Leaf development and the plants iron nutrition status impact chloroplast physiology as well as the iron uptake machinery components PIC1, NiCo, MAR1 and YSL4

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# Table of content

1. INTRODUCTION................................................................................................................... 6

2. BACKGROUND .................................................................................................................... 8
   2.1 Significance of Fe in plant nutrition ................................................................. 8
   2.2 Fe homeostasis in plants ...................................................................................... 9
      2.2.1 Fe acquisition ................................................................................................. 9
      2.2.2 Fe transportation to the shoot tissues .......................................................... 14
      2.2.3 Molecular mechanism of the uptake of Fe to the chloroplasts .................... 17
      2.2.4 The physiological role of Fe in the chloroplasts ............................................. 25
   2.3 Fe must be present in plants in an appropriate amount ........................................ 29
      2.3.1 Fe deficiency in plants .................................................................................. 29
      2.3.2 Fe excess in plants ......................................................................................... 31
      2.3.3 Responses to Fe stress .................................................................................. 33

3. AIMS .................................................................................................................................... 35

4. MATERIALS AND METHODS ........................................................................................... 36
   4.1 Plant material and growth conditions .................................................................. 36
   4.2 Chlorophyll a fluorescence induction .................................................................. 36
   4.3 Determination of chlorophyll content .................................................................. 38
   4.4 Determination of leaf Fe content ......................................................................... 38
   4.5 Determination of chloroplast Fe content .............................................................. 38
   4.6 Expression analysis ............................................................................................... 39
      4.6.1 RNA extraction and cDNA synthesis ............................................................ 39
      4.6.2 Selection of optimal primers for expression analysis ....................................... 40
      4.6.3 Identification of optimal temperature and primer concentration of target genes 40
      4.6.4 BnPIC1, BnNico, BnMAR1 and BnYSL4 expression analysis by quantitative Real
                     Time-PCR ................................................................................................. 42
   4.7 Relative quantification of proteins by immunoblot ............................................... 43
   4.8 Statistical analysis ................................................................................................. 45

5. RESULTS ............................................................................................................................. 46
   5.1 Changes in physiological parameters of leaves .................................................... 46
   5.2 Identification of Brassica orthologs of the Arabidopsis PIC1, NiCo, MAR1 and YSL4 genes
                                                                                           ........................................................................................................ 56
5.3 Changes in the transcript level and protein amount of chloroplast Fe transporters ..........57
5.3.1 Time-scale changes in the expression of BnPICl, BnNico and BnMARl ...............57
5.3.2 Changes in the relative amount of PICl and NiCo proteins ................................59
5.4 Changes in the expression of BnYSL4 in different parts of Brassica napus tissues .....65
5.5 Differential expression of chloroplast Fe uptake related transporters – Interaction between the gene expression of Fe transporters and chloroplast Fe content .......................67
6. DISCUSSION ....................................................................................................................69
7. SUMMARY .......................................................................................................................77
8. ACKNOWLEDGMENTS .................................................................................................80
9. REFERENCES ................................................................................................................82
Appendix ..........................................................................................................................124
I. INTRODUCTION .............................................................................................................124
II. MATERIALS AND METHODS ....................................................................................125
III. RESULTS .......................................................................................................................129
IV. DISCUSSION .................................................................................................................130
V. REFERENCES ...............................................................................................................130
List of abbreviations

2’-deoxymugineic acid (DMA)
3-epihydroxy 2’-deoxymugineic acid (epi-HDMA)
3-epihydroxymugineic acid (epi-HMA)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)
Abscisic Acid (ABA)
*Arabidopsis* H\(^+\) ATPase (AHA2)
*Arabidopsis* H\(^+\) ATPase (AHA7)
Ascorbate Peroxidase (APX)
ATP-Binding Cassette G37/Pleiotropic Drug Resistance 9 (ABCG37/PDR9)
Bathophenanthroline Disulphonate Disodium Salt (BPDS)
Basic Helix-Loop-Helix (bHLH)
Ca\(^{2+}\)-Sensitive Cross-Complementer (CCC1)
Catalase (CAT)
Chlorophyll (Chl)
Class-III Peroxidases (POD)
Complementary DNA (cDNA)
Deoxyribonuclease (DNase)
Deoxyribonucleic Acid (DNA)
Deoxyribonucleotide triphosphate (dNTP)
Diethyl ppyrocarbonate (DEPC)
DMA synthase (DMAS)
Ethylenediaminetetraacetic acid (EDTA)
Fer Like Iron Deficiency-Induced Transcription factor 1 (FIT1)
Ferric Chelate Reductase 7 (FRO7)
Ferric Chelate Reductase Defective 3 (FRD3)
Ferric Reductase Oxidase 2 (FRO2)
Ferric chelate Reductase Defective 1 (frd1)
Ferritin 4 (FER4)
Feruloyl-CoA *ortho*-hydroxylase 1 (F6’H1)
Green Fluorescing Protein- (GFP-)
Horseradish peroxidase- (HRP)
Iron REGulated protein 3 (IREG3)
Iron Regulated Transporter 1 (IRT1)
Iron-inducible \( H^+ \)-ATPase isoform (CsHA1)
Jasmonic acid (JA)
Messenger RNA (mRNA)
MitoFerrin-Like 1 (MFL1)
Mugineic acids (MAs)
Multidrug And Toxic compound Extrusion (MATE)
Multiple Antibiotic Resistance protein (MAR1)
NA aminotransferase (NAAT)
Natural Resistance Associated Macrophage Protein (NRAMP3/4)
Nickel-Cobalt Transporter (NiCo)
Nicotianamine (NA)
Nicotianamine synthase (NAS)
Nicotinamide Adenine Dinucleotide (NAD)
Nicotinamide Adenine Dinucleotide Phosphate (NADPH)
Nitric Oxide (NO)
Non-intrinsic ABC Protein 14 (NAP14)
Oligo Peptide Transporter 3 (OPT3)
Permease In Chloroplast 1 (PIC1)
Photosynthetic Electron Transport (PET)
Photosynthetic Photon Flux Density (PPFD)
Photosystem I (PSI)
Photosystem II (PSII)
Phytosiderophores (PSs)
Polyacrylamide Gel Electrophoresis (PAGE)
Polymerase Chain Reaction (PCR)
PYRabactin Resistance1 (PYR1)/Pyr1-Like (PYL)/Regulatory Components of ABA Receptors (PYR/PYL/RCAR)
Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)
Reactive Oxygen Species (ROS)
Ribonuclease (RNase)
Ribonucleic Acid (RNA)
S-adenosyl-methionine molecules (SAM)
Salicylic acid (SA)
Sodium Dodecyl-Sulphate (SDS)
Superoxide Dismutase (SOD)
The Transporter of Mugineic acid family (TOM1)
Translocase of the Inner Mitochondrial membrane (TIM)
Translocon at the inner envelope membrane of chloroplasts (TIC)
Vacuolar Iron Transporter (VIT1)
Yellow Fluorescing Protein (YFP)
Yellow Stripe 1 (YS1)
Yellow Stripe Like 1 (YSL1)
Yellow Stripe Like 4 (YSL4)
Yellow Stripe Like 6 (YSL6)
Zinc-Iron–regulated transporter (ZIP)
1. INTRODUCTION

Predictions may differ, but there is a consensus in the scientific community in that global climate change is an emerging threat. It leads to unfavorable environmental conditions causing abiotic and biotic stresses (Zhu, 2016) in Arabidopsis thaliana (Yuan et al., 2014; Pineda et al., 2016), Brassica rapa (Kayum et al., 2015), Solanum lycopersicum (tomato) (Diogo and Wydra, 2007), Zea mays (maize) (Kaya et al., 2006), Triticum aestivum (wheat) (Tripathi et al., 2017), Pisum sativum (pea) (Tripathi et al., 2015), Oryza sativa (rice) (Song et al., 2016), having negative impacts on the geographical distribution of plants, affecting the quality of agricultural productivity and threatening food security (Fedoroff et al., 2010). The imbalance of iron (Fe) nutrition is a serious problem since Fe is one of the most important micronutrients which is essential for plant cell functions, especially for photosynthesis (Thomine and Vert, 2013; Zhang et al., 2017). Any alterations in its availability affect the physiological performance and development of plants (Anjum et al., 2015). Both low Fe nutrition and Fe overload are the abiotic factors inducing nutritional stresses that also lead to a strong influence on the global crop yields (Kampfenkel et al., 1995; Mori, 1999; Fourcroy et al., 2016). Excess Fe is highly toxic since it may produce cytotoxic hydroxyl radicals in the presence of hydrogen peroxide via Fenton reaction (Halliwell and Gutteridge, 1992). Worldwide, up to 30% of agricultural soils are alkaline or calcareous with, indeed, low Fe availability for plants as Fe is normally found in the insoluble oxidized ferric form under alkaline conditions (Guerinot and Yi, 1994; Mori, 1999). Fe limitation adversely affects the plant’s life, it induces changes with respect to gene expression (Thimm et al., 2001) and metabolism (Balk and Pilon, 2011) including photosynthesis, respiration, nitrate and sulphate assimilation (Forieri et al., 2016), synthesis of hormones (Puig et al., 2007; Rout and Sahoo, 2015) and Deoxyribonucleic Acid (DNA) (Reichard, 1993). Moreover, the lack of Fe leads to profound alterations not only on the metabolism of plants (Moseley et al., 2002) but also that of human beings (e.g. the prevalence of Fe deficiency in human anemia; Murgia et al., 2012).

In the plant cells, chloroplasts always require a high amount of Fe and contain approximately 80-90% Fe in the leaf cells (Terry and Low, 1982). In chloroplasts, Fe is known as an indispensable element and plays an important role in photosynthetic electron transfer of ferredoxins, cytochrome b6f complex, photosystem I (PSI) and photosystem II (PSII) as well as biogenetic cofactors (heme and Fe–S cluster) also need the participation of Fe in the
photosynthetic apparatus (Briat et al., 2015). Therefore, any adverse alterations in the photosynthetic apparatus depend on the different amounts of Fe under the too low or too high amount of Fe nutritions (Moseley et al., 2002). A significant effect on the photosynthesis is the decrease in the chlorophyll (Chl) \( a \) and other pigments in light harvesting (van Leeuwe and Stefels, 1998). The synthesis of proteins (D1 protein) is strongly affected in the PSII due to the Fe limitation (Vassiliev et al., 1995). The reduction in Fe-sulphur complexes (such as ferredoxin) has a significant impact on the activity of Photosynthetic Electron Transport (PET) chain in the photosynthetic apparatus (McKay et al., 1999). Although Fe content and homeostasis of chloroplasts is essential to maintain elementary physiological functions such as the operation of the photosynthetic electron transport, Fe homeostasis of chloroplasts is hardly known under alterations in the Fe nutrition of plants. Similarly, we also lack information, how the Fe acquisition of chloroplasts alters during the development or aging of the photosynthetic tissues.
2. BACKGROUND

2.1 Significance of Fe in plant nutrition

Macro- and micronutrients play essential roles in the growth and development of plants (White and Brown, 2010; Waraich et al., 2012; Tripathi et al., 2015). To provide the worldwide food web for the human beings, especially in the developing countries, a sufficient and balanced supply of essential nutrients is required at the right time for the proper growth and development of plants (Dordas, 2009; Hänsch and Mendel, 2009; Sarwar et al., 2010; Moharana et al., 2012; Waraich et al., 2012).

Micronutrients (such as Zn, Fe, Mn, B, Cl, Cu, Mo, and Ni) are generally needed in small amounts but play essential roles in plant metabolisms (Welch, 1995). They are essential cofactors in redox enzymes due to their chemical properties to change the valence under biological conditions thus mostly they are found as constituents of prosthetic groups in metalloproteins (Prabhulkar et al., 2012). Particularly, as constituents of prosthetic groups, some micronutrients catalyze redox processes by electron transfer (typically the transition metals: Fe, Mn, Cu, and Mo), they form enzyme-substrate complexes by coupling enzyme and substrate (Fe and Zn, for example) or they enhance enzyme reactions by influencing the molecular configuration of an enzyme or substrate (Welch, 1995).

Fe is a key micronutrient for all living organisms, which is particularly important for plant productivity, product quality (Briat et al., 2015) and human health (Moseley et al., 2002; Zhu et al., 2016). It is the fourth most abundant element and accounts for about 5% in the Earth’s crust (Wedepohl, 1995; Ceballos-Laita et al., 2015). Fe is an essential cofactor in metabolic processes including Chl biosynthesis, respiration, chloroplast development (Kim and Guerinot, 2007), electron-transport or oxygen-transfer chains such as cytochrome P450 monooxygenases and 2-oxoglutarate-dependent dioxygenases (Hänsch and Mendel, 2009; Balk and Pilon, 2011), nitrogen fixation (Kim and Rees, 1992; Briat and Lobréaux, 1997; Muneer et al., 2014; Rout and Sahoo, 2015) and DNA synthesis via the activity of the ribonucleotide reductase (Reichard, 1993). It is also an active cofactor of enzymes involved in plant hormone synthesis (Kerkeb and Connoly, 2006) such as lipoxygenase, 1-aminocyclopropane acid-1-carboxylic oxidase (Siedow, 1991) or abscisic acid (ABA) (those are effective in affecting the activities of plant growth in response to adverse alterations in agricultural environment) (Rout and Sahoo, 2015). Moreover, it serves as a cofactor for a wide range of redox enzymes (e.g. catalase (CAT), class-III
peroxidases (POD), ascorbate peroxidase (APX)) that contain Fe as a prosthetic groups (Sharma and Dubey, 2004; Rout and Sahoo, 2015). An isoform of superoxide dismutase (SOD) which acts as a scavenger of reactive oxygen species (ROS) also contains non-hem Fe (Case, 2017). Since Fe-containing redox enzymes are also involved in the elimination of reactive oxygen species and radicals, the application of Fe as a nutrient supplement has an emerging role in enhancing the tolerance of plants against abiotic stresses. Recent studies pointed out that Fe also plays a significant and/or potential role in alleviating major abiotic stress caused by salinity, drought and the presence of non-essential heavy metals (Emamverdian et al., 2015).

**2.2 Fe homeostasis in plants**

2.2.1 Fe acquisition

All living organisms have developed many mechanisms to acquire mobilized Fe from the environment and distribute available Fe during the plant’s life cycle. To uptake Fe, all land plants have strategies to dispatch from the soil, reduce or chelate Fe and take up into the symplast of root cells under Fe deficiency (Naranjo‐Arcos and Bauer, 2016). There are two distinct strategies: reduction-based Strategy I used by all dicots and non-grass monocots while chelation-based Strategy II used by most species of family Poaceae (Graminae) to enhance efficient Fe acquisition from the rhizosphere (Römheld and Marschner, 1986; Guerinot and Yi, 1994).

2.2.1.1 Reduction-based Fe transport strategy (Strategy I)

The strategy I the model plant of which is *Arabidopsis thaliana* mine valuable Fe from soils by sequential acidification, reduction and transport activities (Walker and Connolly, 2008) combined with the sequestration of coumarin and/or flavin derivatives (Schmidt et al. 2014, Sisó-Terraza et al. 2016). This Fe acquisition strategy includes Fe solubilization, reduction of ferric Fe to ferrous and ferrous import into the plant cells (Morrissey and Guerinot, 2009; Brumbarova et al., 2015). All three processes show increased activities during Fe starvation (Hell and Stephan, 2003).

Plants increase the solubility of Fe in the soil in different ways. In response to Fe deficiency, H⁺-ATPase release protons to lower the soil pH to increase the solubility of ferric Fe complexes in the local rhizosphere (Santi and Schmidt, 2009; Kobayashi and Nishizawa, 2012). In the *Arabidopsis* model, the plasma membrane H⁺-ATPase family includes 12 members under
Fe limitation (Colangelo and Guerinot, 2004). AHA2 is well-known to be responsible for the major acidification of the rhizosphere in response to low Fe, while AHA7 has been shown to support the development of root hairs (Santi and Schmidt, 2009). It causes an increase in the surface area of the root system to enhance the uptake of Fe under Fe starvation (Santi and Schmidt, 2009). In cucumber (Cucumis sativus) model (Santi et al., 2005), the iron-inducible H+ ATPase isoform CsHA1 induced rhizosphere acidification under Fe-deficient root hairs. According to Młodzinska (2012), the transcript levels of CsHA2 and CsHA3 were up-regulated in cucumber roots for the Fe solubilization when plants lack Fe nutrition. The AHA7 (Arabidopsis H+ ATPase) gene was a key factor responsible for the acidification of the rhizosphere in Fe deficiency and its expression depends on Fer Like Iron Deficiency-Induced Transcription factor 1 (FIT1) transcription factor (Colangelo and Guerinot, 2004; Santi and Schmidt, 2009).

In both strategies, roots increase the Fe mobilization by secreting chelating compounds (Römheld and Marschner, 1983) and the types of chelator released depends mostly on individual species (Susín et al., 1994). Among them, flavins and phenolic compounds are found in Strategy I (Yoshino and Murakami, 1998). For example, flavins are expressed for cucumber, tobacco (Nicotiana tabacum) and Medicago truncatula (Vorwieger et al., 2007; Rodriguez-Celma et al., 2011) in response to Fe starvation. The composition of root exudates depends not only on Fe nutritional status but also on soil pH (Rajniak et al., 2018). Phenolic compounds, particularly coumarin subfamily (i.e. plant-derived phenylpropanoids characterized by a 2-hydroxy-1-benzopyrane-2-one backbone) are considered as the main components of root exudates (Jin et al., 2007; Rodríguez-Celma et al., 2013; Fourcroy et al., 2014; Schmid et al., 2014; Sisó-Terraza et al., 2016). Scopoletin is synthesized by the enzyme feruloyl-CoA ortho-hydroxylase 1 (F6’H1) encoding a Fe(II) and 2-oxoglutarate-dependent dioxygenase which diverts the lignin biosynthesis pathway (Kai et al., 2008). Coumarins including esculetin, fraxetin, scopoletin, isofraxidin, and an isofraxidin isomer were participated in the Fe acquisition of Strategy I roots (Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014). Coumarins released into the rhizosphere by ATP-Binding Cassette G37/Pleiotropic Drug Resistance 9 (ABCG37/PDR9; Fourcroy et al., 2014; Ziegler et al., 2016). Both F6’H1 and ABCG37/PDR9 genes are transcriptionally expressed under Fe limitation by the transcription factor FIT1 (Rodríguez-Celma et al., 2013; Schmid et al., 2014; Brumberova et al., 2015; Fourcroy et al., 2016). Besides
the positive effects of phenolic compounds, their specific significance to access metal in the adverse soils is still unclear (Fourcroy et al., 2016).

The second step is the reduction of Fe(III) to Fe(II) by a plasma membrane-bound ferric reductase. Reduction ferric Fe (Fe$^{3+}$) to ferrous Fe (Fe$^{2+}$) is mainly performed by Ferric Reductase Oxidase 2 (FRO2) in Arabidopsis at the root surface (Robinson et al., 1999; Santi and Schmidt, 2009; Ivanov et al., 2012). FRO2 is mainly expressed in the outer layer of root tissues (Mukherjee et al., 2006; Dinneny et al., 2008). The Arabidopsis mutant, ferric-chelate reductase defective 1 (frd1) had no inducible root Fe(III)-chelate reductase activity and developed chlorosis signal (Yi and Guerinot, 1996). The FRO2 gene was found to map at the same location as the frd1 mutation. Transformation of frd1 mutation with the FRO2 gene rescued the frd1 phenotype and indicated that FRO2 encodes the root ferric chelate reductase (Robinson et al., 1999). FRO2 from Arabidopsis thaliana was expressed in soybean transgenic plants, which increased leaf Fe concentration three times, and with 10 percent the Fe in seed under hydroponic growth condition (Vasconcelos et al., 2006).

Finally, the resulting Fe$^{2+}$ is transported into the epidermal cells via the plasma membrane by the divalent Fe transporter Iron Regulated Transporter 1 (IRT1) (Eide et al., 1996; Robinson et al., 1999; Vert et al., 2002; Brumbarova et al., 2015; Connorton et al., 2017). The IRT1 belongs to the Zn-Fe–regulated transporter (ZIP) family of metal transporters firstly identified in Arabidopsis (Eide et al., 1996; Vert et al., 2002; Connolly et al., 2003; Enomoto et al., 2007). IRT1 is mainly localized to the root surface plasma membrane of epidermal cells (Dubeaux et al., 2015). IRT1 has been cloned from Arabidopsis (Dubeaux et al., 2015) and its orthologues have also been cloned from pea and tomato (Eckhardt et al., 2001). The activity and expression of IRT1 were up-regulated in root epidermal cells under Fe limitation that indicated this protein is the main transporter for the uptake of ferrous Fe from soils (Vert et al., 2002; Kim and Guerinot, 2007). In Arabidopsis, irt1 mutant expressed chlorosis, impaired growth and proved to be lethal before setting seeds without a high amount of soluble Fe supply (Henriques et al., 2002; Varotto et al., 2002; Vert et al., 2002). IRT1 also plays an essential role in the transport of other metals such as Zn, Cd, Mn, and Co) (Rogers et al., 2000). In Arabidopsis, gene expression of both FRO2 and IRT1 is regulated by a basic helix-loop-helix (bHLH) family transcriptional factor, FIT1 (Colangelo and Guerinot, 2004). Due to its high affinity for other divalent metals, IRT1 is tightly regulated at both transcriptional and post-transcriptional levels.
(Korshunova et al., 1999; Rogers et al., 2000; Potocki et al., 2013). Reduction based Fe uptake strategy also exist in various plant tissues and intracellular Fe transport processes, since FRO family enzymes are present in multiple membrane systems.

2.2.1.2 Chelation-based Fe transport strategy (Strategy II)

Strategy II (also known as the chelation-based mechanism) is exclusively specific to most of the species of the Poaceae family to acquire Fe from the soil (Nozoye, 2011). Indeed, chelation-based Fe transportation is present in all yet examined plants at cell-to-cell Fe transportation and phloem Fe loading operated by Yellow Stripe-Like (YSL) transporters. The process is different from Strategy I: the root system of Strategy II plants does not acidify the rhizosphere to increase the solubility of ferric Fe or display the increased activity of ferric reductase under Fe-deficient medium. Instead, it is based on the biosynthesis and secretion of mugineic acid-type phytosiderophores (Curie et al., 2001; Murata et al., 2006; Kobayashi and Nishizawa, 2012) – a group of hydroxy- and amino-substituted iminocarboxylic acids synthetised from ornitin via nicotianamine (NA) (Römheld, 1987) to solubilize and uptake ferric compounds by roots (Takagi, 1976). Phytosiderophores are responsible for the chelation of ferric Fe in the rhizosphere (Tripathi et al., 2018). Interestingly, phytosiderophores also solubilize other essential micronutrients such as Mn, Cu, and Zn (Treeby et al., 1989) implicating that phytosiderophores have been released from root to increase the concentration of micronutrients in the rhizosphere in Fe-deficient plants. To acquire Fe from the root surface of Fe-limited availability, graminaceous plants secrete Phytosiderophores (PSs), such as deoxymugineic acids (DMA) – the most abundance phytosiderophore – and exports it by TOM1 (the Transporter of Mugineic acid family), which bind insoluble ferric Fe with high affinity (Takagi, 1976; Takagi et al., 1984; Higuchi et al., 1999; Conte and Walker, 2011; Nozoye et al., 2011).

Different graminaceous species and genotypic differences produce various kinds and different quantities of phytosiderophores (Römheld and Marschner, 1986; Kerkeb and Connolly, 2006). Phytosiderophores were first described in oats and rice by Takagi (1976) and is the member of mugineic acids (MAs) including mugineic acid (MA), 2’-deoxymugineic acid (DMA), 3-epihydroxymugineic acid (epi-HMA), and 3-epihydroxy 2’-deoxymugineic acid (epi-HDMA) (Ma et al., 1995; Mori, 1999; Negishi et al., 2002). Until now, nine different kinds of MAs have been investigated and synthesized from S-adenosyl-L-methionine (Shojima et al.,
The biosynthesis mechanism of MAs forms the precursor NA originated from the condensation of three S-adenosyl-methionine molecules (SAM) was investigated in the past decade (Inoue et al., 2003; Klatte et al., 2009; Bonneau et al., 2016). NA is then converted to a 3’-keto intermediate by NA aminotransferase (NAAT) (Kanazawa et al., 1993; Takahashi et al., 1999). The 3’-keto intermediate is reduced to release deoxymugineic acid by the activity of DMA synthase (DMAS) (Bashir and Nishizawa, 2006; Bashir et al., 2006). DMA is generated into the rhizosphere through the MAs1 transporter (Nozoye et al., 2013). The expression of MAs1 tested in Hordeum vulgare (barley), Oryza sativa (rice), Zea mays (maize) and Triticum aestivum (wheat) (Takahashi et al., 1999; Nakanishi et al., 2000; Kobayashi et al., 2001; Inoue et al., 2003, 2008; Bashir et al., 2006; Bashir and Nishizawa, 2006) was significantly up-regulated in the absence of Fe (Ogo et al., 2011; Kobayashi and Nishizawa, 2012). In graminaceous plants, chelation-based strategy enhances the acquisition of available Fe with the support of the precursors such as Nicotianamine synthase (NAS), NAAT, DMAS, and DMA (Tripathi et al., 2018).

After the acquisition of Fe inside the root, specific transporter proteins transport ferric-PS complexes in the cytosol of the epidermal cells (Römheld and Marschner, 1986). This transporter, Yellow Stripe 1 (YS1)/Yellow Stripe Like 1 (YSL1) family transporter - a protein which is the member of the Oligopeptide Transporter (OPT) family (Conte and Walker, 2011) was first characterized in maize (Curie et al., 2001; Roberts et al., 2004; Curie et al., 2009) and have been studied in several other grass species (Curie et al., 2001; Yen et al., 2001; Murata et al., 2006; Inoue et al., 2008; Lee et al., 2009). YS1 acts as a proton-coupled symporter of Fe(III)-PS complexes (Schaff et al., 2004). In ys1 maize mutants lacking the ability to efficiently use Fe(III)-PS, the yellow symptom was observed in veins of Fe-deficient leaves under Fe-limiting conditions (Jolley and Brown, 1991; von Wiren et al., 1994; von Wiren et al., 1995). In rice, OsYSL15 transporter is responsible for the uptake Fe by Fe(III)-PS (Lee et al., 2009; Inoue et al., 2009; Nozoye et al., 2011). HvYS1 performed the function to complement the Fe uptake defective yeast grown on medium containing Fe(III)-PS complex compared to Fe(II)-NA compound or Fe(III) only, as evidence for being a Fe(III)-PS transporter (Murata et al., 2006). HvYS1 was located in the plasma membrane of epidermal cells by using the immuno-histological staining with an anti-HvYS1 antibody and transient expression of HvYS1::GFP fusion proteins (Murata et al., 2006).
2.2.2 Fe transportation to the shoot tissues

Fe is necessary for many important metabolisms and is active in specific subcellular compartments. Adequate levels of Fe allocation in the different organelles are of great importance for providing the cell function (Roschzttardtz et al., 2011) within the plant body. After Fe is transported from the soil into the root cells, it must be transported to suitable compartments for the Fe utilization in plants by crossing both cellular and organellar compartments (Larbi et al., 2001; Vigani et al., 2013). To be effectively entered into the plant cells, Fe has to bind with chelators when it is present in the form of excessive or low Fe in the soils. When Fe is transported into the plants via xylem, it immediately chelates with siderophores or organic acids (citrate, malate, and succinate) (Shingles et al., 2002; Curie et al., 2009) because Fe is generally found in the oxidized form and should bind with chelators in the formation of complexes when the pH is available around 5.5 – 6.0 (Curie and Briat, 2003). Citrate is perfectly complexed with Fe in the xylem that has been discovered as the ideal chelator to be transported into the plant body (Conte and Walker, 2011). Citrate is thought to be an essential factor in long-distance for Fe transport (Tiffin, 1966). When the plant negatively suffers Fe-deficient nutrition, the amount of chelators significantly increases in the xylem to support plant cells to overcome the adverse agricultural environment (López-Millán et al., 2000). Citrate is known to enter the xylem by Ferric Chelate Reductase Defective 3 (FRD3) found in the root vasculature of Arabidopsis thaliana (Green and Rogers, 2004). FRD3 belongs to the Multidrug And Toxic compound Extrusion (MATE) family and has several members to lessen toxic aluminum via efflux citrate (Magalhaes et al., 2007; Furukawa et al., 2007; Liu et al., 2009). It is discovered to localize in the plasma membrane of cells in the pericycle and vasculature which plays an important role in Fe translocation from the root to shoot by binding with citrate (Jeong and Guerinot, 2009). Under Fe starvation, the expression of FRD3 is up-regulated two-fold higher than that of adequate Fe and the loss of this gene results in the chlorosis in plant tissues (Rogers and Guerinot, 2002). Without citrate, Fe is not efficiently absorbed into the xylem and cannot be used by the shoot tissue. In leaves, Fe is an important micronutrient for photosynthetic apparatus. It is found to locate in the chloroplasts and then is distributed in the leaf mesophyll tissues (Wollman et al., 1999; Morrissey and Guerinot, 2009). Here, Fe continues to enter into the symplast and is reduced into ferrous Fe by the action of FRO proteins and again bind with chelator as Fe²⁺-NA complexes. Both plastids and mitochondria require a high amount of Fe to
maintain their functions in plant life. Specific Fe transporters for each type of organelle have recently been investigated to their distinct roles (Finazzi et al., 2015; Bashir et al., 2016, Vigani et al., 2019). Fe is then transported from the leaf cells to other necessary organelles through the phloem. YSL proteins are known to involve in the long-distance transport of several transition metals such as Fe, Cu, Ni, Co, Mn and Zn which bind with an ideal chelator, NA (Curie et al., 2009). Fe is formed as a Fe$^{2+}$-NA complexes that NA is a non-proteogenic amino acid ubiquitously found in the plant cells for Fe translocation into particular compartments (Stephan and Scholz, 1993; Curie et al., 2009). NA is catalyzed by the condensation of three molecules of S-adenosylmethionine in the NA synthase catalyst and is the strong chelator of various metal transition for both ferrous Fe (Anderegg and Ripperger, 1989) and ferric Fe (von Wiren et al., 1999). NA likely prefers to bind with ferric Fe in high affinity but more stable in the formation of ferrous Fe (von Wiren et al., 1999). The various concentrations of NA greatly impact on the Fe homeostasis on plant species (Takahashi et al., 2003; Douchkov et al., 2005; Kim et al., 2005). However, there are still controversial opinions about whether NA influences the Fe delivery into shoots or the uptake of Fe from the root cells controlled by the formation of novel Fe sinks in the shoot tissues. The expression of YSL2 is found in xylem parenchyma tissues and it is used to maintain the Fe homeostasis in Arabidopsis thaliana, suggesting that this gene plays a role in the lateral Fe movement in the vasculature (DiDonato et al., 2004; Schaaf et al., 2005). YSL1/3 is also located in the vasculature and is the capacity of transporting Fe-NA to the developing tissues and seeds from the phloem to the apoplast (Kumar et al., 2017). The Arabidopsis experiments were carried out to observe the different expression and phenotypes of the single ysl1 and the double ysl1 ysl3 mutant lines show that both YSL1 and YSL3 have the function to the delivery of essential metals such as Fe, Cu and Zn into seed tissues (Waters et al., 2006; Chu et al., 2010; Kumar et al., 2017). In Arabidopsis, Fe is supplied to sink tissues by Oligo Peptide Transporter 3 (OPT3) transporter is close to YSLs family and belongs to the OPT family which plays a dual role by regulating the transmission of a shoot-to-root Fe signal and redistributing Fe to developing tissues such as seeds (Stacey et al., 2008; Mendoza-Cozatl et al., 2014; Zhai et al., 2014). When plants suffer Fe-deficient conditions, OPT3 is up-regulated in the phloem companion cells that Fe is delivered from the xylem to phloem (Khan et al., 2018). The opt3 mutant lines have more Fe that immobilized in leaves and less translocated in other organs such as the seed (Zhai et al., 2014; Mendoza-Cózatl et al., 2014). Knockdown opt3
mutation lines are feasible but high Fe content is found in both shoot and root cells (Stacey et al., 2008; Mendoza-Cozalt et al., 2014; Zhai et al., 2014). Fe has to bind with the NA chelator to form as Fe-complex transported by OPT3 (Lubkowitz, 2011). OPT3 is present in the phloem and has a function to chelate with NA from the mature leaves to developing organs such as seeds and young leaves (Mustroph et al., 2009; Mendoza-Cozalt et al., 2014; Zhai et al., 2014). This investigation shows that OPT3 plays a significant role in Fe retranslocation from the xylem and has a function to connect xylem and phloem (Zhai et al., 2014).

The terminal destination of Fe described as the most important nutrient sink for plants is stored in the seed during the germination stage. The amount of Fe is stored in the seed used for the growth of seedling to develop the young root and uptakes macro- and micro-nutrients from the soil. YSL1 and YSL3 transporters are involved in Fe translocation in seeds (Le Jean et al., 2005; Waters et al., 2006; Chu et al., 2010). In addition, the constitution of seeds are served for the main source of human nutrition, the process of Fe transport and Fe accumulation in seeds has received much attention from many scholars (Clemens, 2014). Both ferric-citrate and ferric-malate complexes were detected in the endosperm of the pea plant and the reduction of ferric Fe was identified in these embryos (Grillet et al., 2014; Curie and Mari, 2017). However, embryos use ascorbate efflux to reduce ferric Fe for easily Fe uptake instead of the usage of a membrane-bound ferric reductase. In Arabidopsis seeds, 5% of total Fe is formed in ferritin (Ravet et al., 2009) while 50% of Fe is found in vacuolar globoids of endodermal and bundle sheath cells (Lanquar et al., 2005; Kim et al., 2006; Roschztattdz et al., 2009; Ramos et al., 2013). Vacuolar Iron Transporter (VIT1) and Natural Resistance Associated Macrophage Protein (NRAMP3/4) have specific functions in vacuolar to maintain Fe homeostasis in plant cells (Lanquar et al., 2005; Kim et al., 2006). The investigation recently revealed that VIT1 and NRAMP3/4 have the connection to affect the effects of different amounts of Fe in the embryo on the germination stage under Fe-deficient nutrition (Mary et al., 2015, Vigani et al., 2019).

Ferritins are Fe-storage proteins and plants reduce their expression when high Fe concentration present in the plant cells (Lobreaux et al., 1992). Ferritins play an important role in the Fe storage and protection of the cells to avoid oxidative stress (Connorton et al., 2017). Ferritins are predominantly present in the mitochondria and plastids with the ability to complex several thousands of Fe atoms when associated in 24-mer multimers (Wade et al., 1993; Zancani
et al., 2004; Kyriacou et al., 2014). Twenty-four subunits form a shell and store up to 4500 ferric ions (Theil, 2011). Ferritins have two main functions: the Fe storage in seeds in germination stage (Kimata and Theil, 1994; Lucas et al., 1998), protection of the cells by sequestering free Fe to avoid oxidative stress against the toxicity under excessive Fe (Theil, 1987; Ravet et al., 2009). The percentage of ferritins stored in seeds is different in plant species, for example, approximately 60% in peas but around 5% in Arabidopsis seeds (Zielińska-Dawidziak, 2015). The lack of ferritin results in a reduction of plant growth and strong defects in flower development (Ravet et al., 2009). There are 4 ferritin genes in the model Arabidopsis thaliana. Three of them have been identified to encode for chloroplast proteins and the (Ferritin 4) FER4 is found in the seed plastids (Petit et al., 2001; Ravet et al., 2009). The amount of chloroplast ferritin proteins is less abundant in mature leaf than seed tissue. However, leaf ferritin is stored in the short-term period in opposition to the long-term period in seeds (Duy et al., 2007). The expression of ferritin is up-regulated (Briat et al., 2010) and ferritin mRNA showed strong induction in all tested plants under Fe excess nutrition (Petit et al., 2001; Majerus et al., 2007).

2.2.3 Molecular mechanism of the uptake of Fe to the chloroplasts

Concerning the chloroplast Fe acquisition, several Fe-uptake related proteins have been identified in the past decade, mostly in the model plant Arabidopsis thaliana. Some Fe transporters have been identified to localize in the chloroplast envelope, most of them are found in the inner envelope (López-Millán et al., 2016). The import of Fe into thylakoid lumen is yet unknown. The only thylakoid membrane integrating, Fe homeostasis related transporters are known from maize (Zea mays; FDR3 & 4; Han et al., 2009; Zhang et al., 2017). Indeed, their proper function has not been revealed yet. Fe-transport and homeostasis proteins were described such as the chloroplast Ferric Chelate Reductase 7 (FRO7) belonging to the reduction-based Fe uptake strategy reducing Fe(III)-complexes to Fe$^{2+}$ (Jeong et al., 2008), and two less characterized proteins, Permease In Chloroplast 1 (PIC1) and Nickel-Cobalt Transporter (NiCo) that proposed to be involved in the transport of free ferrous ions across the chloroplast inner envelope membrane (Duy et al., 2007; 2011; López-Millán et al., 2016). Several components, playing significant roles in Fe translocation, have recently been identified. YSL family Fe-NA complex transporters Yellow Stripe Like 4 and Yellow Stripe Like 6 (YSL4 and YSL6) were described to play a potential role in Fe release from proplastids of seeds (Divol et al., 2013). A Multiple Antibiotic Resistance protein (MAR1 - also known as Iron REGulated protein 3 -
IREG3) is also found in the chloroplast envelope membranes (Conte et al., 2009). In *Arabidopsis*, the Non-intrinsic ABC Protein 14 (NAP14) has the main role in chloroplast Fe homeostasis (Shimoni-Shor et al., 2010), and the presence of MitoFerrin-Like 1 (MFL1) protein is also predicted in chloroplasts.

2.2.3.1 PIC1 participates in chloroplast Fe uptake and plays a significant role in Fe homeostasis

Among chloroplast Fe acquisition-related proteins, the role of PIC1 is proven to be of profound. PIC1 derived from cyanobacterial permeases (Duy et al., 2007; Gross and Bhattacharya, 2009; Fischer, 2011). PIC1 was originally reported to deliver ferrous Fe into the inner envelope membrane of the chloroplast, and essential for the chloroplast Fe homeostasis in *Arabidopsis* plants (Duy et al., 2007; Gong et al., 2015). The PIC1 is thought to transport free ferrous ions, as IRT1 transporter does in the root plasma membrane. According to experiments of Shingles et al. (2002), the translocation of free ferrous ions through chloroplast inner envelope vesicles is driven by an inwardly projecting proton gradient. Following the assumptions, PIC1 is responsible for the proton gradient driven uptake of free ferrous ions for plant use (Vigani et al., 2019). PIC1 contains four membrane-spanning α-helices and is located in the inner envelope (IE) membrane of chloroplast by *in vivo* Green Fluorescing Protein- (GFP-) targeting and

![Figure 1. Fe homeostasis of chloroplasts (from Dr. Ádám Solti)](image-url)
immunoblot analysis (Duy et al., 2007; Fischer, 2011). The integral membrane protein was previously known as the translocon at the inner envelope membrane (Translocon at the inner envelope membrane of chloroplasts, TIC21 has 21 kDa), a member of the inner envelope protein translocon machinery of chloroplasts which involves in the import of nuclear-encoded plastid proteins from the cytosol (Teng et al., 2006; Duy et al., 2007; Kikuchi et al., 2009). The TIC21 protein is the same to PIC1 which was proved by forward genetics to use the screen for mutant lines inadequate in chloroplast protein import (Sun et al., 2001). Teng et al. (2006) mentioned that the function of TIC21/PIC1 acted as essential translocon component in the chloroplast inner envelope based on (i) the accumulation of unprocessed plastid precursor proteins in tic21/pic1 knockout lines, (ii) imperfections in translocation of inner envelope protein found in isolated chloroplasts of a sub-lethal tic21/pic1 mutant lines and (iii) co-immuno-precipitation of TIC21/PIC1 with other major protein import translocon components. However, there was no exact functional proofs of TIC21/PIC1 for protein transport and several studies were carried out on pic1/tic21 mutant lines but no residual precursor proteins could be detected in plastids (Duy et al., 2007). Furthermore, ferritin found in pic1/tic21 plastids shown that PIC1/TIC21 does not have any connection with protein translocation (Duy et al., 2007). Kikuchi et al. (2009) studied TIC21/PIC1 in an about 1 MDa protein complex localized in the inner envelope membrane of chloroplasts including the regular translocon in TIC20. This functional complex is supposed as a widespread TIC protein translocation core complex that TIC21/PIC1 is generally connected with the periphery. However, studies proved that TIC21/PIC1 does not co-purify with this 1 MDa complex (Kikuchi et al., 2013; Nakai, 2015). Thus, the previous studies of PIC1/TIC21 to TIC translocon component proteins like TIC20 may be produced by the higher density of membrane protein in the chloroplast inner envelope than by the interaction of specific proteins. The sequence of PIC1/TIC21 shared a little similarity to protein translocons in the inner envelope membrane of chloroplast, for example TIC20 or Translocase of the Inner Mitochondrial membrane (TIM17 and TIM23) in the inner envelope membrane of mitochondria (Inaba and Schnell, 2008; Balsera et al., 2009). Nevertheless, a possible dual function of PIC1/TIC21 in Fe transport and protein import cannot be excluded considering that the beneficial effect of new functional features (such as protein import) would be an evolutionary implementation to the function of an ancient solute permease of cyanobacterial origin (Gross and Bhattacharya, 2009).
The function of PIC1 transporter was confirmed by the growth promotion of a Fe uptake-deficient yeast strain where PIC1 expression (PIC1ox) caused an increase in the concentration of Fe in yeast cells. Moreover, PIC1ox plants contained approximately 2.5 times more chloroplast Fe than wild-type chloroplasts and lead to adverse signals such as leaf chlorosis, increased level of oxidative stress and finally reduced biomass (Duy et al., 2011). The overexpression of PIC1 negatively affected the Fe content not only in chloroplasts but also in developing tissues such as flowers and seeds (Duy et al., 2011). Ravet et al. (2009) mentioned that flowers of PIC1ox lines contained more Fe but the number of other metals was still unaffected but seeds showed a significant decrease in the concentration of Fe. However, the PIC1ox lines shared a high concentration of Fe as similar as fer knock-out mutant lines in response to excess Fe and showed adverse defects in the development of flower and seed tissues, resulting in a serious decrease in seed yield and percentage of germination stage (Ravet et al., 2009; Duy et al., 2011). The absence of PIC1 expression significantly impacted on plant growth and showed several Fe-deficient signals such as albino phenotype and weak development in the model plant. Moreover, pic1 mutants strongly affected the structure of leaf mesophyll cells and the growth of chloroplasts (Duy et al., 2007). In Arabidopsis plant, Duy et al. (2007) described that chloroplast pic1 knockout accumulates ferritin protein clusters mostly loaded with Fe in the meristems and leaves. The transcript level of PIC1 was also up-regulated in leaf ferritin knockout lines (Ravet et al., 2009). The function of chloroplast PIC1 transporter has been investigated in the study of tobacco PIC1 knockdown and overexpression lines expressed similar phenotypes to the Arabidopsis mutants (Gong et al., 2015). NtPIC1 also complemented the development of Fe deficiency in yeast. Thus, the functional studies in yeast, the loss of function in phenotypes of pic1 mutants and PIC1ox lines were carried out and explained that PIC1 plays an essential role in Fe accumulation in plastid and maintenance of Fe homeostasis (Gong et al., 2015).

In addition to the PIC1 function in Fe accumulation in yeast cells (Duy et al., 2007; Gong et al., 2015) and in Arabidopsis chloroplasts when over-expressed (Duy et al., 2011), PIC1 is described to interact with NiCo protein, a member of Nickel-Cobalt transporter family localized in plastid IE membrane (Eitinger et al., 2005), suggesting its role in Fe transport (Duy et al., 2011). Moreover, At2g16800 expression was one of the two Arabidopsis NiCo genes that showed the high up-regulation in PIC1ox line in Arabidopsis flowers (Duy et al., 2011). To support the function of At2g16800, an in vivo GFP-targeting procedure was done to observe the
typical ring-shaped signals of envelope proteins in chloroplasts. Following the study of Duy et al. (2011), a hypothetical metal translocon including PIC1 and NiCo subunits (PIC-NIC) should exist in the vicinity of the protein translocon complex. NiCo also contains specific metal-binding domains (Eitinger et al., 2005). A possible molecular function of PIC1-NiCo interaction is probably PIC1 may act as ferrous Fe ion permease and NiCo might sense or bind to the metal ions in Arabidopsis IE membranes (López-Millán et al., 2016). Nevertheless, more functional experiments of Fe transport should be investigated to uncover their molecular mechanisms.

2.2.3.2 FRO7 – A ferric chelate reductase in chloroplast inner envelope

Ferric chelate oxidoreductase (FRO) has been described to have an important function in Fe reduction located in the chloroplasts (Mikami et al., 2011; Solti et al., 2014). Function of this enzyme have been approved in the reduction of Fe for Fe accumulation in chloroplasts of Strategy I plants. Plants require a high demand of free ferrous Fe for their optimal growth and development, thus the proper function of FRO7, a ferric chelate reductase enzyme, is significantly necessary for the reduction-based chloroplast Fe uptake (Jain et al., 2014; Solti et al., 2014). However, the ability of the auto-reduction of Fe(III)-complexes may depend on the light conditions to stimulate the effective activity of the FRO enzyme (Jeong et al., 2008; Solti et al., 2012). For example, in response to Fe deficiency, the activity of FRO7 was induced in light conditions to uptake Fe but this enzyme was repressed in the dark conditions (Mikami et al., 2011) in intact barley chloroplasts. In Arabidopsis plant, the FRO7:GFP fusion protein was demonstrated to localize in the chloroplast envelope, and AtFRO7 is responsible for the efficient activity of photosynthesis and the Fe acquisition of proper chloroplast in young seedlings under Fe starvation (Jeong et al., 2008). Any disruption in the organization of FRO7 results in the decrease in chloroplast Fe content and the photosynthesis of Arabidopsis thaliana seedlings (Jeong et al., 2008). Solti et al. (2014) reported that chloroplast ferric-chelate reductase enzyme found in the inner envelope membrane of chloroplasts, and this enzyme played a role in the reduction of Fe(III) complexes and the production of free Fe^{2+} for Fe uptake in isolated Beta vulgaris chloroplast envelope fractions. This enzymatic activity prefers to bind with Nicotinamide Adenine Dinucleotide Phosphate (NADPH) than nicotinamide adenine dinucleotide (NAD) + hydrogen (H) (NADH) and dependently performed the Fe-acquisition mechanism on photosynthetic activity. In addition, the biphasic kinetics and its alteration
suggested the activity of low and high-affinity mechanisms to reduce ferric into ferrous Fe under Fe starvation (López-Millán et al., 2016). The *fro7* loss-of-function mutant lines collected less Fe than that of wild-type plants, around 33% Fe per µg Chl and had the typical signal in Fe limitation such as chlorotic appearance in plants. However, *fro7* mutant lines did not show any visible growth phenotypes under regular Fe condition and the phenotype of Fe-deficient plants was recovered by Fe addition (Mukherjee et al., 2006). Moreover, the staining of FRO7 promoter:GUS reporter lines declared the low expression in mature leaves which confirmed its functionality in early developmental stages (Jeong et al., 2008).

2.2.3.3 Other proteins involved in Fe transport across the chloroplast (inner) envelope

Two transporters from the YSL family, YSL4 and YSL6 are thought to bind with NA as the formation of Fe-NA complexes (Conte et al., 2009; Curie et al., 2009) and play a potential role as plastid Fe-efflux transporters (Divol et al., 2013). There are eight members in the *Arabidopsis* YSL family which can be divided into three groups based on similar sequences. Both YSL4 and YSL6 share up to 84% and 92% similar sequences (Chu, 2010). The organization of YSL4 and YSL6 is also similar since six exons are found in each gene. Moreover, the similarity is observed in each exon length, indicating that they may perform similar functions (Chu, 2010). In *Arabidopsis* plant, the expression of *YSL4* and *YSL6* were up-regulated in proplastids of ripening and germinating seeds, suggesting that they played a significant role in the mobilization and export of Fe from plastids during germination stage (Divol et al., 2013). *AtYSL4* and *AtYSL6* can transport Fe(II)-NA into the specific organ cells. Both of them were also located in the plasma membrane and up-regulated in generative tissues checked by quantitative real-time polymerase chain reaction (qRT-PCR) and YSL promoter:GUS construction. Loss of function of YSL4 and/or YSL6 leads to the alteration of Fe concentrations. Their expression was proved to be up-regulated under excess Fe but YSL6 was found in the chloroplast membrane observed in immunoblot and immunofluorescence techniques (Divol et al., 2013). However, the localization of YSL transporter family present in the outer or the inner envelope membrane of the chloroplasts is still controversial since this matter has yet to be identified by chloroplast proteomic analysis. Both YSL4 and YSL6 did not have the typical characteristic: the N-terminal chloroplast targeting peptide which is easily found in the outer envelope than inner envelope proteins. There is a relative correlation between the expression of *YSLs* and *Fer* genes in embryo tissues and leaf senescence that their physiological functions may
significantly contribute to the Fe detoxification during plastid differentiation in embryogenesis and senescent stage (Divol et al., 2013). Conte et al. (2013) reported that the role of YSL transporters was still controversial since the proteomic data and GFP-targeting were associated with the metal transport across tonoplast and endoplasmatic reticule membranes.

According to Conte et al. (2009), MAR1 - homolog of Ferroportin 1 and 2 – also known as IREG3 is related to chloroplast Fe uptake (Conte and Lloyd, 2010). MAR1/IREG3 is the member of the IREG/ferroportin transporter family that consists of IREG1/FPN1 and IREG2/FPN2 and controls the transport of metal through the plasma membrane in the tonoplast and the stele of the root system (Schaaf et al., 2006; Morrissey et al., 2009). MAR1/IREG3 is not only thought to be an opportunistic gateway of aminoglycoside antibiotics based on the sequence similarity to the metal transporters IREG1 and IREG2 (Schaaf et al., 2006; Morrissey et al., 2009) but also act in the Fe acquisition of the chloroplasts by taking up Fe-NA complexes. Therefore, MAR1/IREG3 plays a significant role in cellular Fe homeostasis in the plant cells (Conte and Lloyd, 2010). Previous studies showed that MAR1/IREG3 is localized to the chloroplast via targeting of a Yellow Fluorescing Protein (YFP) and is expressed in most tissues (Conte et al., 2009). MAR1/IREG3 is also involved in the Fe import from the cytosol under Fe limitation (Yang et al., 2010). MAR1/IREG3 transports aminoglycoside compounds into plastids and has a key function in maintaining the Fe homeostasis (Conte et al., 2009; Conte and Lloyd, 2010). Moreover, aminoglycosides utilize the polyamine to combine with Fe to move in specific compartments (van Bambeke et al., 2000) and NA - one of the main complexed ligands - is used to bind with Fe as Fe-NA complexes (Curie et al., 2009). This evidence leads to the possibility that MAR1/IREG3 might deliver the formation of Fe-NA into the chloroplasts (Conte et al., 2009; Conte and Lloyd, 2010). The expression of MAR1/IREG3 is negatively impacted under Fe starvation whereas MAR1 overexpression showed the adverse signals to affect the plant growth such as leaf chlorosis but phenotype can be recovered by adding Fe nutrition. Taken together, the function of MAR1/IREG3 may be very important in the storage and the sequestration of Fe in plants (Conte et al., 2009).

A putative plastid transporter (NAP14 known as ABCI11) following to ABC transporter nomenclature (Verrier et al., 2008), has been discovered in Arabidopsis thaliana that shares similar sequences to the cyanobacterial FutC unit from the iron transporter named FutABC (Shimon-Shor et al., 2010). NAP14 is known to play an essential role in the plastid transition...
metal homeostasis and its function as a part of the plastid transition Fe transport system (Shimoni-Shor et al., 2010). The \textit{in vivo} green fluorescent protein (GFP) procedure was performed to show evidence in the stroma of chloroplasts which At_CHLORO database connects NAP14 to the inner envelope membrane of chloroplasts, suggesting that NAP14 is a part of the membrane-intrinsic protein (Shimoni-Shor et al., 2010; Ferro et al., 2010). In nap14 loss-of-function mutant lines, a significant increase in the levels of Fe was 18 times in shoot tissues higher than that of wild-type plants, however, a significant reduction was around 50% in root cells in the same mutants (Shimoni-Shor et al., 2010). The nap14 mutant plants showed several adverse signals such as strong chlorosis, weak growth, and development of plants (Shimoni-Shor et al., 2010). In addition, NAP14 may associate with Fe-S cluster and shares the similarity to NAP7/SufC stroma which is found in NBD-NAP protein (Xu and Möller, 2004; Balk and Schaedler, 2014). The analyses of high-throughput proteomes informed the existence of some ABC transporters but their functions have yet to be identified in the inner envelope membrane such as NAP8/ABCB28 and NAP13/ABCI10 that play an essential role in maintaining Fe homeostasis in chloroplasts (Verrier et al., 2008; Ferro et al., 2010; Gutierrez-Carbonell et al., 2014). The existence of the half-size ABC transporter STA1/ATM3/ABCB25 was also analyzed and showed in the envelope of chloroplasts by proteomic studies.

The ideal candidate protein MFL1 is to import Fe into plastids. Ferro et al. (2010) informed that MFL1 is the member of the mitochondrial carrier family annotated as an inner envelope protein in the AT_CHLORO database and detected in the envelope proteome of \textit{Arabidopsis} plants. The expression of \textit{MFL1} highly increased in response to high amounts of Fe but mostly found in rosette leaves which are thought to be consistent with those genes coding for chloroplast proteins like PIC1 (Tarantino et al., 2011). However, the \textit{mlfl} loss-of-function mutant lines had a lower Fe concentration in both leaves and seedling than that of wild-type, indicating a possible role in the maintenance of Fe homeostasis (Tarantino et al., 2011). Taken together, AtMFL1 is identified to have a specific function to response in excess Fe (López-Millán et al., 2016).

2.2.3.4 \textit{Fe trafficking and storage in plastid}

Fe-S clusters are vital components for the activity of photosynthetic apparatus (Balk and Pilon, 2011). However, very little information is found on proteins associated with plastid Fe-S cluster synthesis. One Fe-S protein plays a specific role in controlling Fe homeostasis is NEET in
chloroplasts (Zuris et al., 2011). The NEET family has been identified to involve in various series of biological activities in plant cells (Nechushtai et al., 2012). They organize a unique structure: two protomers intertwine to arrange a two-domain structure, a $\beta$-cap and a unique redox-active unstable 2Fe-2S cluster binding domain (Paddock et al., 2007; Nechushtai et al., 2012). In Arabidopsis plant, GFP-targeting and immunoblot processes were performed to investigate the potential role of NEET and found that NEET protein was associated with Fe-S/Fe cluster (Nechushtai et al., 2012). The study of Ribonucleic Acid (RNA) interference showed the possible functions of this gene in the development of the plant, tissue senescence and balance ROS homeostasis (Nechushtai et al., 2012). In the case of excess Fe, AtNEET knockdown seedlings did not express any signal but showed the sensor responses to low levels of Fe, suggesting this protein is involved in the distribution and translocation of Fe. AtNEET knockdown mutant lines reduced in CAT abundance, a hem enzyme to support the ROS detoxification, presenting a significant role in the heme Fe (Nechushtai et al., 2012). In NEET mutant lines, the suppressed expression decreased in the growth and development of Fe in Fe limitation and the insensitivity of plant growth in response to the high concentrations of Fe, indicating that they are negatively affected in the uptake and distribution of Fe (Nechushtai et al., 2012). Taken together, AtNEET protein plays a key role in Fe homeostasis and the Fe regulation in the cellular metabolism (Mittler and Rizhsky, 2000; Luhua et al., 2008; Nechushtai et al., 2012). Zandalinas et al. (2019) showed that disrupting AtNEET function also disrupts Fe$_2$S$_2$ transfer from plastidial biosynthetic pathway to both plastidial and cytoplasm proteins. They also showed that AtNEET transfers its Fe$_2$S$_2$ clusters to DRE2, which is involved into Fe-S cluster biosynthesis in the cytoplasm. However, the mechanism of action of NEET remained unclear, since Fe$_2$S$_2$ clusters are not stable (Zuris et al., 2011).

2.2.4 The physiological role of Fe in the chloroplasts

In the plant cells, the majority of the Fe content is present in chloroplasts, mitochondria or stored safely in vacuoles (Thomine and Vert, 2013, Vigani et al, 2019). In shoot tissues, approximately 80 – 90% of cellular Fe is typically found in chloroplasts (Terry and Low, 1982; Terry and Abadía, 1986; Morrissey and Guerinot, 2009), 60% of which is bound in thylakoid membranes (Castagna et al., 2009). Chloroplasts are derived from free-living cyanobacteria becoming endosymbiont of mitochondria-containing ancient eukaryotic host cells (Gould et al.,
They are site of photosynthetic electron transport and many essential metabolic pathways (Zhu, 2016). In chloroplasts, Fe is found in all electron transport complexes such as Fe-S cluster-containing proteins in PSI, Rieske protein in the cytochrome *bdf* complex and soluble ferredoxins (Briat et al., 2015; López-Millán et al., 2016; Hantizis et al., 2018) along with heme-containing cytochromes in PSII and the cytochrome *bdf* complex and non-heme Fe cofactor in PSII reaction centre and Fe superoxide dismutase (FeSOD) (Briat et al., 2007; Balk and Schaedler, 2014) as well as necessary for biogenesis cofactors such as heme (cytochrome and P450s), Fe–S cluster (Fe$_2$S$_2$, Fe$_4$S$_4$), mononuclear, di-Fe enzymes, and Fe-bound to ferritin which act as an Fe storage and sequestering Fe to maintain intracellular Fe homeostasis (Tian and DellaPenna, 2004; Gray et al., 2004). Fe-S clusters are essential in controlling the Fe homeostasis since it can behave as an Fe sensor that regulates gene expression among others ferritin (Beinert et al., 1999). Besides being a part of the photosynthetic electron transfer, Fe also participates in Chl biosynthesis (Meguro et al., 2011). Fe is essential for Chl biosynthesis since Fe deficiency switches off the Mg protoporphyrin IX monomethyl ester oxidative cyclase (Spiller et al., 1982) and is also a component of a Rieske-like Fe–S protein of Chl *a* oxygenase in the chloroplast inner envelope (Reinbothe et al., 2006; Hu et al., 2017). Moreover, Fe-S clusters are considered as cofactors of carrier proteins that avoid the toxicity connected with free Fe and allow its delivery at lower intracellular concentrations (Mansy Cowan, 2004). Plant-specific Fe proteins with enzymatic functions include diverse pathways as adenosine-5’-phosphosulfate reductase in sulfate assimilation (Fe$_4$S$_4$), aminocyclopropane-1-carboxylic acid oxidase in ethylene synthesis or the alternative oxidase in respiration (Moseley et al., 2000; Tottey et al., 2003; Mochizuki et al., 2010). Fe is also required for the proper function of nitrate and sulphate assimilation as redox cofactors in essential enzymes such as the nitrate reductase (hem) in the cytosol and nitrite reductase (Fe$_4$S$_4$), glutamate synthase (Fe$_3$S$_4$), adenosine phosphosulphate reductase (Fe$_2$S$_2$) and sulphite reductase (Fe$_4$S$_4$) in the chloroplast (Marschner, 1995; Balk and Lobreaux, 2005; Astolfi et al., 2006; Borlotti et al., 2012). For enzymes such as cytosolic aconitase, aldehyde oxidases and DNA repair enzymes in the nucleus, Fe-S clusters are found to be synthesized by the cytosolic Fe-S cluster assembly apparatus (Balk and Schaedler, 2014).

In case of plants absorb too much Fe, it generates ROS that leads to oxidative damage (Briat et al., 2010). In chloroplasts, this is a serious problem since ROS, e.g. hydrogen peroxide (H$_2$O$_2$) produced by the photosynthetic electron chain (Asada, 1999; Mubarakshina et al., 2010).
Consequently, hydroxyl radicals are formed from free Fe via Fenton reaction (Halliwell and Gutteridge, 1992). Whereas, if chloroplasts are in the lack of Fe, Fe deficiency negatively impacts on Chl biosynthesis and other pigments related to light harvesting, as a result, leaves will turn to typical symptoms of Fe-deficient chlorosis and also affects the photosynthetic apparatus where it requires high amount of Fe (Spiller and Terry, 1980; van Leeuwe and Stefels, 1998; Moseley et al., 2002). Under Fe limitations in the chloroplasts, proper function of PSI containing 12 Fe atoms per monomer is disrupted. In PSII, the synthesis of proteins such as D1-protein is also impacted by Fe starvation (Greene et al., 1992; Vassiliev et al., 1995). Moreover, the significant decrease in Fe-S cluster containing proteins (such as ferredoxin) negatively affects the activity of the photosynthetic electron transport process (Erdner et al., 1999; McKay et al., 1999) which leads to the inefficient photosynthesis (Geider et al., 1993; Davey and Geider, 2001). Therefore, Fe homeostasis should be conserved to provide enough Fe for plant growth and development. Since high levels of Fe are always needed for oxygen-producing chloroplasts, photosynthetic plants not only have to enivolve multifaceted approaches to uptake optimal Fe to avoid the Fe limitation but also prevent the cell damage caused by Fe toxicity (Ivanov et al., 2012). The import and export Fe in plastids as well as storing Fe in plant organelles (e.g. in vacuoles) is needed to be firmly managed (Abadía et al., 2011; Briat et al., 2015). Thus, to uptake bioavailable Fe in adverse conditions, plants have many systems to increase the Fe uptake via soil acidification, reduction of ferric Fe or secretion of phytosiderophores (Puig and Peñarrubia, 2009). When plants suffer the Fe limitation, the very common response has been seen is the upregulation of Fe specific transporters to cope with this serious problem. In contrast to Fe deficiency, Fe has to bind with chelators or active transporters to sequester high amounts of Fe under Fe excess (Pilon et al., 2009).

There are several signals to be recognized in response to an adverse environment. Living organisms have to enivolve many systems to uptake the optimal amount of Fe from the environment via soil acidification, reduction, Fe chelators or up-regulation of Fe transporters and transport Fe through the plant body (Takagi, 1976; Römheld and Marschner, 1986). Under Fe starvation, the up-regulated transcription is expressed as the common signal to accumulate Fe in response to adverse conditions (Kobayashi and Nishizawa, 2012; Hindt and Guerinot, 2012; Rodríguez-Celma et al., 2013). In non-graminaceous and graminaceous plants, the prime transcription factors have been investigated the ability of Fe acquisition in different plant species.
(Kobayashi and Nishizawa, 2012; Hindt and Guerinot, 2012). In the phloem, Fe thought to be chelated with NA and deoxymugineic acid to mobilize Fe to the plant body, both of them are also known as the possible signaling molecules and their concentration is more or less depended on the different plant species (Curie et al., 2009; Kobayashi and Nishizawa, 2012; Nishiyama et al., 2012). For example, a high level of NA increased in the transgenic rice lines, indicating that Fe is entered into the Fe-limiting plant cells (Cheng et al., 2007; Wang et al., 2013; Nozoye et al., 2014). Plants always have the response to adverse conditions and develop many systems to solve either Fe starvation or excess Fe to maintain Fe homeostasis. Chloroplasts also play an important role in a sense of unfavorable agricultural environment. In the chloroplast, hormones (salicylic acid (SA), jasmonates, and ABA), reactive oxygen species, reactive nitrogen species, and redox signals are normally produced to respond to stress conditions (Pfannschmidt and Yang, 2012; Trotta et al., 2014; Bobik and Burch-Smith, 2015). SA is the phenolic compound synthesized in the chloroplasts and has the capacity of defense mechanisms to protect plant species (Bobik and Burch-Smith, 2015). Jasmonic acid (JA) is the product of octadecanoid pathway and is the potential hormone in the chloroplasts to support plant growth and development (Leon, 2013). JA has a connection with another hormone such as ethylene as the signals in plant stress (Kunkel and Brooks, 2002). Among them, ABA is the perfect hormone to have signals and is positively tolerant of different kinds of stresses. This hormone has an essential function in many physiological processes such as stomatal movement and seed dormancy in various plants (Wensuo and Zhang, 2008; Mdel et al., 2009; Kim et al., 2010). In plastids, ABA is thought to be the product of methylerythritol phosphate pathway that generates catotenoids inside the plant cells (Finkelstein, 2013). Many scholars have investigated the possible mechanisms of ABA gene in recent years (Yoshida et al., 2015). ABA receptors are known to belong to the PYRabactin Resistance1 (PYR1)/Pyr1-Like (PYL)/Regulatory Components of ABA Receptors (PYR/PYL/RCAR) family (Park et al., 2009) that can stimulate the transcriptional levels of ABA genes (Kline et al., 2010). However, the gene expression is affected by calcium-dependent kinases and phosphatases due to the downstream of the ABA receptors (Finkelstein, 2013; Yoshida et al., 2015). The relative connection between SA, JA and ABA contribute to supporting the plant resistance in the chloroplasts (Bobik and Burch-Smith, 2015). ROS play an important role in the signaling factor if ROS is in small concentrations but its high amounts of ROS are in danger for damage plant cells in the chloroplasts (Miller et al.,
ROS is produced in the plastids that are activated to change the expression of nuclear genes as the sensor signals in the stress plants (Mor et al., 2014). Another important component - nitric oxide (NO) acts as the second messenger in plant cells and found in the chloroplasts (Mandal et al., 2012; Tewari et al., 2013). NO shows to have positive effects on physiological mechanisms such as germination stage, seed dormancy, the floral transition and respond to the stress environments (Wendehenne et al., 2004; Moreau et al., 2008; Sanz et al., 2015). Moreover, the signaling of NO has an association with other signalings including SA and JA (Wendehenne et al., 2004; Zhou et al., 2015). Taken together, NO is known to play the main role in plant protection under adverse Fe nutrition.

2.3 Fe must be present in plants in an appropriate amount
The uptake of essential elements must be in an optimum range to meet the plant’s nutritional requirements. When any essential micronutrients, especially Fe, are not available in sufficient quantity, plants use highly efficient systems to take it up from the medium. However, activating Fe uptake is facing to double jeopardy (Thomine and Vert, 2013): not only too little Fe leads to the poor plants’ growth (Mora-Macías et al., 2017) and the entry of other potentially metal toxicity (Morrissey and Guerinot, 2009) but also too much Fe itself toxic, and causes damage to plant cells. Therefore, the build-up of Fe reserves within the plant cells is rather complicated and needs a tightly controlled Fe uptake (Guerinot and Yi, 1994; Kampfenkel et al., 1995; Curie and Briat, 2003) to maintain Fe homeostasis for plant growth and development (Jeong and Guerinot, 2009; Zhu et al., 2016; Connorton et al., 2017; Jeong et al., 2017).

2.3.1 Fe deficiency in plants
Fe is quite abundant in soils but it tends to be poorly available to plants (Thomine and Vert, 2013). It can alternate between two oxidation states: reduced, as Fe$^{2+}$ or oxidized, as Fe$^{3+}$ (Shenker and Chen, 2005). In aerated soils Fe$^{3+}$ form is dominant, the fundamental difficulty is the low solubility of ferric oxides and hydroxide minerals, such as goethite and hematite (Halliwell et al., 1992). The general reaction of hydroxide precipitation is presented by the equation: Fe$^{3+}$ + 3(OH)$^-$ $\leftrightarrow$ Fe(OH)$_3$ (solid). Such kind of Fe complexes are found in aerobic conditions and at the neutral or alkaline pH (Guerinot and Yi, 1994). The most abundant Fe species: Fe(OH)$_2$$^+$, Fe(OH)$_3$ and Fe(OH)$_4$$^-$ are found in soil solution with pH 4–9 (Shenker and Chen, 2005). Approximately 30% of global agricultural soils are found to be calcareous with low
Fe (Guerinot and Yi, 1994; Mori, 1999). As a result, plants grown in calcareous soils and high pH, especially non-graminaceous plants, usually suffer from Fe shortage which alters their growth and development (Fourcroy et al., 2016). Thus, Fe deficiency is among the most important challenges of the agriculture but also closely involved in the prevalence of Fe deficiency in human’s anemia (Murgia et al., 2012) because of plants are considered as the principal source of Fe in most diets for the majority of the Earth’s population (Guerinot, 2001; Clemens et al., 2002).

The optimum concentration of Fe required for root uptake and typical plant leaf is ranging from $10^{-4}$ M to $10^{-9}$ M and from 50 to 100 µg g$^{-1}$ dry weight (Guerinot and Yi, 1994) for many so-called Fe-sufficient plants (Jin et al., 2014). However, the concentration of ferrous and ferric Fe in most soils is less than $10^{-15}$ M (Marschner, 1995). Thus, the lack of Fe is one of the serious limiting factors in the metabolism of plants (Moseley et al., 2002). Subcellular organelles, such as chloroplasts and mitochondria, the most active sites of the plant metabolism, require Fe for operating their biosynthetic pathways and stabilization of essential activities. Fe deficiency negatively impacts metabolic functions and leads to the disturbance of Fe homeostasis of both chloroplasts and mitochondria (Vigani et al., 2013, Vigani et al., 2019).

The typical symptom of Fe limitation is the interveinal chlorosis. It is widespread among agricultural plants which initially results in yellow coloration due to the inhibited Chl biosynthesis between the veins of young leaves while the veins remain green. Shoot chlorosis is accompanied by poor root formation. In the final stage, the whole leaf turns to yellow (Bertamini et al., 2001). In some cases, the deficient leaf has white color (due to the cell wall cellulose) with necrotic lesions. The manifestation of the symptoms in young leaves is due to the inability to redistribute Fe within the plant. The phenomenon results in growth retardation, stagnation and plant death (Guerinot and Yi, 1994). Chl content is known to correlate with the amount of Fe in the chloroplasts (Rout and Sahoo, 2015). Fe controls the quantity of $\delta$-aminolevulinic acid (DALA) formation which is the precursor of porphyrins (Miller et al., 1982). During Fe limitation, a reduction occurred in the condensation rate of glycine and succinyl-CoA to form DALA. Therefore, measurement of Chl content is considered as an appropriate indicator of the Fe status of the plant (Thoiron et al., 1997).

The yellow leaf symptom is associated not only with the retardation in the Chl biosynthesis but also affect the biogenesis of the photosynthetic apparatus (Curie and Briat,
Also, the common feature of Fe starvation significantly declines protein synthesis in the chloroplasts (Shetty and Miller, 1966), which is reflected by the decrease in the number of ribosomes, the sites of protein synthesis (Lin and Stocking, 1978). In developed leaves, the composition photosynthetic apparatus remained intact upon Fe deficiency but the number of photosynthetic units significantly decreased (Terry, 1980). To be more specific, the reduction and loss of PSII and inhibition of PSI activity are observed in chlorotic leaves (Bertamini et al., 2001, Basa et al., 2014). Chl $a$ fluorescence induction has been applied as an effective tool to measure the status of photosynthetic apparatus, especially PSII under Fe-deficiency (Kampfenkel et al., 1995; Abadía et al., 1999; Basa et al., 2014). Moreover, chloroplasts suffering from Fe deficiency show an increased heat dissipation and have an impaired CO$_2$ fixation capacity when compared to Fe-sufficient plants (Timperio et al., 2007; Sharma, 2007). The amount of assimilates also decreased under the Fe-limiting condition (Srivastava et al., 1998). The utilization of ribulose-1,5-bisphosphate by Rubisco and its regeneration by the Calvin cycle appear compromised in Fe-deficient plants (Arulanathan et al., 1990).

### 2.3.2 Fe excess in plants

Besides the serious effects of Fe starvation, Fe is also potentially toxic for plants at high concentrations. High soil Fe doses lead to imbalance in the uptake and homeostasis of other essential nutrients (e.g. Mn, P, K, Ca and Mg) in plants due to their decreased absorption which result in variations in permeability, transfer activity, enzyme reactions and lipid phase transition characteristics of the membranes in plants (Adamski et al., 2012). Soil conditions in flooded and acid soils may result in the uptake of an excess of Fe. The excess in the accumulation of Fe can be fatal to plant growth and damages the photosynthetic apparatus (Kampfenkel et al., 1995) which leads to a reduction in the crop yield. Fe stress is common in rice cultures where the first visible symptom of the excess amount of Fe in the plants is the leaf bronzing. Due to the stress generated superoxide radicals and hydrogen peroxide the excess Fe leads to the production of Fe$^{2+}$ by the Haber-Weiss and Fenton reactions (Fe$^{3+}$ + $\cdot$O$_2^-$ $\rightarrow$ Fe$^{2+}$ + O$_2$; Fe$^{3+}$ + H$_2$O$_2$ $\rightarrow$ Fe$^{2+}$ + HO$\cdot$ + HO$^-$), respectively, in the cells (Halliwell and Gutteridge, 1992). This causes intensive oxidative stress damaging most cellular macromolecules (including lipids, nucleic acids, and
proteins) (Rao et al., 2011), Chl and almost every other organic constituent of living cells (Becana et al., 1998; Fang et al., 2001), and finally leads to cell death (Halliwell and Gutteridge, 1984). Even under optimal Fe nutritional conditions, plants frequently face the problems of transporting, storing and assembling Fe into active cofactors and at the same time, protect themselves against the oxidative damage caused by the interaction of Fe and oxygen (Liochev and Fridovich, 1999).

Plants suffered from the toxic free Fe commonly show several typical symptoms (Cook, 1990): leaf bronzing, extensive tissue necrosis (Jeong and Guerinot, 2009), wilting of shoots, yellowing and dieback of oldest leaves (especially from the tips or margins), blackening of leaf tips and stem bases, lack of root branching (Snowden and Wheeler, 1993), blackening of the root (particularly of the apices) (Laan et al., 1991), reduction in leaf size, deepening of green leaf colour (particularly in the youngest leaves), reddening or purpling of stems and older leaves and formation of precipitates on roots (Snowden and Wheeler, 1993). High Fe concentrations also cause a reduction in plant growth (such as height, tillering and an increase in the sterility of the panicle), number and area of leaves, the alteration in photosynthetic efficiency and cellular damages (Briot et al., 2010). For example, Hemalatha and Venkatesan (2011) showed that tea plants suffered typical toxicity symptoms under excess Fe and the abaxial side of leaves turned to coppery red at the later stages. Chl and carotenoid content decreased with increased Fe concentrations (Arunachalam et al., 2009). In addition, ROS-induced oxidation of protein will result in differences in the three-dimensional protein structure, fragmentation and/or cross-linking of the proteins which are proved to be more susceptible to degradation. ROS cause the strand breaks of DNA, removal of nucleotides and serial modifications of the organic bases of the nucleotides which lead to permanent changes or damage to the DNA with potentially detrimental effects for the cell (Sharma et al., 2012). Kampfenkel et al. (1995) reported that Fe overload is accompanied by photoinhibition, imbalance in the activity of the photosystems and increased ROS production (Donnini et al., 2003). A high level of Fe also contributes to a relatively higher cytochrome $b_6/f$ complex level in thylakoids (Suh et al., 2002). The high concentrations of Fe also affect the activity of enzymes. The amylase activity decreased with increased Fe amounts since Fe forms complexes in plants with carbohydrate compounds such as maltose (Hemalatha and Venkatesan, 2011).
2.3.3 Responses to Fe stress

To deal with the high concentrations of Fe, plants have to multi-face with many possible ways to balance Fe homeostasis (Wheeler et al., 1985). Silveira et al. (2007) reported that, in rice EPAGRI 108 cultivar, the concentration of Fe in shoots was 2.5 times lower than that of BR-IRGA409 cultivar under Fe overload status. Another way is to store high amount of Fe depends on the capacity to sequester and store Fe at a safe and bioavailable form by specific metal-binding ligands (Floyd, 1983; Baker and Gebicki, 1986) or complex with amino acids, organic acids and peptides which could be stable at the corresponding pH (Nagajyoti et al., 2010; Álvarez-Fernández et al., 2014). Fe can bind with chelators (citrate and NA) to reduce the high concentration of Fe in shoots (Floyd, 1983; Baker and Gebicki, 1986). Among them, citrate is considered as the main Fe-chelator found in xylem exudates by forming Fe(III)-citrate complexes in Strategy I plants (Cataldo et al., 1988; Rellán-Álvarez et al., 2010). In rice, citrate transporter (OsFRDL1) is responsible for the translocation of Fe and identified in the root pericycle cells (Yokosho et al., 2009). NA is one of the common chelators and plays a central role in the regulation of Fe uptake and its internal transport (Stephan and Scholz, 1993). Although displaying the typical symptoms of Fe deficiency, chloronerva mutants accumulated a high amount of Fe and other metals growing in normal Fe conditions which indicated the important role of NA in Fe transport (Pich and Scholz, 1991). Plant overexpressing NAS genes contained a higher amount of Fe, Mn, and Zn in leaves (Douchkov et al., 2005).

Another way to cope with the very high concentrations of Fe in plants is homeostasis, kinetics and the consequences of Fe uptake (Deiana et al., 1992). Caffeic acid is shown to mobilize Fe at the soil-root interface under such kind of adverse condition (Römheld and Marschner, 1983). Caffeic acid is the phenolic compound produced by plant species (Whitehead et al., 1983; Jin et al., 2008) to reduce ferric Fe and is involved in redox reactions near the rhizosphere and free spaces (Olsen et al., 1981; Deiana et al., 1992).

Excess Fe can also be stored in vacuoles. In cereal grains such as wheat and rice, most Fe is present in cell vacuoles in the aleurone layer which is often removed during grain processing (Kyriacou et al., 2014). VIT1 was first identified in Arabidopsis plant as an orthologue of the yeast Fe transporter Ca2+-Sensitive Cross-Complementer (CCC1) and the loss-of-function mutants in VIT1 disturbed the pattern of Fe distribution (Kim et al., 2006; Roschztartatz et al., 2009). The action of the efflux transporters NRAMP3 and NRAMP4 releases Fe from vacuoles
into the cytosol during germination (Lanquar et al., 2005). Genes from the VIT family are also known to be essential for Fe localization in rice grains and Brassica seeds (Zhang et al., 2012; Zhu et al., 2016). Moreover, excess Fe can be retained in roots which is the aspect of physiological root-based tolerance in rice (Becker and Asch, 2005), thus Fe exclusion or retention in roots played a significant role in protecting plants avoid getting oxidative stress (Mahender et al., 2019).
3. AIMS

Although the Fe homeostasis of chloroplasts is essential to maintain the physiological functions of chloroplasts including operation of the photosynthetic electron transport, the control of chloroplast Fe uptake is hardly known. Similarly, we are also in a lack of information, how the Fe acquisition of chloroplasts alters in the function of the developmental program of the leaves and in that of alterations of the Fe nutrition of plants. Although previous studies indicated that PIC1 and NiCo are essential and substantial components of the chloroplast Fe acquisition machinery that are suggested to be involved into the uptake of free ferrous ions reduced by the chloroplast ferric chelate reductase enzyme, no information exists how the expression and amount of these components change during the developmental program of the leaves and the Fe nutritional status of the plants. YSL4 and MAR1 are also showed previously to have an importance in the chloroplast Fe homeostasis, their role, indeed, is rather doubtful in the Fe uptake of chloroplasts. To clarify the relationship between developmental status, Fe nutrition and chloroplast Fe homeostasis we examined the following questions and hypotheses:

- YSL4 and partly MAR1 are thought to be involved in the transportation of (Fe) NA complexes. May they have a role in the Fe uptake of chloroplasts?
- Chloroplasts should maintain their Fe content in a relatively narrow range to operate physiological functions but to avoid oxidative damage. Does the excess of Fe lead to the downregulation of the chloroplast Fe uptake machinery?
- The majority of Fe in the chloroplasts required by the photosynthetic electron transport chain. Senescence induces the decomposing of the photosynthetic apparatus. How the Fe transportation machinery regulated in senescing leaves? May the Fe uptake components also play a role in the Fe release from the chloroplasts?

To clarify these questions, chloroplast physiological functions (operation of the photosynthetic apparatus), alterations in Fe content, changes in the expression of genes of interest and the number of proteins of interest were measured.
4. MATERIALS AND METHODS

4.1 Plant material and growth conditions

The seeds of oilseed rape (Brassica napus L. cv. DK Exquisite) were a kind gift of Ing. agr. Rudolf Solti) were used in all experiments. Firstly, they were germinated in Petri dishes containing wet papers at moderate light (<100 µmol photons m\(^{-2}\) s\(^{-1}\) Photosynthetic Photon Flux Density; PPFD). Seven-day old seedlings were further grown on half-strength Hoagland solution (2.5 mM Ca(NO\(_3\))\(_2\), 2.5 mM KNO\(_3\), 1.0 mM MgSO\(_4\), 0.5 mM KH\(_2\)PO\(_4\), 0.16 µM CuSO\(_4\), 9.2 µM MnCl\(_2\), 0.38 µM ZnSO\(_4\), 0.24 µM Na\(_2\)MoO\(_4\), 23.12 µM H\(_3\)BO\(_3\) and 20 µM Fe(III)-Ethylenediaminetetraacetic acid (EDTA) as Fe source) in 12 litre containers. Each container includes 5 seedlings under controlled conditions (14 h 120 µmol photons m\(^{-2}\) s\(^{-1}\) PPFD/ 10 h dark periods, 24/22 °C and 75/80% relative humidity) until reaching the four-leaf stage. To induce Fe deficiency, four-leaf-stage plants were transferred to an Fe-free half-strength Hoagland solution containing 0.5% (w/V) CaCO\(_3\). To induce Fe excess, the four-leaf-stage plants were cultivated on half-strength Hoagland solution containing 100 µM Fe(III)-EDTA. As for optimal Fe nutrition, plants were further cultivated according to the pre-cultivation parameters (20 µM Fe(III)-EDTA). Optimal Fe nutrition is also referred to as ‘control’ in the text. During all the time of cultivation, nutrient solutions were constantly aeriated. The half-strength Hoagland solution containing different amounts of Fe treatment was refreshed one time per week. Changes in the parameters of leaves developed partially before the treatments and totally under the treatments (further referred to as 4\(^{th}\) and 6\(^{th}\) leaves, respectively) were monitored during the 35-day treatment time. At time points of weekly regularity (7, 14, 21, 28, and 35 days) leaves were used for physiological measurements and collected for samplings. To let plants go through their full life cycle, plants are grown in soil culture conditions (14 h 120 µmol photons m\(^{-2}\) s\(^{-1}\) PPFD/ 10 h dark periods, 24/22 °C and 75/80% relative humidity) until they have developed around 20 leaves. To induce generative transformation, plants were grown for around 2 months at 120 µmol photons m\(^{-2}\) s\(^{-1}\) PPFD at 4 °C. Flowers, siliques of various developmental stages and germinating seedlings were collected and sampled for later studies.

4.2 Chlorophyll \(a\) fluorescence induction

Chl \(a\) fluorescence induction measurements were carried out on intact selected leaves using a PAM 101-102-103 Chl Fluorometer (Walz, Effeltrich, Germany) equipped with KL 1500
electronic light source (Schott, Mainz, Germany). Selected leaves were firstly dark-adapted before the measurement for 30 min. The $F_0$ level of fluorescence was determined by switching on the measuring light, the modulation frequency of 1.6 kHz and at PPFD less than 1 µmol m$^{-2}$ s$^{-1}$ after 3 s illumination with far-red light to reject reduced electron carriers (Belkhodja et al., 1998). The maximum fluorescence yields, $F_m$ in the dark-adapted state and $F_m'$ in the light-adapted state were measured by applying a 0.7 s pulse of white light (PPFD of 3,500 µmol photon m$^{-2}$ s$^{-1}$, light source: KL 1500 electronic, Schott, Mainz, Germany). The variable fluorescence was determined as $F_v = (F_m - F_0)$. For the analysis of quenching, actinic white light (PPFD of 100 µmol m$^{-2}$ s$^{-1}$, KL 1500 electronic) was provided. Simultaneously with the onset of actinic light, the modulation frequency was switched to 100 kHz. The steady-state fluorescence of light-adapted state ($F_s$) was determined when no change was found in maximal fluorescence values ($F_m'$) between two white light flashes separated by 100 s. All stress factors inhibit the activity of photosynthetic, leading to light stress, the quenching parameters were used to assess the excitation energy allocation in all samples as follows (Hendrickson et al., 2005):

\[
\Phi_{PSII} = \left(1 - \frac{F_s}{F_m'}\right) \cdot \frac{F_v/F_m}{F_vM/F_mM}
\]

\[
\Phi_{NPQ} = \left(\frac{F_s}{F_m'} - \frac{F_s}{F_m}\right) \cdot \frac{F_v/F_m}{F_vM/F_mM}
\]

\[
\Phi_{f,D} = \left(\frac{F_s}{F_m}\right) \cdot \frac{F_v/F_m}{F_vM/F_mM}
\]

\[
\Phi_{NF} = 1 - \frac{F_v/F_m}{F_vM/F_mM}
\]

$\Phi_{PSII}$: the photochemical efficiency of functional PSII centers; $\Phi_{NPQ}$: ΔpH dependent, xanthophyll-cycle coupled non-photochemical quenching; $\Phi_{f,D}$: fluorescence/thermal dissipation of the absorbed energy; $\Phi_{NF}$: the thermal dissipation by inactive PSII centers. $F_vM/F_mM$ was applied as the mean of $F_v/F_m$ values of control (quasi non-inhibited) plants grown under optimal Fe nutrition according to Solti et al. (2014).
4.3 Determination of chlorophyll content

Chl content of fresh selected leaves was determined from 5 leaf disks of 5 mm diameter homogenized quickly in 80% (V/V) of buffered acetone (with 5 mM Tricine-KOH pH 7.8) at low light. The extract was centrifuged at 10,000 ×g for 5 min (4 °C). The absorbance of the supernatants was recorded at 646.6 nm and 663.6 nm by a UV-VIS spectrophotometer (Shimadzu, Japan). Chl \(a\) and \(b\) contents were calculated according to the formula of Porra et al. (1989):

\[
\text{Chl } a \ (\mu g \ cm^2) = (12.25 \times A_{663.6} - 2.55 \times A_{646.6}) \times \text{volume (ml)} \times \text{dilution/surface (cm}^2)\]

\[
\text{Chl } b \ (\mu g \ cm^2) = (20.31 \times A_{646.6} - 4.91 \times A_{663.6}) \times \text{volume (ml)} \times \text{dilution/surface (cm}^2)\]

4.4 Determination of leaf Fe content

Approximately 100 mg of fresh selected leaves were homogenized in liquid nitrogen. One ml washing buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH (pH 7.0), 330 mM sorbitol, 2 mM MgCl\(_2\)) was added and vortexed well, and 250 µl mixture was used for the determination of leaf Fe content (3 replicates). The mixture was solubilized at room temperature for 30 min by adding 1% (w/V) sodium dodecyl-sulphate (SDS; Sigma) and 1% (w/V) dithiothreitol (DTT; Sigma). Non-solubilized material (starch) was removed by centrifugation at 10,000 ×g for 5 min at room temperature. After adding 100 µM ascorbic acid and 300 µM bathophenanthroline disulphonate disodium salt (BPDS; Sigma) to the supernatants, the mixtures were incubated at room temperature in the dark condition for at least 60 min. Fe content was determined by measuring the absorbance of the Fe\(^{II}\)-BPDS complex at 535 nm by UV-VIS spectrophotometer (Shimadzu, Japan) using an extinction coefficient of 22.14 mM\(^{-1}\) cm\(^{-1}\) (Smith et al., 1952).

4.5 Determination of chloroplast Fe content

About 3 g leaves were homogenized in isolating buffer (50 mM HEPES-KOH, pH 7.0, 330 mM sorbitol, 2 mM EDTA, 2 mM MgCl\(_2\), 0.1% (w/V) BSA, 0.1% (w/V) Na-ascorbate) at 4 °C for 2 x 3 s by Waring Blender. The homogenate of selected leaves was then filtered via four layers of gauze and two layers of Miracloth\textsuperscript{TM} (Calbiochem-Novabiochem, San Diego, CA, USA). Chloroplasts were immediately pelleted by centrifugation in a swing-out rotor (1,600 ×g, 5 min, 4 °C). The supernatant was carefully removed, and the pellet was resuspended in 1.2 ml
washing buffer (50 mM HEPES-KOH (pH 7.0), 330 mM sorbitol, 2 mM MgCl₂). The diluted chloroplast suspension was imaged in a Bürker chamber by a light microscope (Nikon Optiphot-2) equipped with a Nikon D70 camera. The number of chloroplasts was counted using the ImageJ software (rsbweb.nih.gov/ij) with a Cell Counter plugin. Iron determination in 250 µl resuspended pellet (3 replicates) was carried out as in 2.4 and the amount of Fe was calculated per chloroplast.

4.6 Expression analysis

4.6.1 RNA extraction and cDNA synthesis

*Brassica napus* germinated seeds, seedlings, target leaves, flowers, and siliques of various developmental stages were used in RNA isolation. Approximately 100 mg of fresh tissue samples immediately frozen in liquid nitrogen and stored at -80 ºC were subjected to total RNA extraction using TRI Reagent® (Sigma). All the steps were carried out in the sterilized chamber. Tissue samples were homogenized in liquid nitrogen, then 1 ml of TRI Reagent was added. Next, 0.2 ml chloroform per ml of TRI Reagent was used to separate messenger RNA (mRNA) according to the manufacturer’s instruction. The mixture was vortexed well and kept at room temperature for 15 min. It was centrifuged at 14,000×g for 15 min at room temperature to separate RNA, DNA, and protein layers. The chloroform phase was carefully collected, and 0.5 ml isopropanol was added to precipitate RNA. The mixture was vortexed well and incubated at -20 ºC for 10 min. After centrifugation for 20 min at 18,000×g (4 ºC), the upper phase was gently removed without disturbing the pellet. The nucleic acid pellet was washed in 1 ml of 75% ethanol (made with Diethyl Pyrocarbonate (DEPC) treated water), and centrifuged for 10 min at 18,000 ×g (4 ºC). The washing step was repeated one or two more times. The pellet was completely dried at -20 ºC for 30 min, then dissolved in 50 µl DEPC treated water completely. The RNA concentration was quantified by a Nanodrop ND-1000 spectrophotometer (Thermo-Fisher Scientific Inc., Waltham, MA, USA).

Preparation of complementary DNA (cDNA) libraries was carried out in the icebox. 20 µl RNA extract was mixed in new Polymerase Chain Reaction (PCR) tube with 2.5 µl Deoxyribonuclease (Dnase) buffer, 0.5 µl Ribonuclease (RNase) inhibitor and 2 µl RNase-free DNase I (Thermo-Fisher Scientific) to eliminate residual genomic DNA contamination. The mixture was vortexed well and incubated at 37 ºC for 60 min. After that, 1.25 µl of 50 mM
EDTA was added to the mixture and incubated at 70 °C for 10 min. Next, 5.5 µl random hexameric oligonucleotides (50 µM) + 5 µl DEPC were added to the sample. It was mixed well and incubated at 65 °C for 5 min. Finally, 5.5 µl deoxyribonucleotide triphosphate (dNTP, 10 mM), 11 µl Reverse Transcriptase buffer, 1 µl RNase inhibitor and 2 µl RevertAid Reverse Transcriptase (Thermo-Fisher Scientific) were supplied to the mixture to transcribe the RNA pool by 1× PCR reaction. The reaction was then incubated for 45 min at 42 °C, and finally for 10 min at 70 °C. The cDNA libraries were divided into small aliquots and stored at -80 °C for further use in qRT-PCR (StepOnePlus Real-Time PCR system, Applied Biosystems).

4.6.2 Selection of optimal primers for expression analysis
According to the target genes, specific primers were designed at exon-exon border sites to avoid genomic DNA contamination. The details of the specific primer sequences are given in Table 1. The optimal primers followed these parameters: T_m around 60 °C and product size range 100 – 200 base pairs, primer sequences with a length of 20 – 22 nucleotides with an optimum at 20 nucleotides and a GC content of 40 – 60%. Primers were checked for the absence of stable hairpins and dimers using OligoAnalyzer, the free software is available here: https://eu.idtdna.com/pages/tools/oligoanalyzer.

4.6.3 Identification of optimal temperature and primer concentration of target genes
50 ng cDNA was used in 10 µl PCR reaction mixture using Taq polymerase (Thermo Fisher Scientific) under the following cycling conditions: an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of 95 °C for 10 s, annealing at 72 °C for 15 s, with a final elongation step of 72 °C for 1 min.
Table 1. Oligonucleotide primers used in the expression analysis of chloroplast Fe metabolism-related transporters

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genbank accession</th>
<th>Arabidopsis ortholog</th>
<th>Primer</th>
<th>Primer sequences</th>
<th>Tm (°C)</th>
<th>Product size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BnMAR1</td>
<td>Bra020559</td>
<td>At5g26820</td>
<td>Fw</td>
<td>5’ - GGCTCTTCTCAGACAATCTCC - 3’</td>
<td>59</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rev</td>
<td>5’ - TGCGAAGCTCCAGACAAACC - 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BnNiCo</td>
<td>Bra037287</td>
<td>At2g16800</td>
<td>Fw</td>
<td>5’ - CTTCCGCAACAATCCTTC - 3’</td>
<td>59</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rev</td>
<td>5’ - CATAACTCCGACAGATCC - 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BnPIC1</td>
<td>Bra036409</td>
<td>At2g15290</td>
<td>Fw</td>
<td>5’ - TCGGGTCACACTCTCTTG - 3’</td>
<td>59</td>
<td>161</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rev</td>
<td>5’ - GATGATGCTCTCTCTCTTC - 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BnYSL4</td>
<td>Bra030327</td>
<td>At5g41000</td>
<td>Fw</td>
<td>5’ - TCAGTCTCGTCCACTCCAG - 3’</td>
<td>57</td>
<td>112</td>
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<tr>
<td></td>
<td></td>
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<td>Rev</td>
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<tr>
<td>18S rRNA</td>
<td>KT225373</td>
<td>At2g16590</td>
<td>Fw</td>
<td>5’ - GCATTCTGTATTCTCATTAGTCAGAGGTG - 3’</td>
<td>61</td>
<td>192</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rev</td>
<td>5’ - CGGAGTCCTAAAGCAACATCC - 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-tubulin</td>
<td>XM_009125342.1</td>
<td>At4g20890</td>
<td>Fw</td>
<td>5’ - TCSATCCAGGARATGTCAGGG - 3’</td>
<td>59</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rev</td>
<td>5’ - ACTCTGCAACAAGATCATCATG - 3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In short, amplification was performed in a 10 µl final volume including 1 µl cDNA template, 1 µl of 10× Taq buffer, 0.5 µl of dNTPs (200 µM), 0.5 µl of forward and 0.5 µl reverse primers, 0.6 µl of MgCl₂ and 0.4 µl Taq polymerase. The annealing temperature of the PCR reaction was varied for each primer set (Table 1). After selecting the optimal annealing temperature, the primer concentration of each gene was performed by traditional PCR to choose the optimal concentration. To confirm the sequences of amplicons, PCR was performed on cDNA for all designed primer pairs. The amplified products (10 µl) were separated by electrophoresis in 2% (w/V) agarose gel stained with ethidium bromide and the expected product size was observed in 2% (w/V) agarose gel.

4.6.4 BnPIC1, BnNico, BnMAR1 and BnYSL4 expression analysis by qRT-PCR

For accurate representation and reliable analysis of target gene expression, a robust normalization of qRT-PCR data with suitable internal control genes is paramount (Czechowski et al., 2005) because the differences in the amount and quality of the starting RNA/cDNA samples can affect the efficiency of qRT-PCR results (Andersen et al., 2004). For accurate analysis of qRT-PCR results, it is highly recommended the use of at least two reference genes (Vandesompele et al., 2002). β-tubulin (XM_009125342.1) and 18s rRNA (KT225373) coding sequences were chosen to correct the non-specific variations. Primer sequences, amplicon sizes and amplification efficiency of all PCR products are listed in Table 1. All target primer pair sequences amplified a single PCR product of the expected size with optimal annealing temperature and primer concentration. Efficiency measurements and qRT-PCR reactions were performed by StepOnePlus Real-Time PCR system (Applied Biosystems) with the StepOne™ v.2.2.3 software. The primer specificity was confirmed by the presence of a sharp peak during the melting curve stage. Also, a single band should be obtained in 2% agarose gel electrophoresis with a molecular weight corresponding to the predicted amplicon size. All qRT-PCR reactions were carried out with three replicates. A standard curve was generated using 7 points of twice-fold serial dilutions of the cDNA template to calculate the specific PCR amplification efficiencies of target genes in qRT-PCR experiments. Based on the threshold values (Ct) for all dilution points in a series, a standard curve was generated using linear regression and the slope. The final Ct was calculated as the average of three replicates. The amplification efficiency of the primer was calculated using the following equation:

\[
\text{Efficiency (\%)} = 10^{(-1/\text{slope})} \times 100.
\]
Ideally, the efficiency of a PCR should be 100% (i.e. the slope of the linear regression = 2, meaning that for each cycle the number of product doubles: $2^n$, where $n$ is the number of the cycles). The calculated slope for each target gene estimated from the linear regression model ranged from 1.87 – 2.08.

To identify the differential expressions among samples by qRT-PCR, 20× – 100× diluted (with RNase-free water) cDNA samples as templates, 0.15 – 0.5 µM gene-specific primers and 2× diluted SYBR Green reagent (Luminaris Color HiGreen High ROX, Thermo-Fisher Scientific) were used in the final volume of 15 µl in an optical 96 - well plate. All cDNA samples were freshly diluted with RNase-free water before qRT-PCR reactions. Samples were mixed from the parallel leaves of two plant individuals. A no-template control (NTC) was also included in each run for each gene, in this study 1.5 µl RNase-free water was used and no specific peaks were detected in the negative control. If there were any specific peaks present in NTC, the experiment should be repeated to get corrected data since the samples got contamination. All measurements were repeated on three technical and two biological replicates for each tissue, and two biological ones to ensure reproducibility of the results. The qRT-PCR program was set up as follows: a predigest step of 50 °C for 2 min, a 95 °C initial denaturation step for 10 min, 40 cycles of 95 °C for 15 s (denaturation), Tm for 20 s (annealing), 72 °C for 20 s (extension), and final melt curve stage. Fluorescence was measured after annealing and extension stages. Transcriptional levels of all target and control genes studies in tissue samples were calculated to perform the expression of each gene. Quantification of the normalized relative transcript level of specific genes was performed according to the method of Pfaffl (2001).

Pfaffl formula:

$$\text{Ratio} = \frac{\text{Efficiency(Target)}^{C_G(\text{target,untreated}) - C_G(\text{target,treated})}}{\text{Efficiency(Reference)}^{C_G(\text{ref,untreated}) - C_G(\text{ref,treated})}}$$

4.7 Relative quantification of proteins by immunoblot

Approximately 50 mg of leaf samples were ground in liquid nitrogen, and solubilized in 500 µl of 62.5 mM Tris-HCl (pH 6.8), 2% (w/V) SDS, 2% (w/V) DTT, 10% (V/V) glycerol. The mixture was then incubated at room temperature for at least 30 min. Non-solubilised material
was removed by centrifugation at 10,000×g for 5 min at room temperature, and 0.001% (w/V) bromophenol blue was added to the supernatant.

30 µg solubilized proteins per lanes were run on 10 – 18% gradient acrylamide gels (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE); Laemmli, 1970) in a MiniProtean apparatus (BioRad). The following electrophoresis using a constant current of 10 mA and a maximal voltage of 200 V per gel slabs (1 cm² surface) at 6 °C for 10 min, the constant current was elevated to 20 mA per slab, and the gels were left to run for 2 h. One of the two gels run with the same samples was used for blotting, the other was stained. Staining was performed overnight with the blue-silver method (Candiano et al., 2004).

For western blotting, proteins separated by SDS-PAGE were transferred to Amersham™ ProtranTM Premium 0.2 µm nitrocellulose blotting membranes (GE Healthcare) in a 25 mM Tris (pH 8.3), 192 mM glycine, 20% (V/V) methanol and 0.02% (w/V) SDS in a BioRad blotting apparatus at 6 °C using constant voltage of 90 V and 400 mA maximal current for 3 h. Ice in a container was used to reduce the heat during running. (After blotting, the gels were also stained with the blue-silver method to see whether the transfer of proteins to the nitrocellulose membrane was total.) Following the horizontal transfer of proteins, nitrocellulose membranes were blocked by 3% (w/V) gelatine dissolved in 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl. Immunoblot against BnPIC1 and BnNiCo proteins was performed with rabbit polyclonal antibodies directed against PIC1 from Arabidopsis (as for reference, see Duy et al., 2007, 2011) and NiCo (against the recombinant, C-terminal part of the PsNiCo protein: amino acids 236 - 375; Pineda Antibody Service) from Pisum sativum. Rabbit polyclonal antibody against the apoprotein of light-harvesting complex II (a gift from Dr. Udo Johanningmeier, Bohum Universität, Germany) was also used to distinguish the similar molecular weight proteins. The mixed and inner chloroplast envelope membranes isolated from Brassica napus leaves were used as positive controls. Antibodies were dissolved in 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1% (w/V) gelatine (1:10,000 dilution). Horseradish peroxidase- (HRP) conjugated goat-anti-rabbit IgG (BioRad, Inc.) was used to detect bands following the manufacturer’s instructions. After treatments with the 1st and 2nd antibodies, the blots were washed 2 times with 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.05% (w/V) Tween 20, and two times with 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl for 20 – 20 min.
Gels and immunoblots were scanned using an Epson Perfection V750 PRO scanner. Densitometry analysis (determination of pixel density on greyscale gel images) was carried out using the Phoretix v. 4.0 image analysis software (Phoretix International, Newcastle-upon-Tyne, UK). The concentration of proteins was counted by comparing the area density of leaf proteins with that of a mixture of standard proteins of known protein amount. The normalization of protein quantification was based on total leaf proteins applied to the gels.

### 4.8 Statistical analysis

Chl, leaf, and chloroplast Fe contents were measured as triplicates (technical replicates) per treatment in three independent experiments (biological replicates). Fluorescence induction measurement was performed throughout the whole time of treatment on the same plants during the experimental period in three biological replicates in every three independent experiments. RNA samples were isolated from two biological replicates of each treatment in three independent experiments. For qRT-PCR analysis samples were processed in technical triplicates to confirm the stable expression of genes of interest. Total leaf proteins were isolated and later processed from two technical replicates of each treatment in three independent experiments.

The standard deviation was determined using Microsoft Excel. Unpaired Student’s t-tests and one-way ANOVAs with Tukey-Kramer *post-hoc* tests were performed on data using InStat v. 3.00 (GraphPad Software, Inc.). The words ‘significantly different’ mean that the similarity of samples is $P<0.05$. Origin v. 6.01 (Origin Lab, Co.) was used to fit mathematical functions on data points.
5. RESULTS

5.1 Changes in physiological parameters of leaves

Developmental pattern of the leaves was dependent on the Fe nutrition status of the plants: Fe deficiency markedly inhibited the leaf area expansion in both 4th and 6th leaves, but superoptimal Fe nutrition did not cause any significant alterations in the area expansion pattern of leaves compared to optimal Fe nutrition (Fig. 1). Under both optimal and superoptimal Fe nutrition, 4th leaves reached their maximal area to the (14–) 21st day of treatment, whereas 4th leaves of Fe deficient plants performed no area growth during the time of treatment. The area expansion of 6th leaves became complete by the 28th day of treatment in all experimental groups.

![Figure 1. Changes in the leaf area during the days of treatments. Grey columns – 4th leaves; open columns – 6th leaves. ΔFe – Fe deficiency, Ctrl – optimal Fe nutrition (control), +Fe – superoptimal Fe nutrition. Error bars represent SD values. To compare the differences, one-way ANOVAs were performed with Tukey-Kramer post-hoc tests on the treatments (P<0.05; n=3×3 [biological×technical]).](image)

Total Chl (Chl a+b) content of leaves was strongly affected by both the age of leaves and the Fe nutrition status of plants. It rose in both 4th and 6th leaves of plants grown under optimal Fe nutrition until it reached the maxima around the 21th/28th day of treatment (Fig. 2A and B).
Figure 2. Changes in the Chl a+b content of 4th (A) and 6th (B) leaves during days of the treatment. ΔFe – Fe deficiency, Ctrl – optimal Fe nutrition (control), +Fe – superoptimal Fe nutrition. Error bars represent SD values. To compare the differences, one-way ANOVAs were performed with Tukey-Kramer post-hoc tests on the treatments (P<0.05; n=3×3 [biological×technical]).
Superoptimal Fe nutrition hardly affected Chl accumulation. Following the full development, a slight but tendentious decrease was found in the Chl content in all leaves grown under optimal or superoptimal Fe nutrition. Compared to the control leaves, Fe deficiency induced significant retardation in the accumulation of Chl in both the 4th and 6th leaves. The Chl content decreased in the 4th leaves due to the stronger inhibition of the relative accumulation of Chl compared to leaf growth, while no significant changes were found in the 6th leaves during the treatment.

Concerning the physiological activity of chloroplasts, alteration in the Fe nutrition led to changes in the operation of the photosynthetic apparatus of the leaves. The differences in the amount of Fe nutrition significantly affect the values of maximum quantum yield of PSII ($F_{V}/F_{M}$) on leaves (Fig. 3A and B). Under three Fe treatments of 4th leaves, there was a peak increase in the values of $F_{V}/F_{M}$ when leaves reached the mature development (21 days). In the next following days, $F_{V}/F_{M}$ values gradually decreased its values in older leaves in Fe deficiency and optimal Fe nutrition whereas it was stable in superoptimal Fe nutrition. In the 6th leaves, the values of $F_{V}/F_{M}$ peaked the highest value in the third week in superoptimal Fe nutrition and the tendency led to the slight decline in $F_{V}/F_{M}$ values in older leaves. The stable increase in $F_{V}/F_{M}$ values was in response to the 6th leaves of optimal Fe nutrition until the three-week treatment before its value was reduced during the rest time of treatment. Whereas, the peak increase in $F_{V}/F_{M}$ values was observed in the first-week experiment of Fe deficiency and it slightly decreased in the total development of the 6th leaves and $F_{V}/F_{M}$ values slightly raised again at the end of experiment.
Figure 3. Changes in the Fv/FM values of 4th (A) and 6th (B) leaves during days of the treatment. ΔFe – Fe deficiency, Ctrl – optimal Fe nutrition (control), +Fe – superoptimal Fe nutrition. Error bars represent SD values. To compare the differences, one-way ANOVAs were performed with Tukey-Kramer post-hoc tests on the treatments (P<0.05; n=3×3 [biological×technical]).
The alterations in Fe nutrition also induced modifications in the tendency of changes in the excitation energy allocation in the leaves (Fig. 4A and B). The high actual photochemical quenching of the PSII reaction centers ($\Phi_{PSII}$) decreased after total leaf development in the 4th leaves of control and Fe deficient plants. It was also stable in the 6th leaves under sufficient Fe supply but was strongly reduced in the 6th leaves of Fe deficient plants during the whole treatment time. The excitation energy quenching of the non-functional PSII reaction centers ($\Phi_{NF}$) increased in the 4th control and the 4th and 6th Fe deficient leaves particularly after 28th–35th days of treatment, probably in connection with the initiation of senescence of the leaves. In the above-mentioned leaves, the fluorescence and constant heat dissipation processes ($\Phi_{f,D}$) behaved similarly to $\Phi_{NF}$, i.e. increased after 28th–35th days. No tendentious significant changes were found in the non-photochemical quenching of the antennae ($\Phi_{NPQ}$). Nevertheless, under superoptimal Fe nutrition, the excitation energy allocation did not change significantly during the whole time of treatment.
Figure 4. Changes in the excitation energy allocation during the time of the treatment in the 4th (A) and 6th (B) leaves. ΔFe – Fe deficiency, Ctrl – optimal Fe nutrition (control), +Fe – superoptimal Fe nutrition. Error bars represent SD values. To compare the differences, one-way ANOVAs were performed with Tukey-Kramer post-hoc tests on the treatments (P<0.05; n=3×3 [biological×technical]).
The Fe content of leaves was affected by both the age of leaves and the Fe nutrition status of plants. Fe content in 4th leaves of plants grown under optimal and superoptimal Fe nutrition did not show significant differences (Fig. 5A and B). Under both growth conditions, the Fe content elevated a little during the development, but it decreased later and it was tendentiously lower on the 35th day than on the 28th day of the treatment. In comparison, there was a gradual increase in the Fe content of 6th leaves in plants grown under optimal and superoptimal Fe nutrition. Moreover, the Fe content of 6th leaves of plants grown under superoptimal Fe nutrition was significantly higher during the time of treatment, 120.2 ± 3.4% of the corresponding control values on the 35th day. At the same time, leaf Fe content was strongly retarded by the limitation of Fe nutrition in both 4th and 6th leaves without any increase during the time of treatment. The Fe contents of 4th and 6th leaves of Fe deficient plants on the 28th day of treatment was 35.8 ± 4.4% and 28.2 ± 1.7% of the corresponding control values.

The Fe content of isolated chloroplasts showed a similar trend of changes to those of leaves with the only exception that in contrast to the Fe content of leaves, under deficient and optimal Fe nutrition, a tendency of decrease in that of the chloroplasts was recorded. Under optimal Fe nutrition, a significant increase in the chloroplast Fe content of 4th and 6th leaves were only found during their development (Fig. 6A and B). In Fe deficient plants, this trend of the increase was less pronounced in the 6th leaves, while the increase stopped at the second week of treatment in the more developed 4th leaves. After reaching the maximum Fe content of chloroplasts on the second and third weeks of treatment, a slight trend of decrease was observed under Fe deficiency and optimal Fe nutrition. Noteworthy that the chloroplast Fe content remained stable even in older leaves under superoptimal Fe nutrition.
Figure 5. Changes in the Fe content of 4th (A) and 6th (B) leaves during days of the treatment. ΔFe – Fe deficiency, Ctrl – optimal Fe nutrition (control), +Fe – superoptimal Fe nutrition. Error bars represent SD values. To compare the differences, one-way ANOVAs were performed with Tukey-Kramer post-hoc tests on the treatments (P<0.05; n=3×3 [biological×technical]).
Figure 6. Changes in the Fe content of chloroplasts isolated from the 4th (A) and 6th leaves (B) during days of the treatment. ΔFe – Fe deficiency, Ctrl – optimal Fe nutrition (control), +Fe – superoptimal Fe nutrition. Error bars represent SD values. To compare the differences, one-way ANOVAs were performed with Tukey-Kramer post-hoc tests on the treatments (P<0.05; n=3×3 [biological×technical]).
To connect chloroplast Fe content to the development and status of the photosynthetic apparatus, correlations of these physiological parameters were calculated. A clear linear correlation was found between Fe and Chl $a+b$ contents of chloroplasts (Fig. 7). The decrease in the chloroplast Fe content also resulted in a significant decrease in the Chl content of chloroplasts independently of the leaf storeys.

Figure 7. Correlation between the Fe and Chl $a+b$ contents of chloroplasts. Linear regression was calculated on 42 averaged individual data pairs where $R^2$ is 0.8769.

Moreover, changes in the actual quantum efficiency of PSII reaction centers were also related to the chloroplast Fe content: during the increase of the chloroplast Fe content, $\Phi_{\text{PSII}}$ only increased to a maximal level leading to a saturation-type relationship of the two physiological parameters (Fig. 8). The increase in $\Phi_{\text{PSII}}$ saturated at $0.742 \pm 0.004$ at $3.12 \pm 0.17$ fmol Fe chloroplast$^{-1}$.
Figure 8. Correlation between the Fe content of chloroplasts and the actual photochemical quenching of PSII reaction centres ($\Phi_{PSII}$). Boltzmann fit was calculated on 33 individual averaged data pair where $R^2$ was 0.8455.

5.2 Identification of Brassica orthologs of the Arabidopsis PIC1, NiCo, MAR1 and YSL4 genes

To identify putative homologs in Brassica napus, AtPIC1 (At2g15290), AtNiCo (At2g16800), AtMAR1 (At5g26820), and AtYSL4 (At5g41000) sequences were used as initial protein queries against Brassica Database (http://aramemnon.botanik.uni-koeln.de) to blast protein sequences. The four candidate genes encoding Brassica orthologues of the Arabidopsis queries were identified as BnPIC1 (Bra036409; score: 461, e$^{-130}$; encoded protein length: 283 amino acids; identities: 249/283 [87%], positives: 259/283 [91%], gaps: 11/283 [3%]), BnNiCo (Bra037287; score: 494; e$^{-140}$; encoded protein length: 376 amino acids; identities: 276/376 [73%], positives: 291/376 [77%], gaps: 11/376 [1%]), BnMAR1 (Bra020559; score: 963; e$^{0}$; encoded protein length: 599 amino acids; identities: 503/599 [83%], positives: 526/599 [87%], gaps: 3/599 [0%]) and BnYSL4 (Bra030327; score: 1149; e$^{0}$; encoded protein length: 665 amino acids; identities: 627/665 [94%], positives: 630/665 [94%], gaps: 31/665 [5%]) the orthologues of At2g15290, At2g16800, At5g26820 and At5g41000 respectively.
5.3 Changes in the transcript level and protein amount of chloroplast Fe transporters

5.3.1 Time-scale changes in the expression of \textit{BnPIC1}, \textit{BnNico} and \textit{BnMAR1}

The expression of \textit{BnPIC1} was strongly dependent on the Fe nutritional status of the plants – more in 4\textsuperscript{th} and less pronounced but also tendentiously in 6\textsuperscript{th} leaves (Fig. 9A). The lower the amount of available Fe was in the nutrient solution, the higher the relative transcript amount of \textit{BnPIC1} was. The relative transcript amount of \textit{BnPIC1} was also dependent on the developmental status of control and Fe deficient 4\textsuperscript{th} leaves: in parallel to the area expansion of the leaves, the relative transcript amount also increased but decreased after reaching the full development (i.e. after 21 days). In contrast, the relative transcript amount showed minor changes in leaves of plants grown under superoptimal Fe nutrition: its value remained stable during the whole experimental period, and similarly low as the values measured at the end of experimental period in leaves of plants grown under Fe deficient and optimal Fe nutrition conditions.

In contrast to \textit{BnPIC1}, the relative transcript level of \textit{BnNico} in 4\textsuperscript{th} leaves increased a little during the leaf area expansion period but remained high or even increased further during the remaining experimental period (Fig. 9B). In 6\textsuperscript{th} leaves, the relative transcript amount was more dependent on the Fe nutrition: the highest expression was found under the lowest Fe nutrition, while not strongly significant alterations were shown under optimal and superoptimal Fe nutrition.

The changes in the tendency of \textit{BnMAR1} expression were mostly affected by the Fe nutritional status. Clearly, the relative transcript amounts of \textit{BnMAR1} were highly expressed under Fe-free nutrition, whereas its expression was down-regulated in both Fe-supplied cases (Fig. 10).

However, the expression of \textit{BnMAR1} was also dependent on the developmental status of the leaves. As the expression pattern of \textit{BnNiCo}, the relative transcript amount gradually increased in the 4\textsuperscript{th} and 6\textsuperscript{th} leaves of Fe deficient and 4\textsuperscript{th} leaves of control plants during the total treatment time, although the transcript levels of \textit{BnMAR1} were different. In contrast, its expression remained more or less constant during the whole experimental period in the 6\textsuperscript{th} leaves of control and 4\textsuperscript{th} and 6\textsuperscript{th} leaves of plants grown under superoptimal Fe nutrition.
Figure 9. Changes in the transcript levels of BnPIC1 (A) and BnNiCo (B) during days of the treatment. Grey columns – 4th leaves; open columns – 6th leaves. ΔFe – Fe deficiency, Ctrl – optimal Fe nutrition (control), +Fe – superoptimal Fe nutrition. Normalized relative quantities (NRQ) are based on the relative transcript levels of 18s rRNA and β-tubulin. Error bars represent SD values. To compare the differences, one-way ANOVAs were performed with Tukey-Kramer post-hoc tests on the treatments (P<0.05; n=2×3 [biological×technical]).
Figure 10. Changes in the transcript levels of BnMAR1 during the days of treatment. Grey columns – 4th leaves; open columns – 6th leaves. ΔFe – Fe deficiency, Ctrl – optimal Fe nutrition (control), +Fe – superoptimal Fe nutrition. Normalized relative quantities (NRQ) are based on the relative transcript levels of 18s rRNA and β-tubulin. Error bars represent SD values. To compare the differences, one-way ANOVAs were performed with Tukey-Kramer post-hoc tests on the treatments (P<0.05; n=2×3 [biological×technical]).

5.3.2 Changes in the relative amount of PIC1 and NiCo proteins

Both PIC1 and NiCo were detected as protein bands of 21 and about 26 kDa, respectively, by immunoblot following SDS-PAGE (Fig. 11). They are low-abundant proteins and their molecular weight is close to that of light-harvesting proteins. We used both positive controls (isolated chloroplast envelopes) and antibody against LHC proteins to be able to identify them.
Figure 11. Identification of BnPIC1 (A) and NiCo (B) proteins. Marks: 1,6,7,13 – molecular weight standards; 2,5,8,12 – total proteins isolated from 4th leaves; 9 – total proteins isolated from 4th leaves according to Duy, D., et al. (Plant Cell 19, 986, 2007) but using 250 μg ml⁻¹ Pefabloc instead of PMSF; 3,10 – chloroplast mixed envelope fraction; 4,11 – chloroplast inner envelope membrane fraction. After blotting, the membranes (A and B) were cut and treated separately with the antibodies against PIC1 (A1-4), NiCo (B7-11) and light-harvesting complex II (LHCII) apoproteins (A5,6 and B12,13). As for molecular weight standard, PageRuler™ Plus Prestained Protein Ladder (Thermo-Fisher Scientific; Lot.: 00463463) was used. Lanes were loaded with 30 μg solubilized protein. Arrowhead is pointing on BnPIC1.

The relative amounts of both PIC1 and NiCo proteins altered to the developmental status of the leaves and also depending on the Fe nutrition of plants (Figs. 11, 12).
Figure 12. Total proteins isolated from 4th and 6th leaves (A-I and J-S, respectively) of plants grown under Fe deficient (A-C; J-L), optimal (D-F; M-O) and superoptimal Fe nutrition (G-I; P-S) conditions. Coomassie stained solubilized proteins on polyacrylamide gel (A; D; G; J; M; P), immunoblot against PIC1 (B; E; H; K; N; R), and immunoblot against NiCo (C; F; I; L; O; S). Marks: 1 – 7th; 2 – 14th; 3 – 21st; 4 – 28th; 5 – 35th day of treatment. Molecular weight standards as in Fig. 10. Lanes on protein gels and immunoblots were loaded with 30 µg solubilized protein.

Both under Fe deficient and optimal Fe nutrition conditions, the relative amount of PIC1 protein increased (stronger in Fe deficient leaves) in parallel with the development of the 4th and 6th leaves, but this increase stopped or even turned into decrease by reaching the full development of leaves (Fig. 13A).
Figure 13. Relative amount (pixel density in the same amount of sum leaf protein) of PIC1 (A) and NiCo (B) based on western blot analysis during days of the treatment. Grey columns – $4^{th}$ leaves; open columns – $6^{th}$ leaves. ΔFe – Fe deficiency, Ctrl – optimal Fe nutrition (control), +Fe – superoptimal Fe nutrition. Error bars represent SD values. To compare the differences, one way ANOVAs were performed with Tukey-Kramer post-hoc tests on the treatment ($P<0.05; n=2\times3$ [biological×technical]).
In comparison, the amount of PIC1 was rather stable in leaves of plants grown under superoptimal Fe nutrition: it was significantly lower compared to other growth conditions in 4\textsuperscript{th} leaves, whereas its amount in 6\textsuperscript{th} leaves was comparable to that of 6\textsuperscript{th} leaves in plants grown under optimal Fe nutrition.

In contrast to PIC1, the relative amount of NiCo (Fig. 13B) did not decrease in 4\textsuperscript{th} leaves of plants grown under Fe deficient and optimal Fe nutrition after leaves reached their full area but even increased further particularly under optimal Fe nutrition. Under superoptimal Fe nutrition, a less significant, but similar changes were measured in 4\textsuperscript{th} leaves. In 6\textsuperscript{th} leaves, the relative amount of NiCo only changed slightly.

Isolated \textit{Brassica napus} chloroplasts were also subjected to total chloroplast protein isolation to get a better detection of NiCo (Fig. 14). In contrast total protein samples obtained from leaf samples, changes in the relative amount of NiCo protein obtained from isolated chloroplasts showed good correlation to its transcript amount particularly in 4\textsuperscript{th} leaves (Fig. 15). Similar to the changes in the NRQ of the \textit{NiCo} transcript, the accumulation of NiCo was also found to be dependent on both the age and Fe nutritional status of the leaves of \textit{Brassica napus} plants. In both 4\textsuperscript{th} and 6\textsuperscript{th} leaves, the amount of NiCo protein increased in parallel to the development and aging of leaves, the accumulation of which also continued, indeed, following the full development of leaves. This accumulation found to be dependent on the iron nutritional status, since the more Fe the plants received, the higher protein density of NiCo was found, and that was more characteristical in older leaves.
Figure 14. Coomassie stained SDS-PAGE pattern of total chloroplast proteins isolated from 4th and 6th leaves (A&C, respectively) and immunoblot against NiCo on the identical samples (B&D, respectively). Lanes on protein gels and immunoblots were loaded with 20 µg protein except lanes belonging to the following samples: 4th leaves (A): ΔFe 21 days (4 µg); ΔFe 35 days (18 µg); +Fe 21 days (18 µg); 6th leaves (C): ΔFe 14 days (12 µg); Ctrl 21 days (11 µg); +Fe 21 days (15 µg), due to low protein concentration in the samples. The 26 kDa band (arrowheads) is identified as BnNiCo. Numbers indicate the time of treatment (days) of the isolates. As for molecular weight standard (MW) PageRuler™ Plus Prestained Protein Ladder (Thermo-Fisher Scientific; Lot.: 00463463) was used.
Figure 15. Relative amount of NiCo based on immunoblot analysis. Grey columns – 4th leaves; open columns – 6th leaves. Error bars represent SD values. To compare the differences, one-way ANOVAs were performed with Tukey-Kramer post-hoc tests on the treatments (P<0.05; n=3×2 [biological×technical]).

5.4 Changes in the expression of *BnYSL4* in different parts of *Brassica napus* tissues

The samples (flowers and siliques of various developmental stages) were collected from 2-month old control plants. The expression of *BnYSL4* was differently affected in the various parts of rapeseed tissues (Fig. 16).
Figure 16. Changes in the transcript levels of BnYSL4 in different parts of Brassica napus tissues. Normalized relative quantities (NRQ) are based on the relative transcript levels of 18s rRNA and β-tubulin. Error bars represent SD values. To compare the differences, one-way ANOVAs were performed with Tukey-Kramer post-hoc tests on the treatments (P<0.05; n=2×3 [biological×technical]).

The relative expression of BnYSL4 was connected to the generative tissues: the highest transcript level was measured during the ripening stage of the silique and showed a significant reduction in other parts of Brassica napus tissues. The expression of BnYSL4 was comparable and low in both developing siliques and cotyledons whereas the transcript amount was nearly doubled in ripened seeds and halved in developed leaves compared to them. Very low expression was found in flower buds and flowers.
5.5 Differential expression of chloroplast Fe uptake related transporters – Interaction between the gene expression of Fe transporters and chloroplast Fe content

In the dicot model plant *Arabidopsis thaliana*, Fe transport-related proteins were identified previously. Four chloroplasts Fe uptake related genes: *BnPIC1*, *BnNiCo*, *BnMAR1* and *BnYSL4* known from *Arabidopsis thaliana* studies were identified in the *Brassica* genome. Except for *BnYSL4* the others were strongly expressed in leaves.

To compare the trend of expression of genes found to play a role in the transport route of Fe in *Brassica* chloroplasts, we calculated the interaction between the three different target genes and chloroplast Fe contents based on the average of 4th and 6th leaves during the development of leaves and under different Fe nutrition (Fig. 17A). It is very clear that the expression of these genes is significantly affected during the leaf development: a gradual increase was observed in the transcript levels of *BnPIC1*, *BnNiCo*, and *BnMAR1* together with the Fe content of chloroplasts independently on the Fe nutrition level of the plants. However, the lower the Fe nutrition of the plants, the higher the expression of *BnPIC1* and *BnMAR1* were. At the same time, a stable increase but low expression of *BnPIC1* and *BnMAR1* were maintained under superoptimal Fe nutrition during the whole time treatments. In contrast, the expression of *BnNiCo* increased more slowly during the development and with the increase in chloroplast Fe content and reached high transcript levels only in mature leaves independently on Fe nutrition. In addition, its expression showed an increasing trend with the increase in Fe nutrition.

It was clear from the decrease in the actual quantum efficiency of PSII reaction centers and Chl content of leaves that the senescence process of leaves started after 21st days of treatment, more specifically in the 4th leaves. It was more pronounced in Fe deficient and control leaves than in the leaves of plants with superoptimal Fe nutrition. To see more clearly the differential expression of the genes of interest only the trends in the expression values of the different genes are compared during this time of treatment and under different Fe nutrition (Fig. 17B).

A gradual decrease was found in the expression of *BnPIC1* in parallel to the Fe content of chloroplasts under Fe deficient and control conditions. At the same time, stable but low expression of *BnPIC1* was maintained under superoptimal Fe nutrition even in the older leaves, and only a small decrease was found in chloroplast Fe. The expression of *BnMAR1* gradually
increased under Fe deficiency but remained quite stable under normal and superoptimal Fe nutrition.

**Figure 17.** Correlation between chloroplast Fe content and the expression of GOIs during (A) and following (B) the development of leaves, based on data of 4th leaves. Treatments as in Figure 1. For the easier comparison of datasets, 21-day Ctrl values were chosen as basis of normalisation (100%). Diamond – chloroplast Fe content; open, grey and closed columns – expression of BnPIC1, BnNiCo nad BnMAR1, respectively.
In contrast, the expression of \textit{BnNiCo} increased and remained high in old leaves under optimal and superoptimal Fe nutrition when Fe was gradually released from chloroplasts, while it was more or less stable in Fe deficient leaves.

6. DISCUSSION

The metabolic processes of mesophyll cells are strongly dependent on the availability of Fe in plants. Since Fe is of great importance in the cellular procedures: photosynthetic activity, nitrogen fixation, respiration, Chl biosynthesis, chloroplast development and DNA synthesis (Reichard, 1993; Kim and Guerinot, 2007; Muneer et al., 2014; Rout and Sahoo, 2015). Fe also involved in protecting plant species from serious stresses such as drought, salinity and heavy metal stresses (Nazar et al., 2012; Emamverdian et al., 2015) due to Fe stimulates the activity of antioxidant enzymes such as CAT, peroxidase and plays a crucial role in superoxide dismutase to protect plant cells avoiding reactive oxygen species (Case, 2017). Insufficient Fe can be fatal in plant development and growth (Boamponsem et al., 2017). Limitations in Fe nutrition through the roots led to a decreased Fe translocation towards the aerial tissues. Since under Fe limitation, the amount of available Fe significantly decrease in the cytoplasm of the mesophyll cells, the compartment that serves as origin of available Fe taken up into the chloroplasts, as a result, the chloroplast Fe content cannot reach that Fe level which sufficient for the development and function of the photosynthetic apparatus and other enzymatic processes. This negative impact also leads to the reduction in the photosynthetic performance and thus that of the biomass production when plants lack available Fe for their body cells (Sharma, 2007; Briat et al., 2015). This may be the result of Fe allocation in the mitochondria for the respiration since both the chloroplast and mitochondria require Fe for the function of photosynthesis and respiration. However, the question is how Fe can allocate between these two components has yet to be undiscovered (Merchant et al., 2006). The surplus level of Fe also negatively affects plant quality and productivity (Anjum et al., 2015). Therefore, adequate and balanced amount of Fe is necessary to be maintained for plant health and good production (Tripathi et al., 2018). In \textit{Brassica napus}, Fe deficiency inhibited the accumulation of Fe content in both target leaves and chloroplasts, reduced the size area of leaves, retarded the accumulation of Chl content, and then hampered the normal functions of the photosynthetic electron transport. The limitation of
available Fe remarkably affects the morphological characteristics such as leaf chlorosis, shoot height, the inhibition of leaf size, leaf number and the formation of new leaves since these parameters have a strong connection with the available Fe nutrition (Fernández et al., 2008). A significant decrease was observed in the area of 4th and 6th leaves due to the lack of available Fe in leaf tissues and led to inhibit the leaf area expansion and plant development. A slight increase was only measured in the Fe content of 6th leaves during the leaf area expansion, the source of which may have been the redistribution of Fe within the plant or Fe translocation from root reservoirs since Fe deficiency treatment was preceded by a pre-cultivation in Fe containing the nutrient solution. This slight greening process indicates that if Fe is highly limited at the individual level, the direction for Fe translocation is preferred towards the developing tissues (Parveen et al., 2019). Nevertheless, the excess of Fe, which is a frequent problem in acidic and anoxic soils, may also damage metabolic processes by facilitating ROS generation via Fenton reaction and decreases the productivity and even survival of plants (Vaahthera et al., 2014; Mignolet-Spruyt et al., 2016). The excess of Fe and thus Fe toxicity most frequently occurs in rice plantations due to the anoxic soil conditions during the vegetative growth of rice plants in culture. In rice, heavy Fe excess promotes early senescence in the leaves by the induction of a WRKY transcription factor, OsWRKY80, linked to the signaling of senescence processes (Ricachenevsky et al., 2010). Since superoptimal Fe nutrition is a less severe agronomical problem in comparison with Fe deficiency and toxicity, we are in a lack of data on how slight excess of Fe alters the Fe metabolism of mesophyll cells and chloroplasts. In our study, superoptimal Fe supply (100 μM initial Fe concentration in the nutrient solution with frequent renewal) neither caused toxic symptoms nor induced early senescence but even retained the activity of the photosynthetic apparatus in aging leaves in Brassica napus plants. In addition, Brassica plants were grown well with green leaves and bushy root systems in superoptimal Fe nutrition. Taxa of the genus Brassica are known to tolerate heavy metal stresses in higher concentrations (Gall et al., 2015). In Brassica rapa (a parental species of Brassica napus) secondary metabolism was shown to be enhanced under a slight excess of Fe (50–100 μM) synthesizing amino acids, phenolic and other sulphur-containing metabolites (Jahangir et al., 2008). Accumulation of these metabolites contributes to the complexation and detoxification of heavy metals. Nevertheless, genes involved in antioxidative defense were also reported to be inducible by the excess of Fe (Vansuyt et al., 1997). Previously, Fe was shown to accumulate in
chloroplasts in *PICI* overexpressing and in *ysl4/6* mutant lines and under superoptimal Fe nutrition in *Arabidopsis thaliana* (Duy et al., 2007; Divol et al., 2013; Briat et al., 2015). Since we neither observed Fe accumulation in the chloroplasts nor any retardation in physiological activity under superoptimal Fe nutrition, we can exclude that 100 μM Fe treatment would have been toxic for the experimental plants. The lack of increase in the chloroplast Fe content (Fig. 6) and only slight increase in the leaf Fe content (Fig. 5) under superoptimal Fe nutrition indicates that Fe retention in the roots may have contributed – by storing the excess of Fe in a form of e.g. ferritin – to the avoidance of Fe toxicity and the alterations in the adverse environments (Wheeler et al., 1985; Becker and Asch, 2005; Pinto et al., 2015; Parveen et al., 2016). Both ferritin and Fe are found in the chloroplasts of leaves when plants are suffered the excessive Fe, the expression of ferritin is up-regulated to store Fe in the ferritin form to protect plants from oxidative stress (Briat et al., 2006; 2010; Darbani et al., 2013).

Since only pieces of information are available in connection with the Fe nutrition-related physiological changes during leaf development (Jacobson and Oertli, 1956), we investigated the variations in the photosynthetic parameters depending on the developmental stage of leaves at different amounts of Fe nutrition. The Fe limitation has a fatal impact on the photosynthetic apparatus, especially in the younger ones. During the total treatment time, direct proportionality was found between the chloroplast Fe and Chl content, while
Avice and Etienne, 2014). In addition, nutrient remobilization occurs when the leaves begin at the senescence stage accompanied by a decrease in Chl levels (Maillard et al., 2015). The remobilization and retranslocation of Fe also take place from the mature green leaves to the older leaves of optimal Fe nutrition but a small amount compared to a serious decrease in superoptimal Fe nutrition at the senescence period. Ye et al. (2015) showed that Fe deficiency increases the transcript of 1-aminocyclopropane-1-carboxylic acid synthase 8 (AtACS8), a biosynthetic enzyme of ethylene in *Arabidopsis* leaves. Thus, earlier decomposition of the photosynthetic apparatus and decrease in the Fe content of chloroplasts of Fe deficient plants can be coupled to an ethylene signaling pathway. Nevertheless, senescing leaves can serve as a significant Fe source for developing tissues (6th leaves of Fe-deficient plants) as well as for generative organs. Although superoptimal Fe nutrition was not toxic, it caused some slight but tendentious alterations in the development and downregulation of the photosynthetic apparatus. It delayed the senescence processes because no severe alteration was found in the Fe content of the chloroplasts and the operation of the photosynthetic apparatus even after the total development of target leaves. Chloroplast-born retrograde signals may have an important role in the regulation of the cellular Fe homeostasis (Vigani et al., 2013; Gong et al., 2015). Since the surplus accumulation of Fe is known to induce NO production in chloroplasts (Touraine et al., 2012) and NO can act as a senescence-delaying signal causing down-regulation of ethylene emission (Leshem et al., 1998; Benavides et al., 2016; Astier et al., 2017). Therefore, these pieces of evidence indicate that the amount of Fe nutrition may contribute to the regulation of Fe redistribution from the chloroplasts to the developing tissues and generative organs.

Concerning the regulation of Fe homeostasis of chloroplasts, the regulation of transporters plays an essential role. At present, large amount of information is available on root cell Fe transporters, but only pieces of information can be found on those of leaf cells and its organelles (Vigani et al., 2013; Curie and Mari, 2017; Hindt et al., 2017, Vigani et al., 2019), none of these really reflects the situation during the leaf development and the developmental frame of the biogenesis of the photosynthetic apparatus. PIC1 was found to be an essential member of the chloroplast Fe uptake machinery (Duy et al., 2007). It is also a key component in the regulation of Fe homeostasis in mesophyll cells (Duy et al., 2011). NiCo protein was first identified in a yeast-based screen for PIC1-interacting proteins, the expression of which appeared to be co-regulated to *PIC1*. Taken together, Duy et al. (2011) speculated that PIC1 and NiCo
might function together in plastid Fe transport (Vigani et al., 2019). Although *PIC1* itself was thought to have a constant expression in all tissues of *Arabidopsis* and transcript levels were not described to correspond to plant Fe deficiency (Duy et al., 2007; Gong et al., 2015), in our study, the expression of both *PIC1* and *NiCo* was found to be dependent on both the developmental stage of leaves and the Fe nutrition status of *Brassica napus* plants (Fig. 9A and B). The expression of *BnPIC1* reached a peak when the leaves got matured but dropped down parallel to aging in both 4<sup>th</sup> and 6<sup>th</sup> leaves under Fe limitation. The transcript and protein amounts of *BnPIC1* correlated well during the treatment time and followed a similar trend of changes in the examined leaves referring to the prime importance of regulation at the expression level. These findings are in agreement with the role of *PIC1* in chloroplast Fe uptake and support the results that the expression of both Ferritins and Fe-SOD decreased in *PIC1*-RNAi down-regulation constructs but increased in *PIC1* overexpressing lines of tobacco in parallel to the changes in the chloroplast Fe content (Gong et al., 2015). Coming to the point of the effects of Fe nutrition, both transcript and protein accumulation levels of *BnPIC1* seemed to be positively regulated by Fe deficiency, whereas they were downregulated by superoptimal Fe nutrition during the development of leaves. This suppression of *PIC1* expression under slight Fe excess implicates that chloroplasts do not contribute to the storage of Fe rather it corresponds to the feedback down-regulation of the Fe uptake of chloroplasts when reaching a certain Fe content under not toxic conditions (Solti et al., 2012). In the initial stage of leaf development, the expression of both *BnPIC1* and *BnNiCo* was lower than at reaching the full maturity, supporting the hypothesis of a Fe-uptake bypass pathway in chloroplasts of young leaves (López-Millán et al., 2016). In contrast to *BnPIC1*, however, the relative transcript and protein amounts of *BnNiCo* did not decrease or even showed a trend of increase after the leaves had reached their full development. Indeed, the relative transcript amount of *BnNiCo* increased (especially in 4<sup>th</sup> leaves) in parallel to the aging of the leaves, that also manifested in the increased relative amount of *BnNiCo* protein under optimal and superoptimal Fe nutrition after the leaves reached their full maturity (Fig. 9, 12). It makes rather improbable that *PIC1* and *NiCo* would contribute in the same way to the Fe transport in aging leaves. Taken together with the changes in the chloroplast Fe content and the relative transcript and protein amounts of *NiCo*, it seems unsupported that *NiCo* would only contribute to the Fe acquisition of chloroplasts but maybe also involved in the Fe sensing, release
and relocation from the chloroplasts. Nevertheless, the parallel decrease in the expression of \textit{PIC1} indicates that PIC1 may not contribute to the Fe release from chloroplasts.

\textbf{MAR1} (IREG3; a sequence homolog of mouse IREG1, Fe efflux mediator in epithelial cells) is suggested to be involved in Fe import from the cytosol (Yang et al., 2010), possibly translocating Fe-NA complexes into chloroplasts (Conte and Lloyd, 2010). MAR1 also specializes in import aminoglycoside antibiotics in the plant cells and has a similar sequence with two metal transporters: IREG1 and IREG2 (Schaaf et al., 2006; Morrissey et al., 2009). In addition, MAR1 is proved to localize in the inner envelope membrane of the chloroplasts (Jarvis, 2008) which has the function of the protein transport and mostly expresses in all plant tissues (Conte et al., 2009). In \textit{Arabidopsis thaliana}, overexpression \textit{AtMAR1} lines have the typical symptoms such as leaf chlorosis which is totally different with the signals found in the NA-free chloronerva mutation of tomato (\textit{Lycopersicon esculentum}) such as interveinal chlorosis on younger leaves, chlorosis appears from the midvein and older cell tissues (Ling et al., 1999; Conte et al., 2009). It can be explained that the remobilization of the cytoplasmic NA tanks to the chloroplasts, thus limiting the role of NA in transporting phloem of Fe and other metals (von Wirén et al., 1999) and also sequester Fe itself to prevent the remobilization of Fe in the whole plant body (Conte et al., 2009). Since the expression of \textit{BnMAR1} remained low during the leaf development under all Fe nutrition treatments, its contribution to the Fe uptake of chloroplasts can be excluded. Moreover, \textit{Brassica} chloroplasts were recently showed not utilizing Fe-NA complexes in their Fe acquisition (Müller et al., 2019). In addition, under Fe deficiency, the increasing expression of \textit{BnMAR1} following the full development of mature leaves together with the parallel decrease in the Fe content of leaves and chloroplasts are tempting to speculate that MAR1 does not involve in the Fe acquisition of leaves and chloroplasts but rather in the Fe release from the plastids and the redistribution or remobilization of Fe in the developing cells. Since under Fe deficiency, the expression of \textit{TAP1}, a hypothetical chloroplast Fe-S cluster exporter was previously shown to be upregulated in parallel to \textit{MAR1} (Yang et al., 2010), redistribution of Fe content not only covers the export of Fe (complexes) but also Fe containing cofactors. According to the Conte et al. (2009), the expression of \textit{AtMAR1} in \textit{Arabidopsis} plant was down-regulated which is different from the up-regulation expression in \textit{Brassica napus} in our study under Fe limitation. There was no increase in the \textit{AtMAR1} expression under Fe excess in \textit{Arabidopsis} plants due to the adverse impact of excessive NA (Cassin et al., 2009). In
contrast, the expression of *BnMAR1* showed slight alterations in the transcript level during the time treatment of 4\textsuperscript{th} and 6\textsuperscript{th} leaves under superoptimal Fe nutrition but the amount of transcript was much smaller than that of optimal Fe nutrition. This can be explained that MAR1 may have the function as the Fe transport protein in the chloroplasts, it may act like AtSBL, to import Fe into the chloroplasts as the ferritin form (Wintz et al., 2003). Another different point is that the superoptimal Fe nutrition did not cause any typical chlorosis in the target leaves of *Brassica napus* whereas the chlorosis symptoms were observed in *Arabidopsis thaliana* leaves resulted in the oxidative damage generated from the overaccumulation of Fe in the chloroplasts (Conte et al., 2009). Although Fe nutritional status and the leaf age seem to contribute to the regulation of chloroplast Fe remodeling, the signaling of chloroplast Fe uptake and homeostasis related proteins needs further investigations to distinguish the native functions of MAR1 in Fe homeostasis.

Plants require to get adequate Fe distribution to maintain Fe homeostasis in all tissues. Many root transporters have been identified but very little information is available on how plants can transport Fe from root epidermis into the xylem, move the Fe from the xylem into cells or subcellular compartments in leaves and on Fe remobilization from fully developed leaves into seeds. To investigate the control of the Fe distribution from root to seeds, YSL family members are the candidates. Both *YSL4* and *YSL6* genes were planned to use for studying their expression tendencies in generative tissues. However, we were not able to identify any putative *Brassica* homolog with *ArYSL6* (*At3g27020*), therefore, we only focused on the investigation of *BnYSL4* expression. According to the result of Chu et al. (2010), *YSL4* can transport Fe(II)-NA which plays a key role in the translocation of Fe using yeast functional complementation in *Arabidopsis* plants. NA is known as an Fe chelator used to distribute Fe from organs to organs within the plant species (Schuler et al., 2012), indicating *YSL4* is involved in this process. *YSL4* is located in the plastid envelope presented only in (pro)plastids of ripening and germinating seedlings (Divol et al., 2013). In our experiment, the highest expression of *BnYSL4* was found in the ripening state of siliques using qRT-PCR (Divol et al., 2013). The other parts of plant tissues (ripened seed, developing siliques and cotyledon) showed similar medium expression except for bud and flower tissues, where its expression was hardly detected (Fig. 15). These expression patterns support that *BnYSL4* expression may be linked with the development of generative tissues (Müller et al., 2019). Interestingly, the result of Chu et al. (2010) reported that the high expression of *YSL4* was
found in the *Arabidopsis* flower which was completely contradictory with our result in *Brassica* flower. Vacuoles are very important components for the storage of metals, approximately 50% of the Fe in the cell sequestered in the vacuoles (Lanquar et al., 2010). In *Arabidopsis* plants, VIT1 transports ferrous Fe into the vacuole and is expressed in the vasculature, mostly during embryo and seed development (Kim et al., 2006). The AtNRAMP4 is known to be related to Fe remobilization during germination (Lanquar et al., 2005) and co-localizes with γ-TIP (Bolte et al., 2011). γ-TIP label structures inside the protein storage vacuole (PSV; Bolte et al., 2011) and associates with globoid structures (Jiang et al., 2001). Thus, YSL4 is suggested to function in the release of metal from the vacuole. Conte et al. (2013) used GFP-tagged AtYSL6 to observe green fluorescence signals associated with chloroplasts but they never detected any other places than the tonoplast membrane. AtYSL4 and AtYSL6 share high sequence similarity and may have identical activities (Chu et al., 2010). Taking into account its expression in leaf samples in our experiment, the transcript amount of *BnYSL4* is quite low in fully developed leaves, thus we hypothesize that this component is not involved in the Fe acquisition into the chloroplasts of leaves. To be clearer, the expression of the *BnYSL4* gene is found in the marginal of leaf tissue showing that the transport of Fe–NA complexes across the chloroplast envelope membrane may only be associated with the development of the generative tissues, except photosynthetic apparatus of fully mature leaves.

In conclusion, the developmental status of leaves and the Fe nutrition status of the plants together have strong regulatory role on the Fe homeostasis of chloroplasts where the Fe uptake transporter PIC1 is up-regulated by Fe starvation in fully developing leaves but down-regulated after leaves reached their full maturity and possibly upon senescence signals, whereas NiCo seems to stay under different regulation and shown a distinct pattern of changes in the expression. Thus, there may be a partial overlapping in their function only. Based on the increased expression of MAR1, it assumed to play a key role of Fe-NA chelator in the chloroplasts and related with the stabilization of deliberated Fe of Fe release from the chloroplasts to the older leaves. Nevertheless, its pattern of expression does not support its participation in Fe acquisition rather in Fe release processes (Fig. 16). Its expression is mostly dependent on the developmental status of leaves and different amounts of Fe nutrition. YSL4 seems not to be involved in the Fe acquisition of chloroplasts and thus in the development of photosynthetic activity since the expression of *BnYSL4* is quite low in examined leaves.
compared with *BnPIC1*, *BnNiCo*, and *BnMAR1*. Indeed, it may be involved in the Fe loading and unloading in seed tissues and/or distribution Fe in the generative tissues. Fe deficiency occurs together with early senescence, induces the remodeling of Fe content of plant cell tissues during the development of plants manifested in a decreasing Fe content of chloroplasts without any significant change in the Fe content of leaves. The excess of Fe is connected to the downregulation of the chloroplast Fe acquisition machinery and the delay of senescence while the Fe status of the chloroplasts together with the operation of the photosynthetic electron transport chain remains rather stable following the full development of leaves.

7. SUMMARY

Fe is necessary for all plant species since it is used as a cofactor in numerous enzymes, especially in the photosynthetic apparatus. It localizes mainly in the chloroplasts in the mesophyll. Thus, chloroplast Fe homeostasis plays a key role in maintaining the physiological function of plants. Complex regulatory pathways control the alterations in the gene expression related to Fe homeostasis. The activation or repression of chloroplast Fe uptake related genes may also depend on the Fe supply conditions but only a few pieces of information are available on the regulation of protein expression related to Fe transport. PIC1 was the first protein found to be related to Fe acquisition which is targeted in the inner envelope of chloroplasts and mediates Fe accumulation within cells together with NiCo protein, forming a functional complex for the transport of free Fe$^{2+}$ ions. MAR1/IREG3 may be an opportunistic gateway of multiple antibiotics but it may also act in the Fe homeostasis of chloroplasts by transporting NA, or Fe complexes, such as Fe-NA. YSL4 plays a key role in plastid Fe-efflux and functions as an NA and/or Fe-NA complex transporter.

Oilseed rape (*Brassica napus* L. cv. DK Exquisite) plants were grown hydroponically on half-strength Hoagland nutrient solution (Fe source: 20 μM Fe(III)-EDTA). To induce Fe deficiency, plants were grown Fe-free and CaCO$_3$ containing nutrient solution. To induce superoptimal Fe, plants were supplied with 100 μM Fe(III)-EDTA. Leaves developed before and during the time of treatment were labelled as 4$^{th}$ and 6$^{th}$ leaves, respectively. Fe deficiency induced significant retardation in the accumulation of Chl content. A slight but tendentious
reduction was observed in leaf Chl content of plants grown under optimal and superoptimal Fe nutrition after reaching the full development. Aging caused a slight reduction in the photosynthetic activity of both optimal Fe grown and Fe deficient leaves but it was stable in leaves grown superoptimal Fe nutrition. Leaf Fe content of optimal and superoptimal Fe grown plants did not show any significant differences in 4\textsuperscript{th} leaves but showed a gradual increase in the 6\textsuperscript{th} leaves. Under Fe deficiency, leaf Fe content gradually decreased in both 4\textsuperscript{th} and 6\textsuperscript{th} leaves tendentiously during the time of treatment. Chloroplast Fe content showed more remarkable changes; it increased during the development but decreased by the aging of leaves with a peak between 14 and 21-day-old leaves. It remained the stable under superoptimal Fe nutrition.

The relative transcript amount of \textit{BnPIC1}, \textit{BnNiCo}, \textit{BnMAR1} and \textit{BnYSL4} genes were studied by qRT-PCR based on 18sRNA and $\beta$-tubulin as internal control genes. The relative transcript level of \textit{BnPIC1} and \textit{BnNiCo} was found to depend on the Fe nutrition status and the area expansion of 4\textsuperscript{th} leaves. The highest expression was observed in the developed leaves but decreased thereafter in Fe deficient and optimal Fe nutrition grown plants but proved to be low but rather stable in superoptimal Fe grown leaves. The transcript level of \textit{BnNiCo} showed a gradual increase in leaf area expansion and was found to depend on Fe nutrition. In 6\textsuperscript{th} leaves, its value, in contrast to \textit{BnPIC1}, remained stable after reaching the full development. The relative amount of PIC1 protein increased in parallel with the development of target leaves but decreased after reaching the full development of leaves of Fe deficient and optimal Fe plants leaves. It remained, indeed, stable under superoptimal Fe nutrition. In contrast, the relative amount of NiCo did not decrease following full development of 4\textsuperscript{th} leaves but increased further particularly under optimal Fe nutrition. The relative transcript amounts of \textit{BnMAR1} were highly expressed under Fe deficiency, whereas optimal and superoptimal Fe nutrition induced lesser induction in its expression. Also, \textit{BnMAR1} expression was dependent on the developmental status of leaves when the peak transcript amount was found in older leaves. The relative expression of \textit{BnYSL4} was connected to the generative tissues: the highest expression was transcribed in the ripening stage of the siliques and reduced in leaf, flower and flower bud. Four chloroplasts Fe uptake related genes: \textit{BnPIC1}, \textit{BnNiCo}, \textit{BnMAR1}, and \textit{BnYSL4} were found to a significant increase in leaf tissue. The first three target genes were strongly expressed in the leaf tissue in a various amount of Fe nutrition, except \textit{BnYSL4}. 
In conclusion, the developmental status of leaves and the Fe nutrition status of the plants together have strong regulatory role on the Fe homeostasis of chloroplasts where the Fe uptake transporter PIC1 is up-regulated by Fe starvation in fully developing leaves but down-regulated after leaves reached their full maturity and possibly upon senescence signals, whereas NiCo seems to stay under different regulation and shown a distinct pattern of changes in the expression. Thus, there may be a partial overlapping in their function only, but our results do not support their exclusive collaboration in the Fe acquisition processes. According to the results, MAR1 seems not participate in Fe acquisition but rather in Fe release processes. Moreover, neither YSL4 seems to be involved in the Fe acquisition of chloroplasts.
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Budapest, Hungary

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Appendix

The expression of *Haberlea rhodopensis* mitochondrial alternative oxidase under low-temperature stress

I. INTRODUCTION

*Haberlea rhodopensis* Friv. (Gesneriaceae) is a perennial herbaceous poikilohydric homoiochlorophyllous resurrection plant with remarkable resistance to withstand severe desiccation, and revive from an air-dry condition (Georgieva et al., 2007; Ivanova et al., 2017). It is a tertiary relict species, and nowadays an endemic taxon only found in Balkan and Rhodope Mountains, where it also faces and able to withstand severe cold stress (Benina et al., 2013). Thus, it is a unique model plant for studying the mechanisms underlying tolerance to different stresses.

Exposure to cold stress leads to serious damage to the plant cell membranes and inhibition of metabolic processes, particularly photosynthesis (Allen and Ort, 2001; Liu et al., 2018). Primarily, biochemical reactions are slowed down by low temperatures. In chloroplasts, the inhibition of the Calvin cycle elevates the amount of unconsumed NADPH and ATP leading to an increase in the excitation pressure of PSII which enhances the production of reactive oxygen species. Cold tolerant plants have numerous protective mechanisms/metabolites to avoid the above-mentioned harmful effects (Heidarvand and Amiri, 2010). *H. rhodopensis* was shown to have a great potential for metabolic defense including sugars, polyols, and organic acids against cold stress (Benina et al., 2013) but other data about its cold tolerance are not available.

In higher plants, the mitochondrial alternative oxidase (AOX) also plays an important role in stress protection (Vanlerberghe, 2013; Saha et al., 2016). It is a 32–36 kDa dimeric non-heme di-iron carboxylate family of protein localised on the matrix side of the inner mitochondrial membrane, and is involved in an alternative (to the cytochrome pathway) electron transport route that couples the oxidation of ubiquinol to the reduction of O$_2$ to water without ATP production but releasing the energy as heat (Rogov et al., 2014). The alternative oxidation
pathway also works with non-energy conserving external alternate dehydrogenases (Clifton et al., 2005), and activated biochemically by specific organic acids including pyruvate, malate, succinate (Millar et al., 1993). The physiological importance of this alternative route is to rapidly adjust the cellular demands for ATP, pyridine nucleotides, and carbon intermediates, and this way it controls the carbon, energy and redox status of the cell (Vanlerberghe, 2013). Accordingly, it reduces the potential for the production of reactive oxygen species when the Calvin cycle in the chloroplasts or the cytochrome pathway in the mitochondria are inhibited, and thus also protect the photosynthetic apparatus against photodamage (Zhang et al., 2012).

The AOX expression was observed in a wide range of environmental stress conditions, particularly under low-temperature conditions (Clifton et al., 2005; Vanlerberghe, 2013). Depending on the different sequences (Considine et al., 2002), nuclear-encoded AOX1 and AOX2 genes are distinguished in higher plants (Arabidopsis thaliana). To be more specific, AOX1 genes have been found in both monocots and eudicots while AOX2 genes have only been observed in eudicots (Considine et al., 2002; Costa et al., 2014). A recent detailed investigation of AOX protein sequences pointed out that the AOX2 subfamily is divided into two types: constitutively expressed AOX2a–c and stress-responsive AOX2d (Cavalcanti et al., 2013; Costa et al., 2014). In wild-type cucumber, AOX2 was transcriptionally up-regulated in leaves and male flowers by cold stress indicating that it carries stress-related functions (Mróz et al., 2015). Moreover, only one AOX2 has been expressed in Cucurbitales (Costa et al., 2014). Thus, the response of AOX forms to stresses is probably species-dependent.

This study aimed to find out whether AOX was connected with the cold stress tolerance of H. rhodopensis. Therefore, the effect of low temperatures on the expression profiling of AOX2A was investigated in the leaves of this plant.

II. MATERIALS AND METHODS

To investigate the expression of mitochondrial alternative oxidase at various temperatures, H. rhodopensis shade ecotype (grown at 30 µmol m⁻² s⁻¹ PPFD) was used as plant material. Plants were collected from their natural habitat in Rhodope Mountains, South-West Bulgaria. The population was sustained in the ex situ conservation collection of the Eötvös Loránd University Botanical Garden. They were exposed to a light intensity of approximately 25
µmol m$^{-2}$ s$^{-1}$ at midday in June which resulted in a leaf temperature of 21–25 °C. The plants were examined at 20 °C – control, 5–10 °C – cold stress, -10 °C – frost stress, and at 4 °C – post-frost regeneration state. The fully developed leaves were collected and immediately deep-frozen in liquid nitrogen for expression analysis.

High-quality total-RNA samples were prepared for mRNA sequencing using Trizol method following the manufacturer’s instructions. The extracted RNA was subjected to DNase I (Thermo-Fisher Scientific) treatment to obtain DNA-free RNA. The RNA concentration was quantified by a Nanodrop ND-1000 spectrophotometer (Thermo-Fisher Scientific Inc., Waltham, MA, USA). Reverse transcription was performed using random hexameric oligonucleotides and RevertAid Reverse Transcriptase (Thermo-Fisher Scientific). The cDNA libraries were stored at -80 °C for further use in qRT-PCR.

Primers for the qRT-PCR analysis were designed using Primer3 software using the contig data published in Apostolova et al. (2012). The following contigs and corresponding primers were used for the analysis: (1) target gene - mitochondrial *HrAOX2a* (*Contig_002889*) and (2) two genes as for internal control with stable expression: *Heat Shock Protein 90* (*HrHSP90; Contig_000452*) and *Ubiquitin* (*HrUBC; Contig_009712*) (Wan et al., 2010; Gechev et al., 2013). The detailed primers were listed in Table 2. The gradient PCR protocol was performed to choose optimal annealing temperature and primer concentration: an initial denaturation step at 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s, annealing at 72 °C for 15 s, with a final elongation step at 72 °C for 1 min. The PCR fragments were separated on 2 % (w/V) agarose gel with the expected product size.

The qRT-PCR analysis was performed using a StepOnePlus Real-Time PCR system, Applied Biosystems. Expression data were normalized using *HSP90* and *UBC*. The efficiency of each gene was estimated from the slope of a linear regression model and ranged from 1.96 – 2.19. All reactions contained 7.5 µl (2× diluted SYBR Green reagent (Luminaris Color HiGreen High ROX, Thermo-Fisher Scientific)), 1.5 µl (10× – 100×) diluted cDNA, 0.375 – 0.625 µM gene-specific primers in a final volume of 15 µl. The qRT-PCR program was set up as follows: a predigest step at 50 °C for 2 min, a 95 °C initial denaturation step for 10 min, 40 cycles at 95 °C for 15 s (denaturation), T$_{m}$ for 20 s (annealing), 72 °C for 20 s (extension), and final melt curve stage. Quantification of the normalized relative transcript level of specific genes was performed.
according to the method of Pfaffl (2001). Quantitative RT-PCR analysis of genes and samples was processed in technical triplicates to confirm the stable expression of genes of interest.
Table A1. Oligonucleotide primers used in the expression analysis of *mitochondrial alternative oxidase 2a* (*AOX2a*) related transporters

<table>
<thead>
<tr>
<th>Gene</th>
<th>Contig Accession</th>
<th>Primer</th>
<th>Primer sequences</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
<th>Product size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HrAOX2a</td>
<td>002889</td>
<td>Fw</td>
<td>5'-GCTGCCGTTCTGTTATGGTA-3'</td>
<td>64</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev</td>
<td>5'-CCACTTTGGCTGCACAAGCTC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HrHSP90</td>
<td>000452</td>
<td>Fw</td>
<td>5' - GTAAGAAACTCGTATCTGCTACC - 3'</td>
<td>59</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev</td>
<td>5' - CGGCTAGAAACCACCTACCT - 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HrUBC</td>
<td>009712</td>
<td>Fw</td>
<td>5' - GCCGAAGAAGATCAAGC - 3'</td>
<td>59</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev</td>
<td>5' - GCCGCACTCAAGATTAGG - 3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Unpaired Student’s t-tests and one-way ANOVAs with Tukey-Kramer *post-hoc* tests were performed on data using InStat v.3.00 (GraphPad Software, Inc.). The term ‘significantly different’ means that the similarity of samples is *P*<0.05.
III. RESULTS

The expression level of *HrAOX2a* was analyzed in mature leaves of *H. rhodopensis* exposed to different temperature treatments. The qRT-PCR results confirmed that the expression of *HrAOX2a* is upregulated by cold stress. A peak transcript level was measured during cold treatment (Fig. A1). Compared to cold, the transcript abundance of *HrAOX2a* performed a slight reduction at frost temperature. In the recovery period, the transcript level fell under the value of the control stage.

![Figure A1. Changes in the transcript levels of HrAOX2a in Haberlea rhodopensis leaves at various temperatures. Control: 20 °C, Cold: 5-10 °C, Frost: -10°C, Recovery: 4 °C. Normalized relative quantities (NRQ) are based on the relative transcript levels of HrHSP90 and HrUBC. Error bars represent SD values. To compare the differences, one-way ANOVAs were performed with Tukey-Kramer post-hoc tests on the treatments (P<0.05; n=1×3 [biological×technical]).](image-url)
IV. DISCUSSION

Cold stress negatively impacts on plant metabolism which induces remarkable changes in their transcriptome (Chinnusamy et al., 2007). Under low and non-freezing temperatures, i.e. cold acclimation, the expression of many transcription factors is induced to enhance the ability of plants to tolerate the adverse environmental conditions (Zhu, 2016). The analysis of transcriptome data provides valuable insights into the molecular mechanisms involved in the tolerance to desiccation of *H. rhodopensis* (Gechev et al., 2013). However, transcriptome changes induced by cold acclimation of this resurrection plant are not available. According to our results, *H. rhodopensis* AOX2a is upregulated by cold and frost stresses in fully developed leaves. The experiment of Costa et al. (2014) revealed that only AOX2d genes are involved in stress response. Nevertheless, Mróz et al. (2015) found that AOX2a–c subtype genes are upregulated by cold stress in three mosaic mitochondrial mutants of cucumber. Therefore, stress-related AOX genes may be different in different plants and under different stressful conditions.

In conclusion, the increased expressional changes of the *HrAOX2a* transcript in *H. rhodopensis* implies that alternative oxidase may play a role during low-temperature stress acclimation of this plant. The full understanding of the specific role of AOX in low-temperature tolerance will contribute to the discovery of novel stress-responsive genes as potential targets for the improvement of crops.

V. REFERENCES


