

Figure 2. Experimental design.

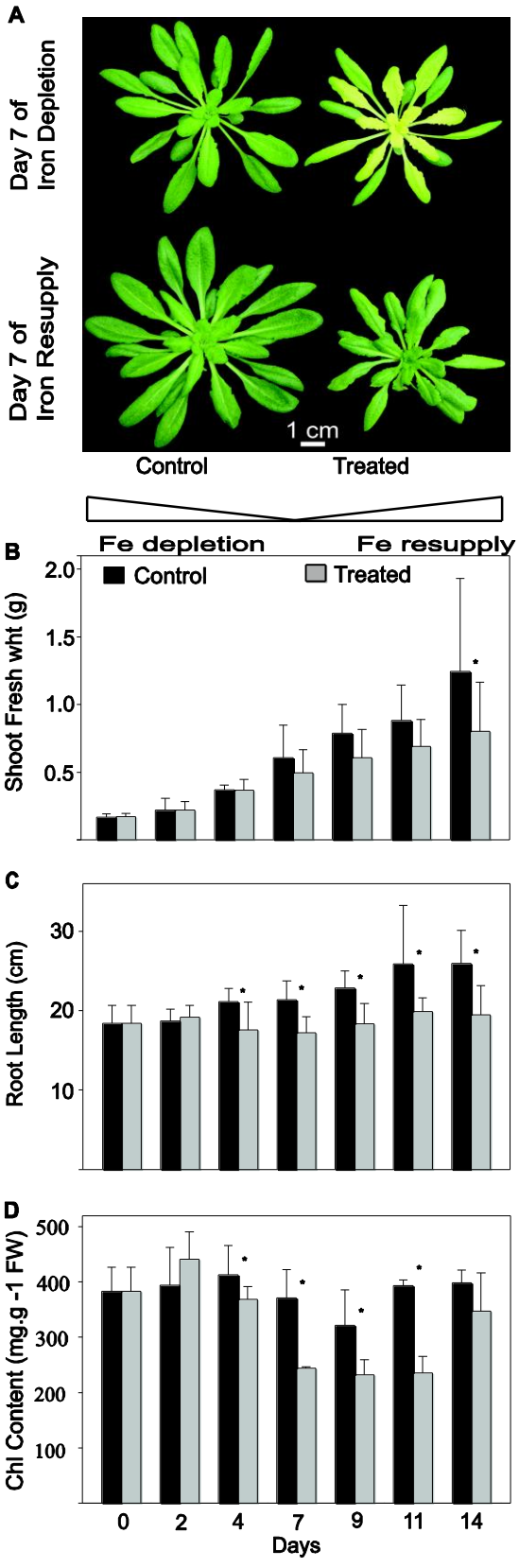

A
Day 7 of Iron Depletion
Day 7 of Iron Resupply
1 cm
Control
Treated
Fe depletion
Fe resupply
B
Shoot Fresh wht (g)
2.0
1.5
1.0
0.5
Control
Treated
C
Root Length (cm)
30
20
10
D
Chl Content (mg.g -1 FW)
500
400
300
200
100
0
2
4
7
9
11
14
Days

Figure 3. Symptoms of iron depletion. *Arabidopsis thaliana* were grown in hydroponics with sufficient iron, 10 μM Fe-EDTA. At four weeks test plants were exposed to seven days of iron depletion, 10 nM Fe-EDTA. Panel A. Representative images of rosettes from control, right, and Fe depleted plants, left. Top image is of plants at T7. Bottom image is of plants at T14.
For the time course sampling the first samples were taken before initiation of Fe depletion (T0) and again two (T2), four (T4) and seven (T7) days later. After sampling on T7 Fe was resupplied, and samples were taken two days (T9), four days (T11) and seven days (T14) later. Black bars represent control plants and grey represents treated plants. Significant differences between control and treated plants was determined by Student's t-test *. $P< 0.05$. Results are shown as means $\pm$ standard deviation of min. of 4 plants. Panel B. Shoot fresh weight. Fresh shoots were harvested on days indicated and weighed immediately. Weight is given in grams. Panel C. Root length of plants harvested on indicated days. Root length is given in centimeters. Panel D. Chlorophyll a and b concentration. Chlorophyll content was determined for ground frozen shoots sampled on indicated days and expressed as milligrams of chlorophyll per gram of fresh weight.

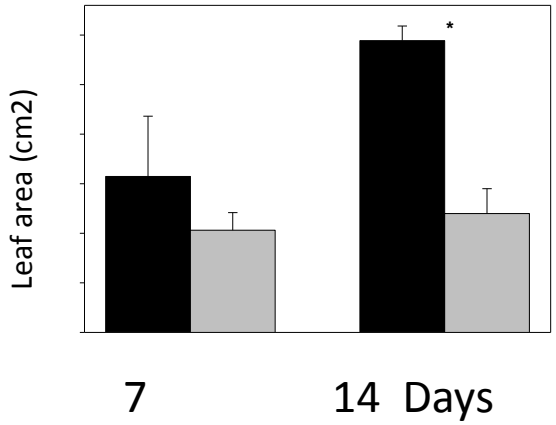


Figure 4. Effects of iron depletion on leaf area. *A. thaliana* were grown in hydroponics with sufficient iron, 10 μM Fe-EDTA. At four weeks test plants were exposed to seven days of iron depletion, 10 nM Fe-EDTA. Control plants were maintained at 10 μM Fe-EDTA. Leaf area of whole rosettes was calculated after seven days of Fe depletion (T7) and again seven days after Fe resupply. Black bars represent control plants and grey bars represent treated plants. Significant differences between control and treated plants was determined by Student's t-test *. $P<0.05$. Results as means $\pm$ standard deviation of min. of 5 biological replicates.

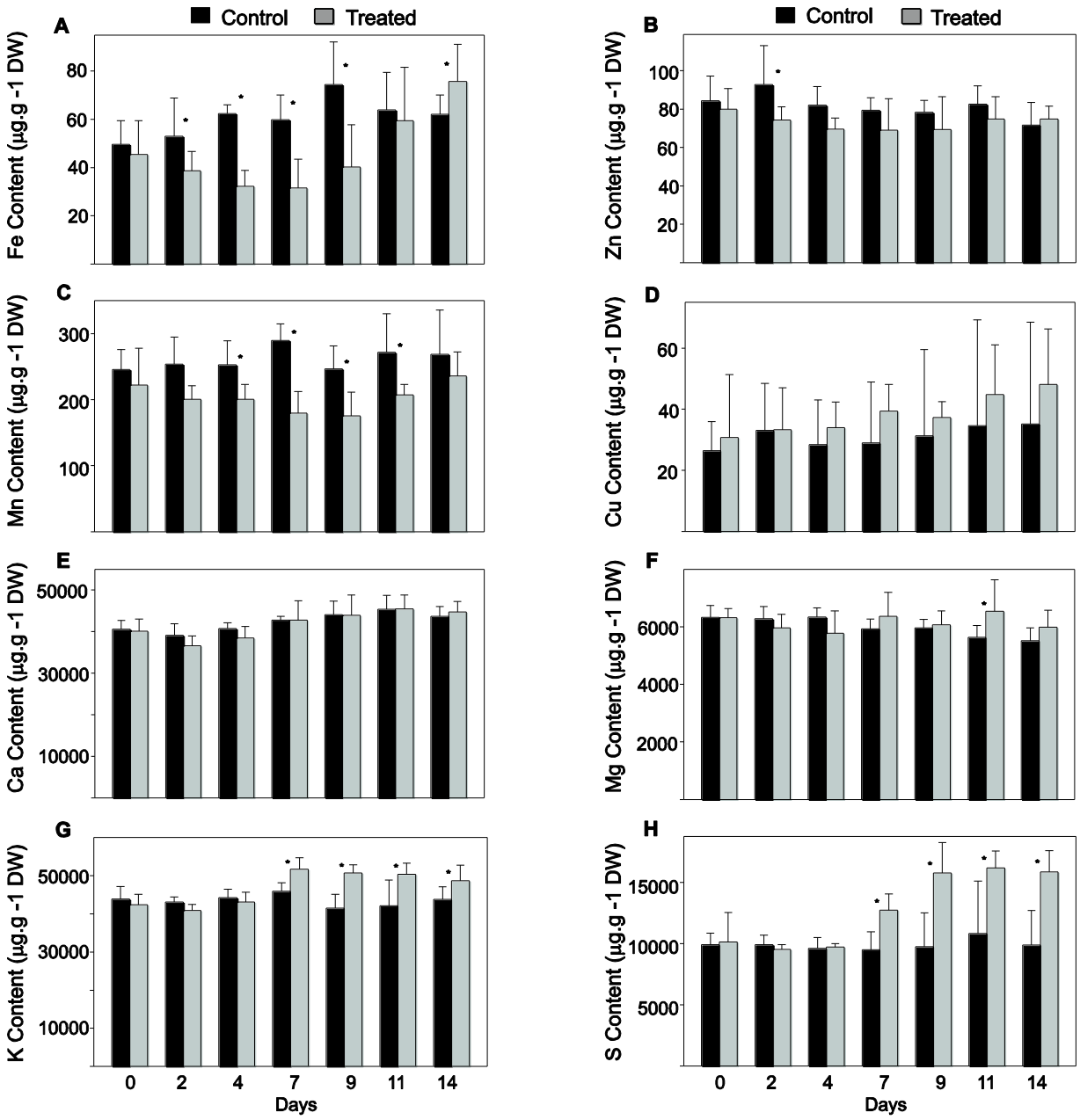


Figure 5. Content of minerals in shoots.

Arabidopsis thaliana were grown in hydroponics with sufficient iron, 10 µM Fe-EDTA. At four weeks test plants were exposed to seven days of iron depletion, 10 nM Fe-EDTA. The first samples were taken before initiation of Fe depletion (T0) and again two (T2), four (T4) and seven (T7) days later. After sampling on T7 Fe was resupplied, and samples were taken two days (T9), four days (T11) and seven days (T14) later. Fresh shoots were harvested on indicated days and dried. Concentrations were determined using ICP-OES. Values shown are micrograms per gram of dry shoot material. Black bars represent control plants while grey represents treated plants. Significant differences between control and treated plants was determined by Student's t-test *. $P<0.05$. Results are shown as means $\pm$ standard deviation of min. of 5 plants.

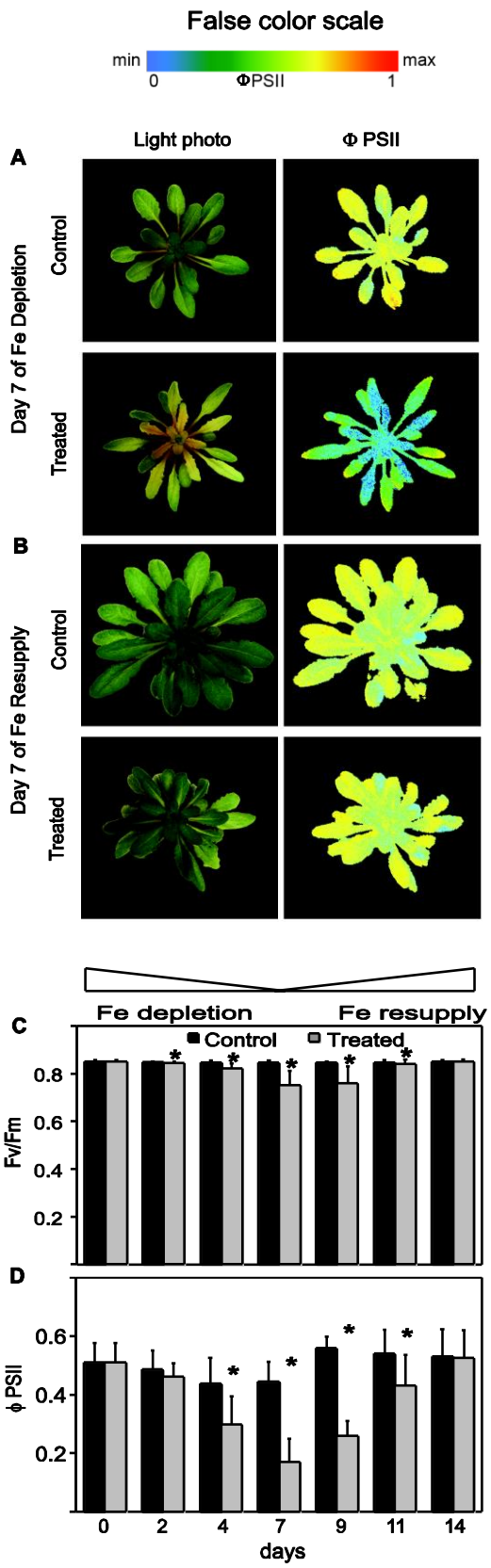

False color scale
min
max
0
ΦPSII
1
Light photo
Φ PSII
A
Day 7 of Fe Depletion
Control
Treated
B
Day 7 of Fe Resupply
Control
Treated
Fe depletion
Fe resupply
C
Control
Treated
Fv/Fm
0.8
0.6
0.4
0.2
D
ϕ PSII
0.6
0.4
0.2
0
2
4
7
9
11
14
days

Figure 6. Photosynthetic parameters. *Arabidopsis thaliana* were grown in hydroponics with sufficient iron, 10 μM Fe-EDTA. At four weeks test plants were exposed to seven days of iron depletion, 10 nM Fe-EDTA. The first samples were taken before initiation of Fe depletion (T0) and again two (T2), four (T4) and seven (T7) days later. After sampling on T7 Fe was resupplied, and samples were taken two days (T9), four days (T11) and seven days (T14) later. Black bars represent control plants and grey represents treated plants. False image coloring represents a value scale for Φ PSII as indicated. Significant differences between control and treated plants was determined by Student's t-test *. $P<0.05$. Results shown are means ± standard deviation of min of 8 plants. Panel A and B. FluorCam images of whole rosettes allow for spatial analysis of the effect of iron depletion. Shown on the left visual chlorosis and on the right Φ PSII, chlorophyll fluorescence at an actinic light intensity of 120 μmol m^{-2} s^{-1}. Panel A, representative images of plants at T7. Panel B, representative images of plants at T14. Panel C and D. Quantitative analysis of fluorescence parameters with an FMS system. Panel C. Fv/FM from dark adapted plants indicates photosynthetic performance. Panel D. Φ PSII for an actinic light intensity of 350 μmol m^{-2} s^{-1}.

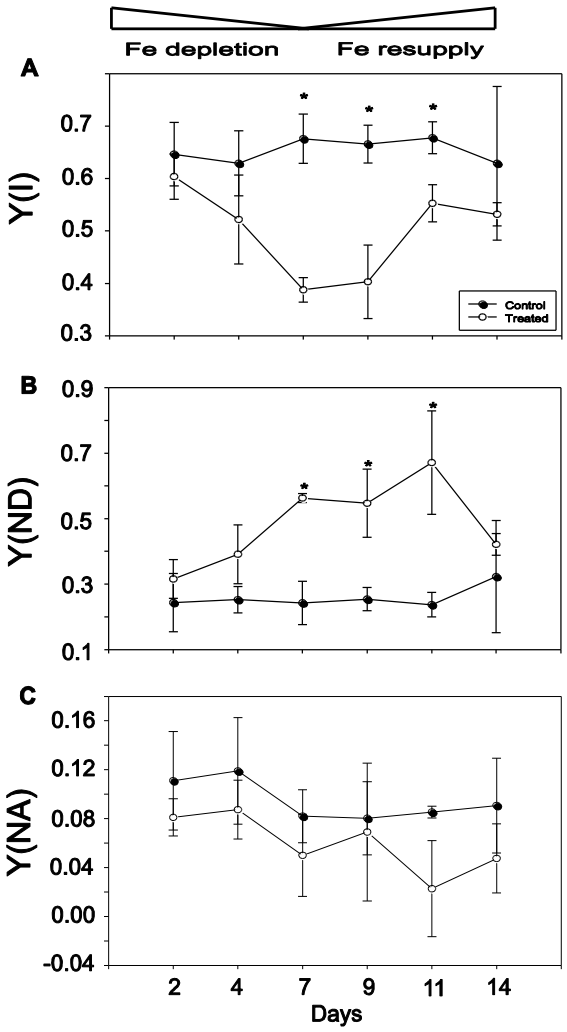


Figure 7. Oxidation-reduction levels of PSI. *Arabidopsis thaliana* were grown in hydroponics with sufficient iron, 10 μM Fe-EDTA. At four weeks test plants were exposed to seven days of iron depletion, 10 nM Fe-EDTA. The plants were tested at 354 μmol $m^{-2}s^{-1}$two days after the initiation of iron depletion (T2) as well as four (T4) and seven (T7) days later. After sampling on T7 Fe was resupplied, and plants were tested two days (T9), four days (T11) and seven days (T14) later. Black circles represent control plants and open circles represent treated plants. Significant differences between control and treated plants was determined by Student's t-test *. $P < 0.05$. Results shown are means ± standard deviation of min. of 3 plants. Panel A. Y(I) indicates the quantum yield of PSI represented as the fraction of P700 that is reduced and cannot be oxidized. Panel B. Y(ND) indicates the fraction of P700 that remains oxidized. Panel C. Y(NA) indicates the fraction of reduced P700 that is not oxidizable.

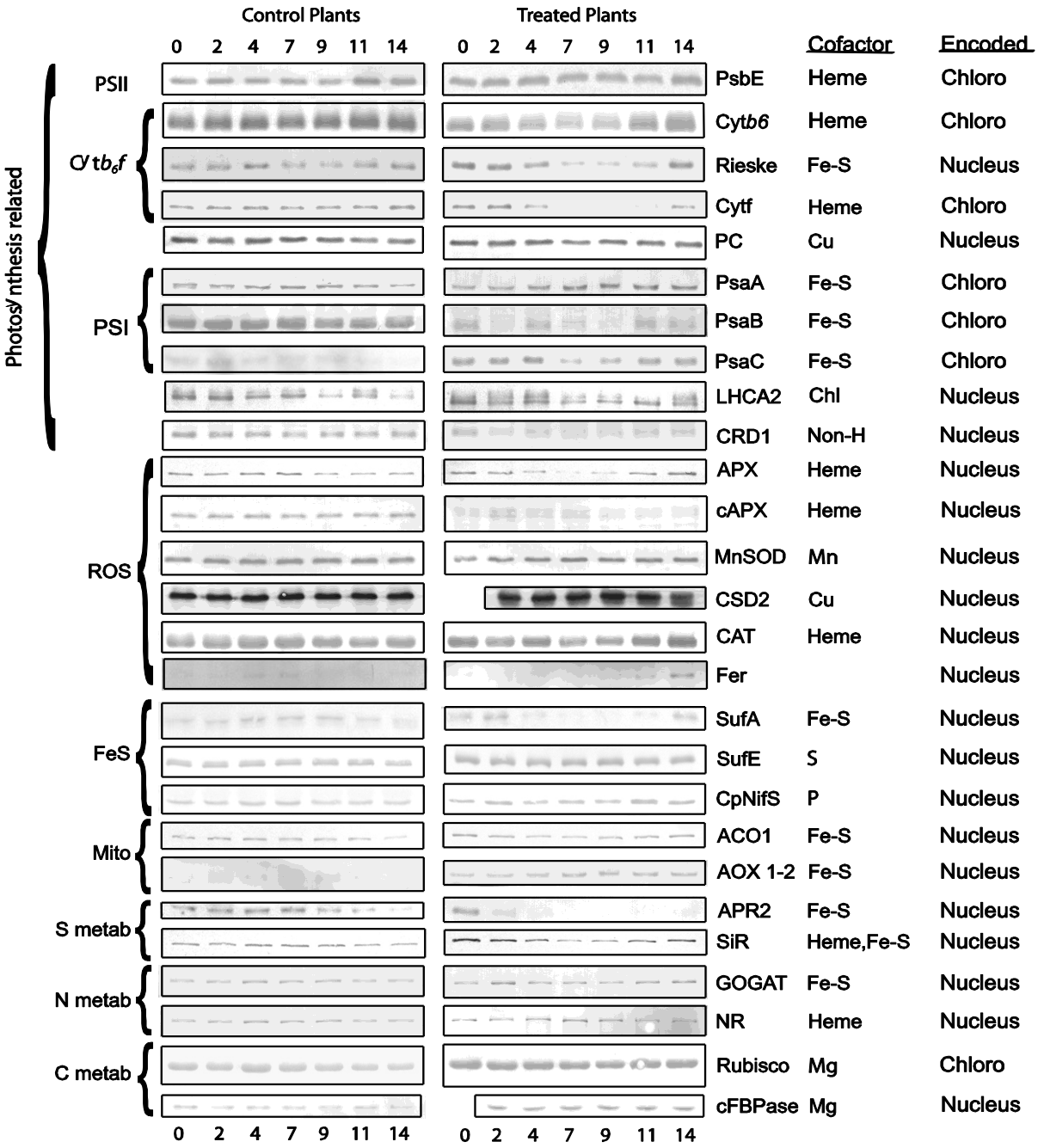


Figure 8. Effects of iron deficiency on the abundance levels of iron-dependent proteins. Immunoblots tracking protein abundance in the shoots through iron depletion and recovery. *A. thaliana* were grown in hydroponics with sufficient iron, 10 μM Fe-EDTA. At four weeks test plants were exposed to seven days of iron depletion, 10 nM Fe-EDTA. The first plants sampled before initiation of Fe depletion (T0) and again two (T2), four (T4) and seven (T7) days later. After sampling on T7 Fe was resupplied, and samples were taken two days (T9), four days (T11) and seven days (T14) later. Images shown are representative of min. of two biological replicates. The amount of protein loaded was 20 μg for all proteins except PsbE and Cyt*f* where 30 and 10 μg were loaded respectively. Proteins were separated on 15% polyacrylamide gels.

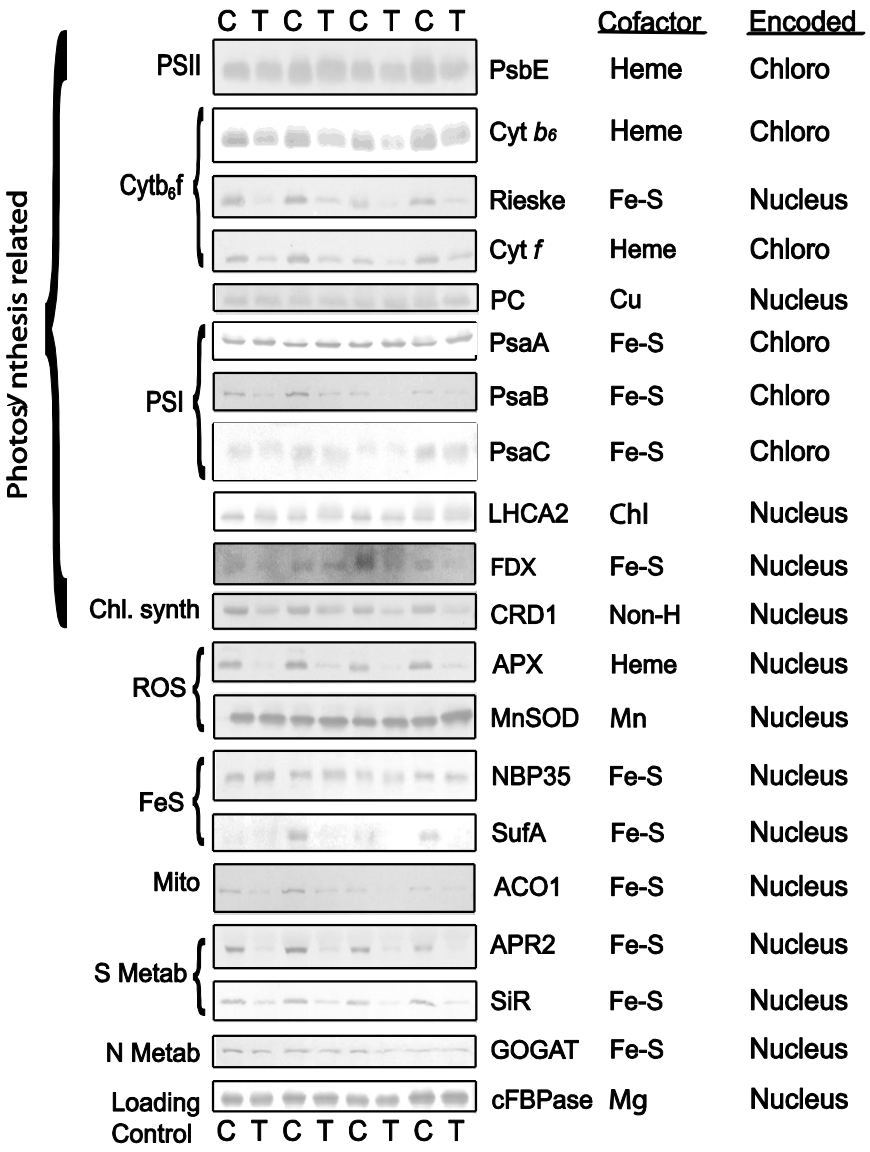


Figure 9. Effects of iron deficiency on the abundance levels of iron-dependent proteins. Immunoblots of proteins from shoots of control and treated plants exposed to seven days of iron depletion. *A. thaliana* were grown in hydroponics with sufficient iron, 10 μM Fe-EDTA. At four weeks test plants, T, were exposed to seven days of iron depletion, 10 nM Fe-EDTA, while the control plants, C, were maintained at 10 μM Fe-EDTA. Plants were sampled after seven days of treatment, T7. 20 mg of protein was loaded onto 15% polyacrylamide gels.

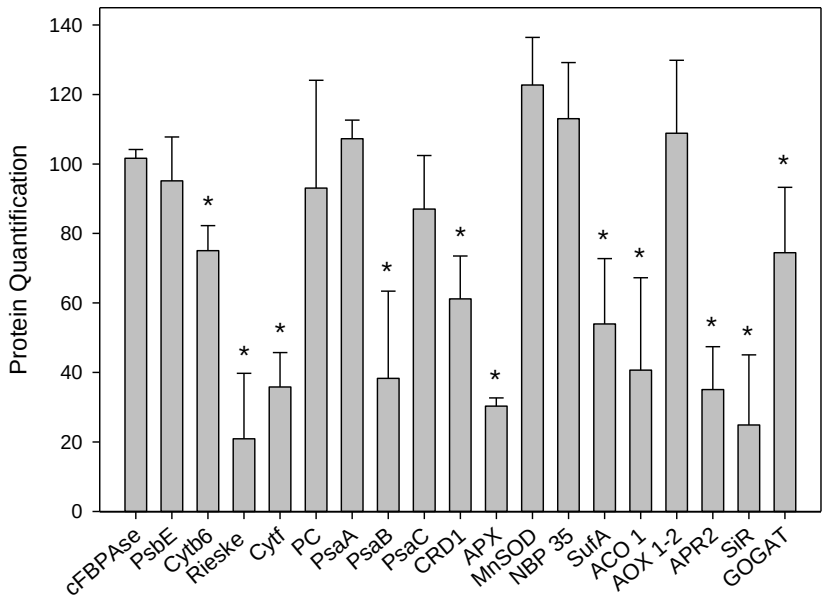


Figure 10. Quantification of protein abundance after seven days if iron depletion. Protein abundance quantified from immunoblots. *A. thaliana* were grown in hydroponics with sufficient iron, 10 μM Fe-EDTA. At four weeks test plants were exposed to seven days of iron depletion, 10 nM Fe-EDTA. Abundance was calculated using imageJ. Abundance ratios of control and treated plants was calculated and then normalized to cFBPase. The abundance of cFBPase was arbitrarily set to 100 %. Significant differences in abundance between each specific protein and cFBPase was determined by Student's t-test *. $P<0.05$. Results as means $\pm$ standard deviation of min. of 3 biological replicates.

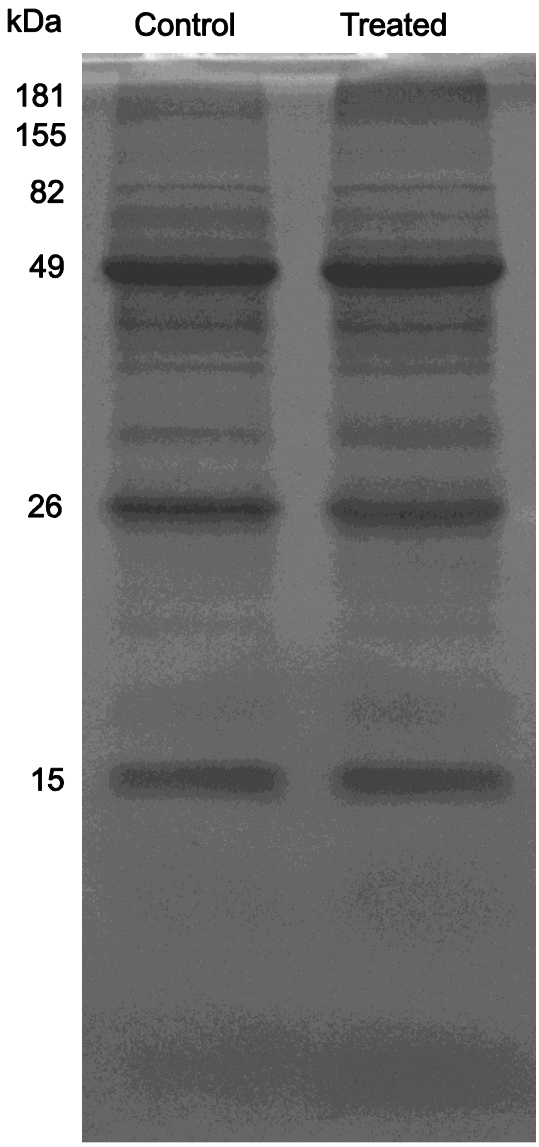


Figure 11. Protein profile of proteins from isolated chloroplasts. *A. thaliana* were grown in hydroponics with sufficient iron, 10 μM Fe-EDTA. At four weeks test plants were exposed to seven days of iron depletion, 10 nM Fe-EDTA. Control plants were maintained at 10 μM Fe-EDTA. Chloroplasts were isolated from of the fresh leaves of plants four days after initiation of Fe depletion (T4). The amount of protein loaded was 10 μl. Proteins were separated by SDS-PAGE (15% gel) and visualized with Coomassie Brilliant Blue staining.

Table 1. Gas exchange. *A. thaliana* were grown in hydroponics with sufficient iron, 10 μM Fe-EDTA. At four weeks test plants were exposed to seven days of iron depletion, 10 nM Fe-EDTA. Control plants were maintained at 10 μM Fe-EDTA. Gas exchange of whole rosettes was calculated after seven days of Fe depletion (T7) and again seven days after Fe resupply except for O_2 which was only tested for at T7.Significant differences between control and treated plants was determined by Student's t-test *. $P<0.05$. Results as means ± standard deviation of min. of 3 biological replicates.

	Day 7 of Iron Depletion		Day 7 of Iron Resupply	
Parameter	Control	Treated	Control	Treated
ci	25.69 ± 0.6	25.88 ± 0.6	419.84 ± 14	421.14 ± 14
gsw	6.9^{-3} ± 9^{-4}	9.5 ± 9^{-4}	0.13 ± 0.11	0.11 ± 0.13
Rd	0.056 ± 9^{-3}	0.080 ± 9^{-3}	1.08 ± 0.45	1.63 ± 0.45
O_2	-26.47 ± 9.3	-12.26 ± 8.4		

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