Final report (NKFI K-124159)
Plant ferric chelate reductases: Key components of the reduction-based iron uptake

Ferric Reductase Oxidase (FRO) family proteins are key components of the reduction-based iron (Fe) transport strategy in plants, fungi, and animals. The majority of angiosperm plants apply the reduction based Fe uptake strategy (also called Strategy I) operated by FRO enzymes, those not only support the absorption of Fe from the rhizosphere but also the plastidial and mitochondrial Fe loading. Although multiple details on the regulation of the reduction-based Fe uptake of the roots have been revealed, the operation of plat ferric chelate reductases, the key enzymes of the reduction-based Fe uptake and transport remained unclear, among others their importance in the plastidial and foliar Fe uptake. Although several Strategy I taxa were indicated to secrete coumarin derivatives (such as *Arabidopsis thaliana*) or flavin derivatives (among others *Cucumis sativus*, *Beta vulgaris*, *Medicago truncatula*) as for Fe deficiency responses, no direct evidences how these compounds supplement the operation of ferric chelate reductases. Therefore, we aimed to answer the following questions in the project:

- Are flavin and coumarin compounds involved in the ferric chelate reduction as interacting partners of the ferric chelate reductases enzymes / carriers of reducing power?
- Is the reduction of Fe compounds important in the foliar Fe uptake?
- Is the reduction based Fe uptake of chloroplasts dependent on a specific source of Fe?
- How do developmental processes interfere with the reduction based Fe uptake of chloroplasts?
- Does the reduction based Fe uptake determine the Fe acquisition process of the chloroplasts?

To answer these questions, we conducted investigations in 8 research topics and we applied *Cucumis sativus* as flavin secreting model taxon and *Brassica* spp. that lack the ability of the extracellular secretion of flavin derivatives. *Brassica* spp. were also used as for model in chloroplast studies as close relative of the well-established plant model *Arabidopsis thaliana* but enables harvesting a large amount of material for purification studies.

<u>Topic 1:</u> Remote Fe mobilisation and adjoined Fe deficiency responses in the expression of FRO and riboflavin biosynthesis genes support the contribution of apoplast flavins to the root ferric chelate reductase activity

Poorly soluble ferric oxides such as ferrihydrite, goethite (α-FeOOH) and haematite (α-Fe_2O_3) commonly occur under environmental conditions, thus abundant in multiple soil types. At near-neutral pH, higher ionic strength and higher temperature, the formation of haematite is favoured, but ferrihydrate also undergoes a slow phase transformation resulted in the formation of haematite primarily. These 10–100 nm sized nanoparticles occur attached to the surface of soil components like clay minerals in the nature. Although nanoparticles of natural

occurrence are important sources of Fe for plants, little is known about the sites of utilisation. Moreover, the size exclusion property of the cell walls in the roots question whether these particles would reach the plasma membrane of the plant cells and thus get in contact with directly. Although H^+ extrusion is a common Fe deficiency response among Strategy I plants, haematite particles are generally resistant to low pH, acidification of the rhizosphere does not provide sufficient amount of soluble Fe. Based on literature evidences (e.g. Sisó-Terraza *et al*. 2016, *New Phytol* 209:733) that suggested a redox shuttle involved in the reduction based Fe uptake of flavin secreting Strategy I plants, we set up the primarily hypotheses that Fe liberation from nanoparticle size Fe sources is based on a remote action of plant cells and the enhanced biosynthesis of flavin derivatives and flavin secretion is an inclusive Fe deficiency response.

To verify the first hypotheses we applied nanocrystalline hematite colloid suspension (particle size of 10…20 nm) as for Fe source of *Cucumis sativus* plants grown under Fe deficient conditions (complete depletion of Fe). First we characterised the haematite nanoparticle suspension by transmission electron microscopy (TEM), selected area electron diffraction (SAED) and ^{57}Fe Mössbauer spectroscopy to address the proposed biological mobilisation of Fe from the particles. We applied haematite particles in the nutrient solution of Fe deficient plants and attempted to detect the naoparticles in the apoplast of root cells. Thus we applied high resolution (HR) TEM and energy-dispersive X-ray spectroscopy (EDS) at 200 keV accelerating voltage allowing 0.08 nm resolution in HRTEM for atomic resolution imaging of the nanoparticles and 1.6 nm resolution in STEM using a high angle annular dark field (HAADF) detector for average atomic number contrast imaging. Detecting Fe Kα peak, elemental distribution map showed elevation coincides with the middle lamella in the two-cell junction of root cells. HRTEM analysis indicated the presence of multiple separate electron dense particles in this region. Atomic resolution of individual particles proved crystalline with a periodicity of 2.51 Å typical to haematite d(110) interplanar spacing. Fourier transformation of the atomic resolution images indicated haematite nanoparticles with [001] zone axis orientation. Comparing this results with the TEM and SAED results on the original nanohaematite suspension we revealed a significant Fe mobilisation from the particles. Nevertheless, particles accumulated in the middle lamellae in the cell walls exclusively indicating the strict pore filtering property of the apoplast excluded the possibility of any direct contact of the particles with the plasma membrane localized ferric chelate reductase enzymes. Therefore, Fe mobilisation was a remote action of the cells.

To verify the second hypothesis that enhanced flavin biosynthesis is a direct Fe deficiency response in *Cucumis sativus*, we investigated the relaxation of the Fe deficiency induced expression of the three root ferric chelate reductases (*CsFRO1*: *AY590765*.1; *CsFRO2*: *Cucsa.260380.1* and *CsFRO3*: *Cucsa.108040*.1*)* together with the riboflavin biosynthesis enzyme GTP cyclohydrolase II (*CsRIBA1*: *Csa4M111580*). As for reference, we applied *CsAct* (*AB010922*) and *CsEF1A* (*XM_004149499.2*). We have demonstrated that expression of both *CsFRO* and *CsRIBA1* genes have a circadian periodicity indicating the rhythm of Fe deficiency responses around the clock. Clear morning and afternoon peaks were distinguished. In the ferric chelate reductase (FCR) activity of roots we also detected a clear

circadian rhythmicity. In response of nanohaematite treatment, the relaxation profile in the expression of *CsFRO1, CsRIBA1* and *CsFRO3* in multiple aspects highly overlapped: completely relaxation required 24 hours of treatment. In contrast, the relaxing of *CsFRO2* expression required 48 hours. However, based on bioinformatics and the expression pattern of CsFRO2 we have excluded that FRO2 would operate as a plasma membrane ferric chelate reductase, in contrast of the data of Marastoni *et al*. (2019; *Plant Physiol Biochem* 136:118). The presence of a 20 amino acid signal sequence was indicated that target the translated protein into the secretory system very likely. Relaxing of the Fe deficiency response in the FCR activity was delayed in comparison to the decline in the expression of *CsFRO1&3* and *CsRIBA1* indicating post-translational effects in the suppression of the root FRC activity. Parallel decrease in the expression of *CsFRO1&3* and *CsRIBA1* indicates a co-regulation that support the redox equivalent shuttle role of flavin derivatives that operate the remote Fe mobilisation form apoplast stacked Fe particles.

→ In conclusion, we approved the *a priori* hypothesis. Fe liberation from the nanoparticles does not require their contact to the plasma membrane. Parallel relaxation of *CsFRO* and *CsRIBA1* expression support the importance of flavins in the remote ferric Fe utilisation. Results were presented on conferences [18; 21; 24]. A manuscript (Singh *et al.* [6]) based on these results is under review/published soon.

Topic 2: Riboflavin and fraxetin are able to mobilize Fe under abiotic conditions that support the reduction based Fe uptake

Flavin secreting Strategy I plants release various riboflavin derivatives (among others 4'-ketoriboflavin, riboflavin 3'- and 5'-sulphate, 7-hydroxy-riboflavin, 7α-hydroxy-riboflavin, 7-carboxy-riboflavin) into the rhizosphere that all proved to be important in the Fe uptake under Fe limited or alkaline conditions. Since in literature studies riboflavin derivatives proved to be essential in this action, we set up the hypothesis that riboflavin has inherent capacity to mobilise Fe from insoluble compounds.

In order to reveal the interaction between Fe oxides and flavins, we tested the dissolution of stable nanoscale α-Fe_2O_3 haematite in the presence of equimolar (0.5 mM) amount of riboflavin both at pH 5.0 (buffered by 500 mM MES-KOH) and at pH 8.5 (alkaline conditions, buffered by 8 mM K_2CO_3 and 800 mM $KHCO_3$). To avoid any photoreduction event by exciting either riboflavin molecules or Fe compounds, experiments were performed under 630 nm red illumination. Nanoscale Fe particles were removed by high-speed centrifugation and Fe content of the supernatant was measured by ICP-MS as 'soluble Fe'. The initial solubility of the applied α-Fe_2O_3 haematite nanoparticle suspension was 3.269±0.096% and 0.044±0.009% of the total Fe content at pH 5.0 and 8.5, respectively. The addition of riboflavin did not change the dissolution of Fe neither at pH 5.0, nor at pH 8.5 (dissolution was 3.188±0.320% and 0.016±0.009%, respectively). In consequence, under abiotic conditions riboflavin is not effective at solubilizing Fe. Potential interactions between riboflavin and Fe complexes were also investigated by ^{57}Fe Mössbauer spectroscopy.

Although Mössbauer spectroscopy requires an Fe concentration of at least 5 mM, riboflavin can be only kept dissolved in a water solution with dimethyl sulfoxide (DMSO) at 1–10 mM concentration range. Indeed, DMSO is able to form a stable complex with Fe. Application of DMSO dissolved riboflavin caused no alteration in the Fe species (Fig. 1). To avoid DMSO to Fe interactions, we also applied water dissolved riboflavin solution at the maximum of its solubility at 25 °C. To dissolve riboflavin, we also applied alkaline conditions. None of these environments caused any measureable alterations in the Fe species (Fig. 2).

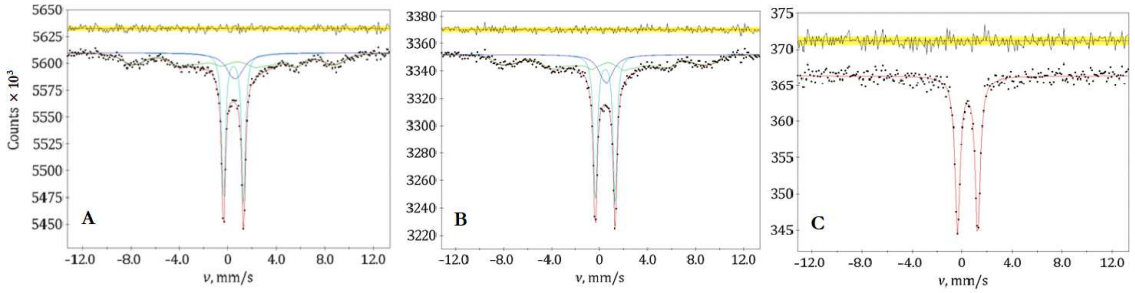

Figure 1. ^{57}Fe Mössbauer spectra of 10 mM ^{57}Fe(III)-EDTA (A) in the presence of saturated riboflavin solution in water (solid riboflavin applied in a nominal concentration of 20 mM; B) and in the presence of 20 mM dissolved riboflavin at pH 12 (C). A, B and C samples are characterised by one doublet, indicated by the red line and described by isomer shift of 0.4603; 0.4580 and 0.4543, respectively and quadruple splitting of 1.6405; 1.6283; 1.5813, respectively.

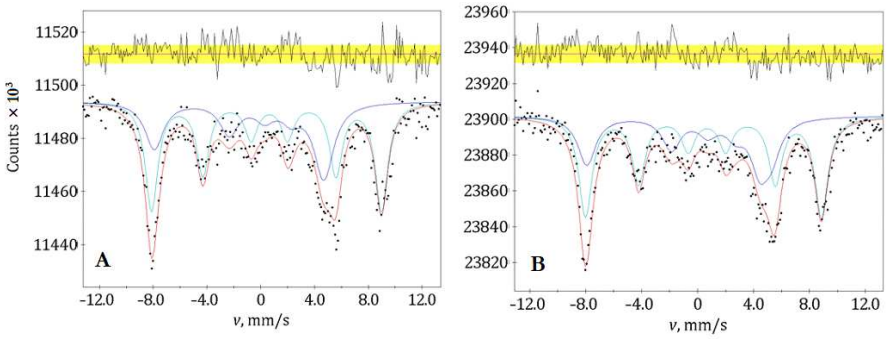

Figure 2. Complex ^{57}Fe Mössbauer spectra of 10 mM ^{57}Fe(III)-EDTA in the presence of 50% (V/V) DMSO (A) and in the presence of 10 mM riboflavin (Fe:riboflavin=1:1) dissolved in DMSO diluted to 50% (V/V) final concentration (B). Multiplex arrangement originates from DMSO to Fe interaction without any apparent effect of riboflavin.

→ In conclusion, we rejected the *a priori* hypothesis. Riboflavin does not interact to ferric complexes under abiotic conditions in the absence of light excitation. Riboflavin is not readily able to mobilise Fe from insoluble fractions. Initial results were included into the submitted manuscript of Singh *et al.* [6]; detailed analysis will be published in the manuscript under preparation Singh *et al.* [10]

Among the best characterised plan models *Arabidopsis thaliana* release fraxetin (7,8-dihydroxy-6-methoxycoumarin), a hydroxycoumarine aglycon into the rhizosphere as an Fe deficiency response by PDR9 plasma membrane coumarine transporter. Literature studies indicated that the effective Fe uptake of A. thaliana plants require fraxetin in the rhizosphere. The mechanism that enables a better Fe availability when fraxetin is released remained, indeed, unresolved. Since hydroxycoumarines can participate in redox reaction, but considering complexation chemistry principles, we set up the hypothesis that fraxetin can coordinate and reduce ferric Fe to make it available for the Fe uptake.

In order to reveal the abiotic action of fraxetin, we studied Fe speciation in the presence of soluble fraxetin. Thus we mixed $^{57}FeCl_3$ (pH~1) with fraxetin, dissolved in DMSO. Mixture was immediately frozen and investigated by means of Mössbauer spectroscopy (Fig. 3). Obtained spectrum indicated approx. 10% of ferrous Fe formation, whereas the major Fe species was determined as $FeCl_3$ remained intact. We detected no complexation of Fe by fraxetin. In contrast to Mössbauer spectroscopy results, the application of ferrous Fe complexing bathophenantroline disulfonate (BPDS) in the fraxetin – $^{57}FeCl_3$ mixture indicated the presence of a significant amount of ferrous Fe (2.944±0.296 mM Fe(II), whereas in the 10 mM Fe(III)-citrate solution, BPDS assay indicated the presence of 20.7+0.7 µM Fe(II) only).

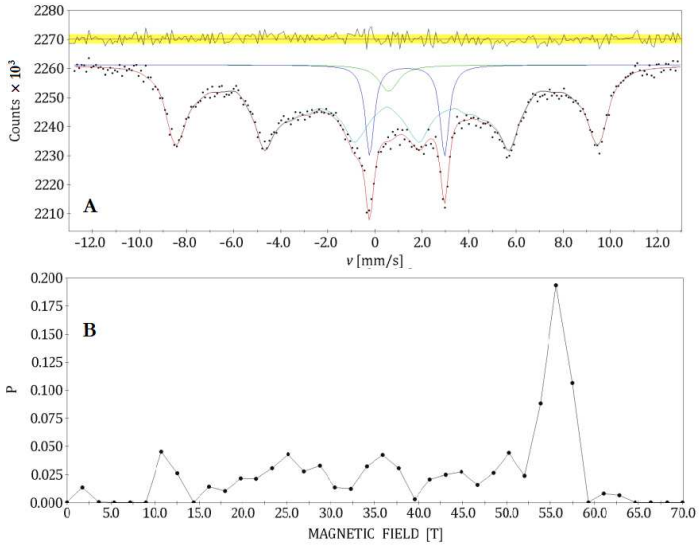

Figure 3. ^{57}Fe Mössbauer spectrum of 10 mM $^{57}FeCl_3$ in the presence of fraxetin dissolved in DMSO (A). The doublet component indicated by blue line with parameters: isomer shift of 1.3726 and quadruple splitting of 3.1814, covering 10.60% together with the paramagnetic characteristics (B) indicate the formation of Fe(II).

To test the effect of fraxetin of ferric complexes that are also stable at higher pH, we analysed ^{57}Fe(III)-citrate (pH~5) mixed with standard fraxetin according to the before mentioned conditions. Mössbauer spectroscopy results indicated neither complexation nor any signs of ferric Fe reduction either applying immediate freezing of the mixture or freezing it after half an hour of incubation at room temperature (Fig. 4).

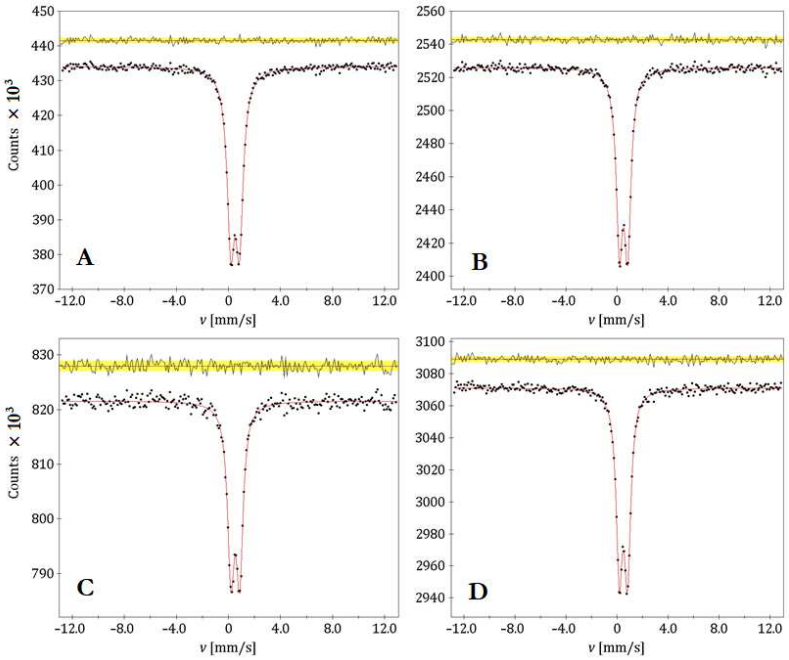

Figure 4. ^{57}Fe Mössbauer spectra of 10 mM ^{57}Fe(III)-citrate at pH 4.8 (A) in the presence of 50 % (V/V) DMSO (B), and in the presence of 10 mM fraxetin and 50% (V/V) DMSO frozen immediately after mixing (C) and frozen after 30 min incubation at RT (D).

In contrast, BPDS test for ferrous Fe indication was strongly positive. Taken together the results we set a model that in fraxetin the ortho-dihydroxi form (hydroquinone or reduced form, Frax(OH)) is oxidized to the quinone form (Frax(O)). Thus we suggest the following equilibria:

$$\text{Frax(OH)} + \text{Fe(III)} \leftrightarrow [\text{Frax(OH)-Frax(O)}] + \text{Fe(II)}; \text{ and}$$

$$[\text{Frax(OH)-Frax(O)}] + \text{Fe(III)} \leftrightarrow \text{Frax(O)} + \text{Fe(II)}.$$

By the addition of BPDS, this equilibrium shifts to:

$$\text{Fe(II)} + \text{BPDS} \rightarrow [\text{Fe-BPDS}]$$

based on the strong ferrous Fe complexing property of BPDS. This equilibrium (depending on the pH, since the reduction of Frax(O) to Frax(OH) is preferred at low pH) can shift towards the Fe(III) form, but once ferrous Fe is removed by e.g. the addition of strong ligand BPDS in a comparable amount to Fe, the equilibrium shifts towards the ferrous Fe formation by withdrawing Fe(II) from the system. The fact of ferrous Fe formation for $FeCl_3$ in comparison to Fe(III)-citrate indicates uncoordinated Fe^{3+} ions react with Frax(OH) but the complexation equilibrium with citrate maintains a very low free Fe(III) concentration, that remains the detection threshold of Mössbauer spectroscopy.

→ In conclusion, we verified the *a priori* hypothesis. Since under biological conditions membrane transport also removes ferrous Fe from the equilibrium, interaction of fraxetin to ferric Fe contributes to the reduction based Fe uptake not depending on ferric chelate reductase activity. A manuscript (Gracheva *et al*. [8]) based on these results will be submitted for publication until June, 2022.

Topic 3: Riboflavin and fraxetin enhance the ferric chelate reductase activity of plants

Strategy I plant *Cucumis sativus* is known to release among others 4'-ketoriboflavin into the rhizosphere upon Fe deficiency. According to the results of Topic 2 that flavins do not have an inherent Fe ligation or ferric Fe reduction property, we set up a hypothesis that flavins operate as reducing power shuttles between the plasma membrane FRO enzymes and the soluble ferric Fe compounds and in this manner, flavins are substrates of the FRO enzymes. To test this hypothesis, we applied a multiple model arrangement in respect of plant taxa / *in vivo* / *in vitro* systems.

To address FCR activity measurements, first we measured the spectrum and the concentration of the secreted flavin metabolites released in the nutrient solution and retained in the apoplast of roots. We applied *Cucumis sativus* cv. Joker F1 as for plant model. We induced Fe deficiency by the complete removal of Fe to avoid the application and thus the doubtful effects of carbonate. From the nutrient solution of Fe deficient plants, two compounds were separated by UHPLC. Compounds were detected at λ=270 nm, retention times are 2.11 and 2.68 min, respectively. Using standards, the R_t=2.11 min compound was identified as riboflavin. Deprotonated riboflavin and 4'-ketoriboflavin were identified with molecular masses of 375.13013 and 373.11435, respectively, whereas and formiate adducts of both compounds were detected with molecular masses of 421.13562 and 419.11981, respectively using HR-MS with negative ionisation. The concentration of riboflavin and 4'-ketoriboflavin in the nutrient solution proved to be 0.21±0.1 ng μL^{-1} and 0.33±0.3 ng μL^{-1}, respectively, after incubating three plants in a 400 mL pot filled with nutrient solution for two weeks, while water loss caused by water uptake and transpiration was complemented by the addition of deionized water. To reveal flavin derivatives infiltrating the apoplast, roots of plants gown on optimum Fe nutrition and on Fe deficient medium were also involved into the analysis. Measurements indicated that only the amount of 4'-ketoriboflavin increased under Fe deficiency whereas riboflavin and a riboflavin isomer did not show variance among the treatments (Fig. 5).

To test if secretion of flavin derivatives also exist in *Brassica napus*, nutrient solution of plants grown under Fe deficient conditions were also collected and analysed. Concentration of flavin derivatives remained below the limit of detection, thus *Brassica napus* was stated as non-flavin type Strategy I taxon. In addition, we were unable to detect traces of fraxetin or any related coumarine compounds in the nutrient solution of *Brassica napus*, thus in contrast to *Arabidopsis thaliana*, it is considered neither secreting coumarin derivatives as an Fe deficiency response. Based on the obtained data on the secretion of Fe deficiency related compounds, we involved both *Cucumis sativus* (flavin positive, coumarin negative) and *Brassica napus* (flavin negative, coumarin negative) models into the FCR activity assays. Since flavin derivatives are infiltrating the root apoplast of *Cucumis sativus* under Fe deficiency, their effect was taken into account at evaluating in vivo FCR activity data. Based on preliminary saturation kinetics assays, 400 µM Fe(III)-EDTA was applied at pH 6.0 in the *in vivo* FCR assays.

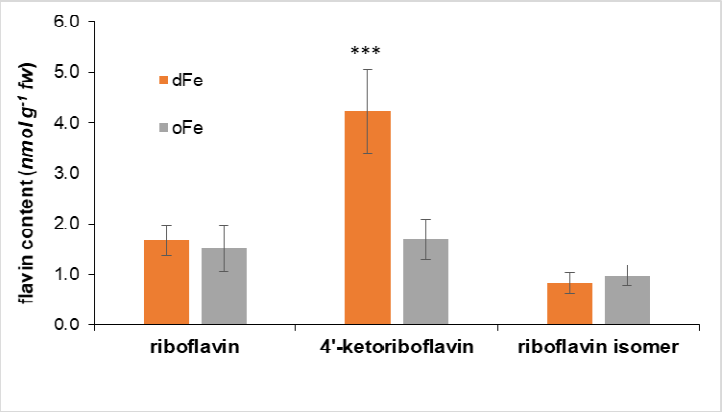

Figure 5. Flavin content in roots of *Cucumis sativus* plants grown under optimal Fe nutrition (oFe) and under Fe deficient conditions (dFe). Asterisk represent significant difference at $P<0.05$ according to Student's t-test.

The application of riboflavin in the *in vivo* FCR assay using *Cucumis sativus* model resulted in a multiphasic kinetics with two phases of decrease ($K_{M;I}$: 0.057±0.009 µM riboflavin; $v_{min;I}$: 18.7±1.5 nmol Fe min^{-1} g^{-1} fw and $K_{M;II}$: 5.49±0.68 µM riboflavin; $v_{min;II}$: 3.8±0.5 nmol Fe min^{-1} g^{-1} fw respectively) in the FCR activity and one enhancing phase ($K_{M;III}$: 54.9±20.4 µM riboflavin; $v_{max;III}$: 32.0±2.6 nmol Fe min^{-1} g^{-1} fw) (Fig. 6). According to our model, the decreasing phases represent a redox reaction between the apoplast infiltrating flavins and the externally applied riboflavin where riboflavin has a higher potential to receive reduction power than ferric Fe. According to this model, equilibrium at 33.65±1.78 µM riboflavin represent an equimolar balance between the externally applied and the apoplast flavins. Restoration of FCR activity at high riboflavin concentration suggest that riboflavin contributes to ferric Fe reduction and able to replace apoplast flavins.

Presence of additional riboflavin also altered the *in vivo* root FCR activity of the *Brassica napus* model where no apoplast flavins were detected (Fig. 7). We measured a clear enhancement on the FCR activity using the model, in parallel with the increasing externally applied riboflavin concentrations (kinetical parameters were: $K_{M;I}$: 0.080±0.006 µM riboflavin; $v_{max;I}$: 123.4±3.1 nmol Fe min^{-1} g^{-1} fw; $K_{M;II}$: 0.506±0.060 µM riboflavin; $v_{max;II}$: 158.9±7.3 nmol Fe min^{-1} g^{-1} fw; $K_{M;III}$: 42.6±6.4 µM riboflavin; $v_{max;III}$: 378.8±3.2 nmol Fe min^{-1} g^{-1} fw). Note that $K_{M;III}$ both in the cucumber and the rapeseed model fall in a similar range.

Comparing the results gained on flavin-type model *Cucumis sativus* and non-flavin type model *Brassica napus*, we concluded that flavins enhance the in vivo FCR activity, but the apoplast infiltrating flavins highly influence the in vivo measurements and thus to gain a clear enhancement kinetics, purified system, absent of multiple riboflavin derivatives is required.

To model the FRO enzyme – flavins – ferric Fe compound interaction, we involved in vitro activity measurements with purified root plasma membrane fractions, isolated from both the *Cucumis sativus* and the *Brassica napus* models. We applied aqueous two-phase separation of

membranes isolated from the root tips of the plant models, where pure root plasma membrane fractions were collected in the Dextran phase of the PEG/Dextran two-phase system. Multiple purification steps were applied to gain high-purity samples. The identity of plasma membrane vesicles was verified by western blot against plasma membrane H^+-ATPase. Fractions positive against H^+-ATPase but negative against mitochondrial marker AOX1/2 were applied in the in vitro FCR assay (Fig. 8). FCR activity was also identified in the samples using non-ionic detergents and isoelectric focusing (Fig. 9).

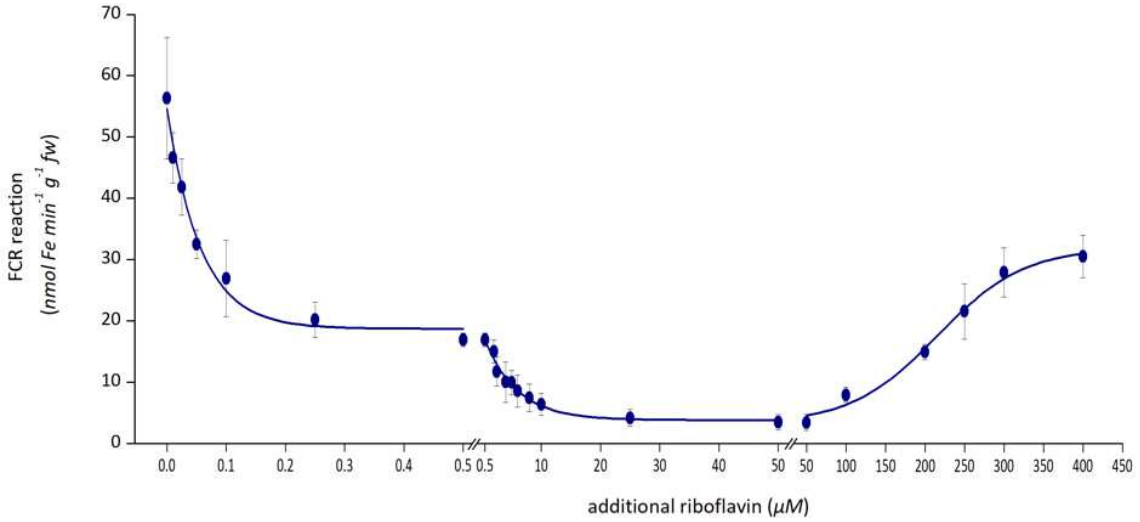

Figure 6. Ferric chelate reductase activity of roots of Fe deficient *Cucumis sativus* at saturating 400 µM Fe(III)-EDTA Fe source in function of the concentration of riboflavin applied in the assay (plotted on multiple logarithmic scales). Parameters indicated in the text.

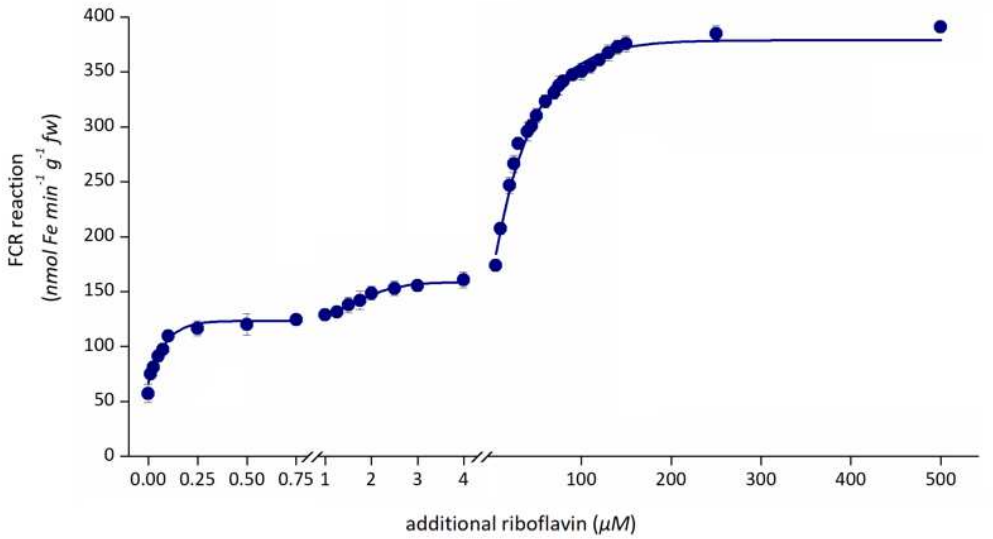

Figure 7. Ferric chelate reductase activity of roots of Fe deficient *Brassica napus* at saturating 400 µM Fe(III)-EDTA Fe source in function of the concentration of riboflavin applied in the assay (plotted on multiple logarithmic scales). Parameters indicated in the text.

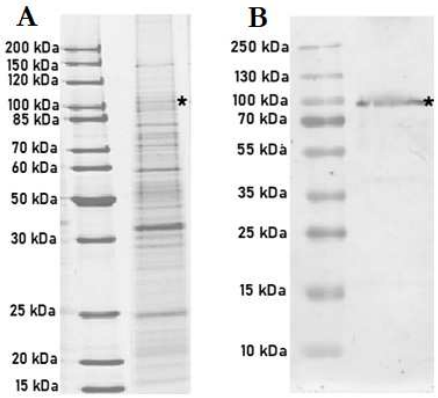

Figure 8. SDS-PAGE of proteins (A) and immunoblotting against plasma membrane H^+-ATPase (B) in a representative *Cucumis sativus* plasma membrane sample. Asterisk indicate the signal of H^+-ATPase at ca. 95 kDa.

Figure 9. FCR activity staining by BPDS assay on *Cucumis sativus* purified root plasma membrane sample treated by non-ionic detergents prior to focusing. Proteins were separated on pH 3–10 linear strip in BioRad IEF cell. Asterisk indicate the single identified activity band with pI of approx. 5.68.

In vitro FCR assays were performed on slightly solubilised samples achieved by the application of Triton-X-100 that enabled the retaining of the enzyme activity but made the cytosolic FAD and NADH binding domains readily available in the assay for the cofactors. FCR assay were performed using plasma membrane fractions obtained from *Cucumis sativus* with the application of the aforementioned 400 µM Fe(III)-EDTA as for ferric Fe substrate. Since as for cytosolic cofactor, FRO enzymes require FAD bound on the cytosolic domain, first we clarified whether riboflavin can replace FAD in the enzyme activity. Since elimination of FAD from the assay in the presence of riboflavin also vanished the enzyme activity we approved that FAD and riboflavin are not interchangeable at the cytosolic site of the enzyme. Application of riboflavin in the assay resulted in an enhancement of the FCR activity on a concentration dependent manner, where double saturation saturation kinetics was measured with parameters are of $K_{M;I}$: 4.61±0.83 µM riboflavin; $v_{max;I}$: 0.63±0.02 pmol Fe min^{-1} μg^{-1} protein and $K_{M;II}$: 39.00±4.70 µM riboflavin; $v_{max;II}$: 0.079±0.02 pmol Fe min^{-1} μg^{-1} protein (Fig. 10). Taken into consideration of the result of Topic 1 that the *Cucumis sativus* model express two FRO genes associated with plasma membrane FCR activity, results suggests that both of these enzymes are capable for interaction to riboflavin, although minor structural differences may affect the interaction.

Plasma membrane vesicles isolated from *Brassica napus* were also subjected to FCR activity analysis. Nevertheless, technical difficulties in the plasma membrane isolation has not allowed to gain enough data points to calculate enzyme kinetics yet, thus supplementary measurements are in progress. Already existing data suggests a saturation in the 140–200 µM external riboflavin range. Monophasic saturation is suggested. We expect that measurements on the *Brassica napus* model will be completed by May, 2022.

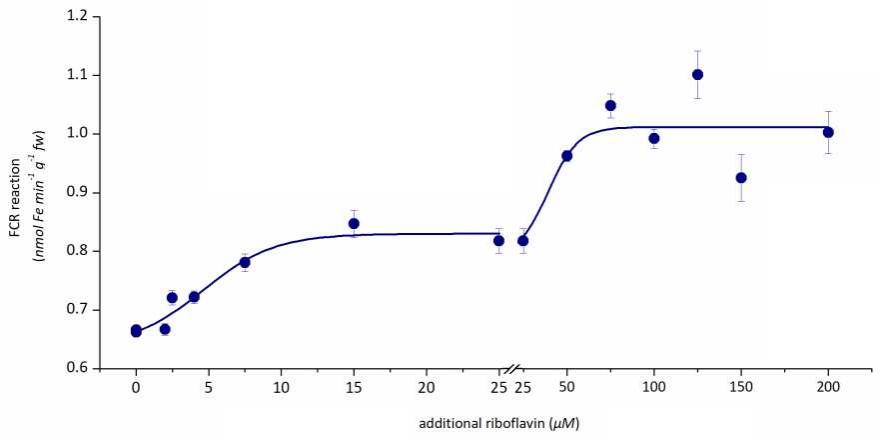

Figure 10. Ferric chelate reductase activity of plasma membrane isolated from *Cucumis sativus* at saturating 400 µM Fe(III)-EDTA Fe source in function of the concentration of riboflavin applied in the assay (plotted on multiple scales). Parameters indicated in the text.

→ Taking all results together, data verify our *a priory* hypothesis. Flavins operate as redox shuttle between plasma membrane localised FRO enzymes and ferric Fe compounds that enable a remote reduction of ferric Fe. The riboflavin concentration dependent enhancement on the FCR reaction indicates that riboflavin can transmit the reducing power to ferric Fe compounds that mechanism is an evolutionary advantage of flavin secreting species to cope with Fe limitations in the rhizosphere. Preliminary results were presented on conferences [16; 17]. A manuscript based on these data is in preparation [10].

Strategy I plant *Arabidopsis thaliana* is known by literature evidence to release fraxetin into the rhizosphere upon Fe deficiency. According to the results of Topic 2 that fraxetin maintain an equilibrium that shifts towards ferrous Fe formation but does not reduce ferric Fe directly, although reduction of fraxetin cannot be excluded, we set up a hypothesis that similarly as flavins the coumarine fraxetin can also operate as reducing power shuttle. To test this hypothesis, instead of *Cucumis sativus* we have involved *Arabidopsis thaliana* along with the *Brassica napus* model. Based on UHPLC measurements, we could no detect traces of fraxetin in the roots and nutrient solution of *Brassica napus* grown under Fe limiting condition thus it was considered as non-secreting species for fraxetin.
We applied the in vivo FCR assay using excised root tips at saturating Fe concentration of 400 µM Fe(III)-EDTA, pH 6.0. Using the *Brassica napus* model in the *in vivo* assays, fraxetin enhanced the FCR activity in the low concentration range. Enzyme activity parameters were K_M: 2.60±0.79 µM fraxetin and v_{max}: 5.07±1.68 nmol Fe min^{-1} g^{-1} fw. Exceeding 100.13±39.98 µM concentration of fraxetin in the assay, FCR activity get limited and indicate a decline. Using *Arbidopsis thaliana* model, we got a similar result where *in vivo* FCR activity was enhanced by the presence of fraxetin on a concentration dependent manner (parameters are: K_M: 3.61±0.62 µM fraxetin and v_{max}: 7.80±2.21 nmol Fe min^{-1} g^{-1} fw.), and the reaction get limited in the 50–125 µM fraxetin range. Although these results indicate that fraxetin, similarly to riboflavin, is capable to enhance the root FCR activity. Nevertheless, the equilibrium that maintained between ferric and ferrous Fe by fraxetin should be taken into

account at the interpretation of the results. As we presented at Topic 2, adding BPDS to the fraxetin:Fe(III) system shifts the equilibrium towards Fe(II) that results in a $Fe(II)(BPDS)_3$ stable complex formation. Since in the *in vivo* FCR assay ferrous Fe formation can be measured by specific Fe(II) ligands only, determination of the effect of fraxetin on the FCR activity is limited. Since non-invasive techniques such as Mössbauer spectroscopy does not give a signal in the 400 µM Fe concentration range or below, we reached an unforeseen difficulty in the experimental setup. To overcome on these difficulties we keep searching for microscale analytical techniques to determine FCR activity on a BPDS independent manner.

Nevertheless preliminary data enable to state that fraxetin also enhance FCR activity at least in low concentration. Taken together the results with data obtained in Topic 2, the equilibrium maintained by fraxetin also support the reduction based Fe uptake. Therefore, fraxetin seems to have a dual role in enhanging Fe availability.

Topic 4: Ferric-citrate is susceptible to photoreduction that contributes to the foliar Fe uptake

Light has a special important in the chemistry of Fe compounds that also affect the biological utilisation and stability of Fe. Since both ligands of Fe and Fe complexes can absorb in the visible range of the spectrum, both excitation can impact Fe reduction. Thus we set up a hypothesis that light is a significant factor in Fe nutrition that support the reduction based Fe transport in plants.

To verify the hypothesis, photocatalysed reduction of Fe by riboflavin and fraxetin were tested in a 100 µM Fe(III)-EDTA solution. 10 min 80 µmol m^{-2} s^{-1} white light led to a significant reduction of ferric Fe: according to BPDS measurement, concentration of Fe(II) were 2.0±0.1 and 8.4±0.9 µM in samples kept in darkness and on light, respectively. In contrast to riboflavin, fraxetin induced similar extent of Fe reduction both on light and in darkness, the reasons of which is discussed at Topic 2.
Light is also known to promote the reduction of ferric Fe by oxidative decarboxylation of carboxylate ligands. The quantum yield and the final products highly depend on the pH of the solution, ligand-to-metal ratio and the type of the light source. Although Fe photoreduction also impact the organic ligand, the composition of the final products of reoxidation was questionable. Previous data remained doubtful since spectrophotometric determination of Fe(II) also requires the application of measuring light, which can be considered as additional irradiation of the sample resulting in photoreduction during the measurement. To overcome these complications, we applied ^{57}Fe Mössbauer spectroscopy, which is a powerful tool to investigate electronic and magnetic properties of Fe compounds and their transformation. As among the he most important Fe compounds in the apoplastic spaces of leaves (potentially light exposed plant tissues; Sági-Kazár *et al.* 2022. *J Exp Bot* 73:1717) we applied Fe(III)-citrate in the photodegradation studies. Fe compounds formed during the photodegradation of ferric citrate was studied in immediate frozen solutions. Depending on the time of irradiation of four model systems of different pH values (1.4, 3.3, 5.5 and 7.0, respectively) with Fe(III)

to citrate molar ratio equals to 1:1.1, the extent of ferric Fe photoreduction highly varied. In the strongly acidic solution complete reduction of Fe was revealed with formation of Fe(II) citrate and hexaaqua complexes in equal concentration. At higher pH the only product of photodegradation was Fe(II) citrate, which was later reoxidized and polymerized, resulting in the formation of polynuclear stable ferric Fe compound. Fe(III)-citrate was also applied as foliar treatment on leaves of *Brassica oleracea* plants developed under Fe limiting conditions. Fe species detected in the unwashed leaves were identical to those observed in the solution exposed to the same illumination conditions indicating that processes on the leaf surface can be modelled by the processes in the iron containing solutions. The dominant iron species found in the washed leaves indicate, that the majority of Fe nuclei taken up by the foliage of Fe deficient plants are allocated to the chloroplasts, to symplastic Fe transport routes and most probably to vacuolar storage.

→ Our results verified the *a priori* hypothesis. Results were presented on conference [11] and published in Gracheva *et al*. (2022, *Photochem Photobiol Sci* DOI: 10.1007/s43630-022-00188-1 [1]).

<u>Topic 5</u>: Chloroplast ferric chelate reductase prefers ferric citrate as for substrate

FRO enzymes are characterized to contain a ferric reductase domain on the external membrane side of the membrane intrinsic proteins. Although FRO enzymes, especially the best characterized root FROs are capable to reduce ferric Fe compounds in an enormous variability, the natural substrates of FRO enzymes remained poorly characterized. In spite of root plasma membrane FRO enzyme, intracellular members of the family associate transmembrane intracellular Fe transport by reducing ferric Fe forms available in the labile Fe pool of the cells. Although pieces of information is available on the chemical forms of Fe involved in the foliar and intracellular Fe homeostasis of plants, the *in vivo* form of Fe pool had not been properly characterised in the cytoplasm before, since i) no cytoplasm isolation techniques retaining the *in vivo* microenvironment of Fe are available, and ii) the concentration of Fe in the cytoplasm is generally below the detection limit of techniques that can assess Fe speciation such as Mössbauer spectroscopy or μXANES. Taking into consideration literature evidence and the proposed pH of the inter membrane space in the chloroplast envelope, we set up a <u>hypothesis</u> that the chloroplast inner envelope membrane localised FRO7 utilize Fe(III)-citrate in the FCR reaction.
To test this hypothesis, we characterises the preference of Fe species of the chloroplast Fe acquisition machinery, the operation of which was previously shown to be based on the activity of FRO7. We applied low-temperature, high-magnetic-field Mössbauer spectroscopy to investigate the chemical forms present at the pH of the cytoplasm in solutions of the complexes Fe(III)-citrate, Fe(III)-nicotianamine and Fe(II)-nicotianamine. To test the substrate preference, we involved these natural Fe compounds along with artificial Fe chelates Fe(III)-EDTA and Fe(III)-*o*,*o*'EDDHA in Fe uptake assay using intact *Brassica napus* chloroplasts.

Fe(III)-nicotianamine was characterized to contain a minor paramagnetic Fe(II) component in the ^{57}Fe Mössbauer spectrum (ca. 9% of the Fe content), a paramagnetic Fe(III) component exhibiting dimeric or oligomeric structure (ca. 20% of the Fe content), and a Fe(III) complex, likely being a monomeric structure, which undergoes slow electronic relaxation at 5 K (dominant component, ca. 61%). Fe(II)-nicotianamine contained more than one similar chemical Fe(II) environments whereas no sign of Fe(III) components was identified, approving that nicotianamine form stable complexes with Fe(II) that retains the reduced status. Fe uptake of chloroplasts utilized Fe(III)-citrate complexes only, whereas Fe(III)-nicotianamine and Fe(II)-nicotianamine, Fe(III)-EDTA and Fe(III)-*o*,*o*'EDDHA were only able to cross the chloroplast outer envelope membrane but was not utilised into the Fe uptake into the chloroplast stroma. Since no Fe-nicotianamine complex species were found in the intact chloroplasts, nicotianamine was rejected operating as primary Fe ligand in chloroplasts.
→ We concluded that Fe(III)-citrate is the natural substrate of chloroplast FRO7. Results were presented on conferences [22; 23] and published in Müller *et al.* (2019, *Planta* 249:751 [5]).

→ We summarized the recently gained knowledge on the intracellular Fe transport and homeostasis in a review paper (Vigani *et al.* 2019, *Plant Cell Physiol* 60:1420 [4]). Along with all already known component of the intracellular Fe homeostasis in plants, we discussed the characteristics of FRO enzymes with a special focus of FRO enzymes in the foliage and in the generative tissues.

Topic 7: Suppression of chloroplast ferric chelate reductase is essential to avoid excess Fe accumulation in chloroplasts

Reduction based Fe uptake of chloroplasts was previously stated as the primary route of the plastidial Fe acquisition. Literature evidence indicated that knock down mutation of chloroplast FRO7 highly increase the sensitivity against alkaline soil conditions and plants are only able to survive on a way dependent on Fe photoreduction. Nevertheless, chloroplast FCR activity only enables liberated Fe(II) for the transmembrane Fe transport across the chloroplast inner envelope membrane. Indeed, mutation of the chloroplast Fe(II) transporter PIC1 is lethal, thus characterization remained unresolved. Literature evidence indicated a co-expression of PIC1 to NiCo, orthologue of cyanobacterial Ni and Co transporter. Thus, NiCo had been stated as an interacting partner of PIC1 in the transmembrane ferrous Fe transport in chloroplasts. Since direct evidence was missing, we set up a hypothesis that an exclusive collaboration of PIC1 and NiCo requires a synchronized expression and protein content independent on the Fe nutrition and developmental status.
To test this hypothesis, we analysed PIC1 and NiCo at transcript and protein levels along with the organellar Fe unloading component MAR1/FPN3 in leaves of *Brassica napus* model. We applied deficient, optimal and supraoptimal, non-toxic Fe nutrition schemes. Characteristics of leaves were followed throughout their lifespan from development to senescence. In parallel with the increasing Fe supply and aging of the leaves expression of *BnPIC1* became repressed, in parallel with the limited Fe accumulation in the plastids. This was concluded as a

protective mechanism against toxic Fe accumulation. In contrast, transcript and protein amount of *BnNiCo* tendentiously increased during the leaf development and senescence initialization, with a markedly upregulation in aging leaves. To compare, relative transcript amount of *BnMAR1* increased mainly in aging leaves facing Fe deficiency.
→ Taken together chloroplast physiology, Fe content and transcript amount data, we concluded that the participation of NiCo in the chloroplast Fe acquisition is not supported, thus we rejected the *a priori* hypothesis of exclusive PIC1-NiCo collaboration. Results were presented on conference [20] and published in published in Pham *et al.* (2020, *Planta* 251:1 [3]).

Topic 7: Suppression of chloroplast ferric chelate reductase is essential to avoid excess Fe accumulation in chloroplasts

Chloroplast FRO7 is the key enzyme of the operating chloroplast inner envelope membrane FCR activity. Knocking down FRO7 or limiting its function results in a diminished plastidial Fe homeostasis according to literature evidence. Fe in the chloroplasts is required for the biosynthesis and the function of the photosynthetic apparatus, thus plastidial Fe content represent over 80% of the Fe content of the mesophyll. Nevertheless, constant ligation of Fe is essential since any dysfunction in the foliar Fe homeostasis and Fe liberation results in a massive oxidative stress due to the redox active nature of Fe ions and the initiation of Fenton reactions, especially in the presence of hydrogen peroxide. In consequence, proper functioning of the plastidial Fe homeostasis is crucial. Taking together, we set up the hypothesis, that FRO7 chloroplast FCR enzyme is the key component in the Fe homeostasis of chloroplasts and the expression and enzyme activity level regulations of FRO7 are responsible for the balanced Fe content of the chloroplasts that enables the biosynthesis of the photosynthetic apparatus but eliminates the risk of chloroplast Fe overloading.

To test this hypothesis we used the *Brassica napus* model as a close relative of the already characterized *Arabidopsis thaliana* that enables to harvest a large amount of foliar material for chloroplast and membrane purification studies. Nevertheless, to reveal transcript level regulations on the chloroplast FRO, first we had to identify *Brassica napus* orthologue of *Arabidopsis thaliana FRO7.* A sequence encoding *Brassica* orthologue of the *Arabidopsis* query was identified in the *B. napus* genome in the *B. rapa* parental genome as *FRO7* orthologue (*GSBRNA2T00048061001; BnaAnng20940D*; reciprocal best hit: *At5g49740.1*). Protein sequence alignments of AtFRO7 and BnFFO7 also indicated a high similarity. Chloroplast transit peptide was identified in the sequence.
To validate the identification of *Brassica FRO7*, a GFP labelled fusion construct was created together with promoter (identified as 1875 bp sequence upstream to the start codon of *GSBRNA2T00048061001*; application of primers used for cloning of the promoter sequence on genomic DNA isolated from *Brassica napus* cv. Darmor resulted in a 1900 bp product; identity approved by Sanger sequencing), 5' UTR and 3' UTR sequences that were identified in the *Brassica* genome. The edited sequence, containing coding, linker, marker tag and 3' UTR sequences was commercially synthesized. Transformation cassettes (pCambia2301

vector) were assembled containing the synthesized *FRO7* cDNA and its 3' UTR (encoding FRO7-GFP tag fuson). Alongside, FRO7-3xFLAG tag construct was also created. To test the localisation of the FRO7-GFP construct, transient expression studies were carried out by infiltration of *Rhizobium radiobacter* AGL1 strain carrying the construct into young but already developed leaves of common bean (*Phaseolus vulgaris* L. var. *nanus* cv. Borlotto). Intact chloroplasts were purified from positive spots of bean leaves. Localisation of FRO7-GFP was confirmed by laser-confocal microscope in the chloroplast envelope membrane.

We also established transformant lines expressing FRO7-GFP and FRO7-3xFLAG tag. Since transformation of *Brassica napus* cv. DK Exquisite (autumn cultivar) and cv. Westar (spring cultivar) both found to be complicated, we introduced *Brassica oleracea* subsp. *alboglabra* AG DH 1012 in transformation studies. T_0 individuals identified carrying the FRO7-FLAG construct failed to produce transgenic seeds. Stable T_1 lines carrying the FRO7-GFP transgene were identified, indeed. Expression of the transgene was validated by RT-PCR and western blot on purified chloroplast envelope membranes against the GFP fusion tag. To test the protein complex localisation of the FRO7-GFP construct, T_1 individuals were grown in hydroponic cultures to produce enough plant material for envelope isolation for BN-PAGE studies. To prepare one envelope protein sample for BN-PAGE 10–50 g of fresh developing leaves is required. Chloroplast envelope isolation techniques were optimised for the model plant to reduce the plant material needed for the further protein studies. So far, FRO7-GFP construct was unable to detect by immunoblotting on Blue Native/SDS 2D PAGE gels. Thus we assume that Coomassie marks GFP epitopes. To avoid this complication, work remained ongoing to optimize Clear Native page on chloroplast inner envelope membrane fractions as for first dimension separation of native chloroplast envelope protein complexes. Based on the stable lines carrying FRO7-GFP, further studies are planned, thus established value will be also applied in upcoming projects.

Based on the identification of *FRO7* in *Brassica napus*, we applied the same experimental setup as in Topic 6 to test developmental and Fe nutrition scale alteration in the expression of *FRO7*. Parallel to the quantitative transcript analysis, we measured the Fe content in both leaves and chloroplasts. Although supraoptimal Fe nutrition caused an accumulation of Fe, especially in older leaves of plants cultivated on supraoptimal Fe nutrition, Fe content of the chloroplasts did not show any signs of Fe accumulation. Nevertheless, Fe content of the chloroplasts in these plants remained stable also with aging. To test the Fe uptake characteristics of isolated chloroplasts, we measured the light-driven utilisation of Fe(III)-citrate Fe source in *in vitro* assay. Supraoptimal Fe nutrition caused a loss in the substrate affinity of the plastidial Fe uptake (K_M were 46.37±13.26; 47.0±2.60; and 30.22±5.43 μmol Fe(III)-citrate in chloroplasts of plants grown under deficient, optimal and supraoptimal Fe nutrition, respectively) whereas Fe uptake both of the chloroplasts of Fe deficient and supraoptimal Fe grown plants remained below the optimal Fe nutrition (v_{max} were 0.68±0.15; 7.81±0.16; and 3.38±0.01 fmol Fe chloroplast^{-1} in in chloroplasts of plants grown under deficient, optimal and supraoptimal Fe nutrition, respectively), the reason on which in Fe deficient chloroplasts was the improper function of the photosynthetic apparatus based on previous data. The enzyme activity of FRO7 was measured in in vitro assays on purified

chloroplast inner envelope membrane fractions. Purity of chloroplast inner envelope membranes was tested by western blot against: TPT, IEP37, PIC1 (inner envelope membrane), TOC75, OEP16 (outer envelope membrane), LHCII (thylakoid) and AOX1/2 (mitochondrial) markers. TPT/IEP37/PIC1 positive, TOC75/OEP16/LHCII/AOX1/2 negative samples were identified as pure fractions. Similarly to that of the characteristics of the Fe uptake of chloroplasts, in the in vitro FCR reaction we also find a significantly lowered substrate affinity in samples of plants grown under supraoptimal Fe nutrition (KM were 23.46±3.03; 22.07±2.87; and52.52±2.36 μmol Fe(III)-EDTA in chloroplasts of plants grown under deficient, optimal and supraoptimal Fe nutrition, respectively) whereas saturating of the enzyme activity remained intact (v_{max} were 12.54±0.40; 11.99±0.34; and 12.58±0.53 pmol Fe min^{-1} μg $protein^{-1}$ in chloroplasts of plants grown under deficient, optimal and supraoptimal Fe nutrition, respectively). Regarding the expression of *FRO7*, supraoptimal Fe nutrition induced a rapid decline (within a 4-day time) in the relative transcript amount. A negative correlation was found between Fe nutrition and the highest relative transcript abundance of *FRO7*: the higher the Fe nutrition the lower the relative transcript amount at its peak was found.

→ In conclusion, data verified our *a priori* hypothesis. Both Fe deficiency and supraoptimal Fe nutrition induce a holistic suppression of the reduction-based Fe uptake of chloroplasts thus regulation of *FRO7* fundamentally differs from that of the root *FROs*. In contrast to root tissues where Fe deicieincy response resulted in a peak expression and activity of the FROs, regulation of FRO7 is less sensitive to the deprivation of Fe. In contrast, saturation of the Fe requirement suppress the reduction based Fe uptake of chloroplasts by regulating FRO7 that mechanisms is a crucial mechanisms that protects chloroplasts against Fe overloading and thus against oxidative damages. Results were presented on conferences [14; 16; 19] and published in published in Sági-Kazár *et al*. (2021, *Front Plant Sci* 12:748 [2]).

Topic 8: The plastidial reduction based Fe acquisition is in a reciprocal connection with the operation of the photosynthetic machinery

Fe uptake of chloroplasts is highly dependent on the reduction based strategy operated by the plastidial FRO enzyme. Previous data evidenced that plastidial FRO enzyme require reduced NADPH as for source of the reducing power. Since NADPH is produced in the operation of the photosynthetic electron transport system in chloroplasts, NADPH dependence make plastidial Fe uptake directly dependent on the operation of the photosynthetic apparatus. This is in accordance with the findings of Topic 7 that Fe deficiency and thus improper biosynthesis of the photosynthetic machinery cause a feedback inhibition on the plastidial Fe uptake. Nevertheless over Fe deficiency, multiple environmental stressors and conditions affect the development and the operation of the photosynthetic electron transport system. Although plastidial Fe acquisition can be affected by these factors, it has not been characterized so far. Since heavy metal stresses such as exposure of cadmium (Cd) and zinc (Zn) are known to affect the Fe homeostasis of plants from literature evidence, we set up a hypothesis that heavy metal stresses also affect the plastidial Fe uptake by impacting the photosynthetic apparatus and thus NADPH in the chloroplast stroma.

To verify the hypothesis, we applied 10 µM Cd and 200 µM Zn stress on previously characterised *Beta vulgaris* model that has a high sensitivity against heavy metal stresses (in comparison to *Brassica napus*). As for reference, we also involved plants grown under optimal Fe nutrition and Fe deficiency conditions. Fe content of the chloroplasts proved to be sensitive to al applied treatments (plastidial Fe contents were 0.984±0.171; 0.407±0.091; 0.494±0.076; and 0.539±0.070 fmol Fe chloroplast^{-1} in plastids of plants grown under optimum and deficient Fe nutrition, Cd and Zn stress, respectively). Although Fe uptake of chloroplasts from an external Fe(III)-citrate pool were significantly lowered in all treatments, too (342.0±41.5; 278.9±21.0 and 288.7±55.5 amol Fe chloroplast^{-1} in chloroplasts of Fe deficient; Cd and Zn treated plants, respectively) compared to that of chloroplasts of plants grown under optimum Fe nutrition (570.7±102.6 amol Fe chloroplast^{-1}), a direct inhibition of chloroplast Fe uptake is unlikely based on literature evidence. Cd treatment induced a strong dysfunction of the photosynthetic apparatus indicated by the increase in the proportion of non-functional photosystem II reaction centres (Φ_{NPQ} of 0.251±0.021 and 0.000±0.011 in leaves of Cd treated and optimum Fe supply grown plants, respectively). Zn treatment not induced a decrease in the photochemical efficiency. In turn, in Zn treated plants the concentration of malondialdehyde, indicating oxidative membrane damages, was found to be increased 4.86±0.08-fold compared to plants grown under optimum Fe supply, without treatment. Thus we tested the status of the antioxidative defence in the chloroplasts as potential competing processes with Fe uptake for reducing power source NADPH. To measure the activity of antioxidative defence enzymes, intact chloroplasts were purified. The activity of superoxide dismutase (SOD) and ascorbate peroxidase (APX) isoforms as primary antioxidant enzymes in chloroplast, was determined by activity assay followed by the separation of native proteins by native PAGE. Both SOD and APX activity increased in the chloroplasts of plants grown under Fe deficient conditions (SOD activity were 412.0±33.9; and 774.1±88.0 µg^{-1} protein, and APX activity were 3.41±0.12; and 8.82±0.38 µg^{-1} protein in chloroplasts of plants grown under optimum and deficient Fe nutrition, respectively). In Cd and Zn stressed plants, the SOD activity indicated no induction (activities were 392.0±174.2; and 445.3±190.8 µg^{-1} protein in chloroplasts of Cd and Zn stressed plants, respectively), and the activity of plastidial APXs were strongly suppressed (0.23±0.08; and 0.05±0.01 µg^{-1} protein in chloroplasts of Cd and Zn stressed plants, respectively). The activity of glutathione reductase (GR) also indicated similar alterations: chloroplasts of plants grown under Fe deficiency showed elevated, whereas those of Cd and Zn stressed plants showed suppressed GR activity compared to chloroplasts of optimum Fe grown plants (activities were 335.4±8.8; 605.5±28.2; 9.6±1.6; and 87.9±3.2 pmol reduced NADPH µg^{-1} protein in chloroplasts of plants grown under optimum and deficient Fe nutrition, Zn and Cd stress, respectively). Preliminary measurements on the amount of reduced GSH, ascorbate and NADPH in the chloroplasts requires repeated measurements. We expect that measurements will be completed by May, 2022.

→ <u>In conclusion</u>, the decreased activity of antioxidative enzymes contributes to the oxidative damages, whereas decreased amount of reduced ascorbate, GSH and NADPH indicate an impaired biosynthesis/oxidation balance. Thus, the reduction based Fe uptake of chloroplasts

is in competition with all other metabolic processes that decrease the ratio of reduced NADPH as reducing power source. After approval, a manuscript of Zelenyánszki *et al.* [7] based on these data will be submitted.

Over environmental stressors such as heavy metals, the developmental program also affect the photosynthetic apparatus. In higher plants the biogenesis of the photosynthetic apparatus and biosynthesis of chlorophylls are light dependent processes. In the absence of light chloroplast development remains incomplete resulted in the formation of etioplasts containing prolamellar body, a special internal membrane structure. Etioplasts thus lack the photosynthetic apparatus and in consequence NADPH production by photosynthesis. Nevertheless, biogenesis of the photosynthetic apparatus, but housekeeping function of non-photosynthetic plastids also require the presence of Fe cofactors. Thus we asked the question, whether the reduction based Fe uptake route operates in non-photosynthetic plastids?

To reveal this question, we applied etioplast model in tissues that are already dedicated towards became photosynthetically active but in the absence of light, etiolation syndrome developed. As for plant model, we applied *Brassica oleracea* var. *sabauda* (kale), the overlapping leaves of which model induce an etiolation syndrome in the inner leaves (central leaves of the "head"). Regarding the Fe content of leaves Fe was mainly found to be associated with veins in etiolated leaves (average Fe contents were 34±4.16 and 14±2.56 µg Fe g^{-1} dw in veins and lamina, respectively). To study plastidial Fe content, the status of the photosynthetic apparatus and FCR activity of the plastidial inner envelope membrane, etioplasts and etio-chloroplasts were isolated. Minor Fe content was determined in the etioplasts (5.9±2.06 amol Fe $plastid^{-1}$) (Fig. 11). According to thylakoid proteome and chlorophyll fluorescence decay analysis, components of the photosynthetic apparatus cannot be detected until Fe content of etio-chloroplasts reach 44.69±14.35 amol Fe $plastid^{-1}$ in middle leaves of the head, suggesting plastidial Fe uptake occurs prior to the development of the photosynthetic apparatus. Fe uptake of both etioplasts and chloroplasts were studied. Although Fe uptake of chloroplasts proved to be a magnitude higher, light induced Fe uptake was also measured in etioplasts (Fig. 12). Nevertheless, important to mention that in vitro chloroplast Fe uptake assay includes a 30-min light incubation on 120 µmol photons m^{-2} s^{-1} photosynthetic photon flux density that also impact de-etiolation processes.

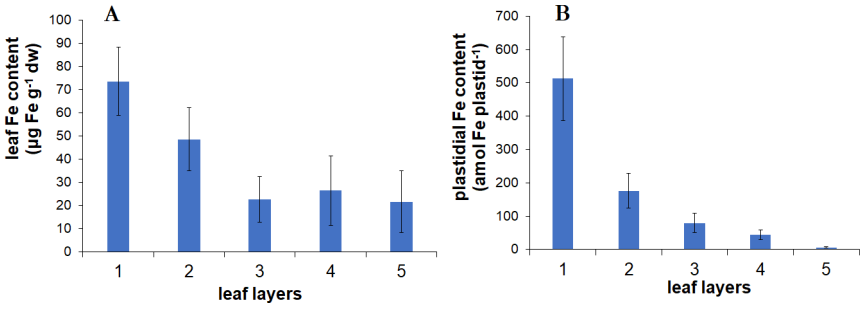

Figure 11. Average Fe content in the leaf lamina (A) and in the plastids (B) of different leaf storeys where storey 1 represents the outermost leaf layer of the kale's head and layer 5 is the innermost one.

Although *in vitro* plastidial Fe uptake assay might have been compromised by the application of illumination, we tested the FCR activity of both chloro- and etioplasts. For that, we have purified inner envelope membrane vesicles that were subjected to in vitro activity assays. Since in in vitro FCR assay NADPH and FAD is provided, activity is only determined by the status of the FRO enzymes. No differences were found between the FCR activity between the samples (Fig. 13) indicating the presence of FRO7 in the inner envelope of etioplasts.

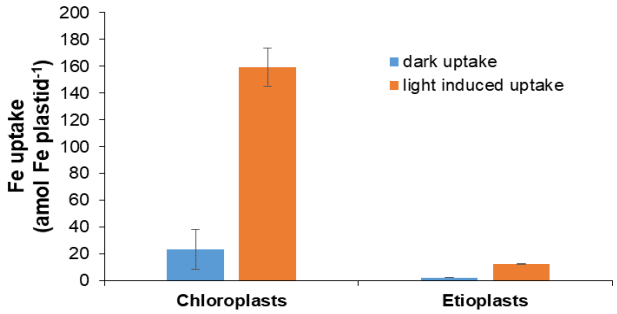

Figure 12. Light-induced and darkness Fe uptake of chloroplasts and etioplasts isolated from *Brassica oleracea* leaves. As for Fe source, 100 µM Fe(III)-citrate was applies as standard substrate of the chloroplast Fe uptake assays.

To address the differences between the results of Fe uptake and FCR activity results, we measured the content of reduced NADPH in the plastids of the leaf layers in the kale head (Fig. 14). The concentration of reduced NADPH was clearly dependent on the stage of etiolation where the NADPH content remained below the measurement threshold of the spectrophotometric measurement. Although photosynthetic apparatus was absent in the inner layers, oxidative pentose phosphate cycle is also able to produce NADPH for the anabolic metabolism.

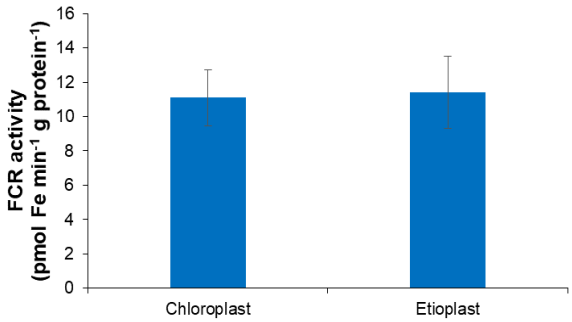

Figure 13. Ferric chelate reductase (FCR) activity of inner envelope membranes isolated from chloroplasts and etioplasts of *Brassica oleracea*.

To approve the presence of the reduction based Fe uptake in etioplasts, we measured variations in the relative transcript amount of *FRO7* between the leaf layers. In order to get a holistic overview on the Fe homeostasis of plastids, we also include into the analysis *MFL1* (proposed Fe transporter), *PIC1* (Fe(II) transporter in the chloroplast inner envelope), *ABCI8* (SUF machinery component), *MAR1*/*FPN3* (plasditial/mitochondrial dual localised putative

nicotianamine transporter), *YSL4* (Fe/Fe-nicotianamine transporter), and *YSL6* (Fe/Fe-nicotianamine transporter) (Fig. 15).

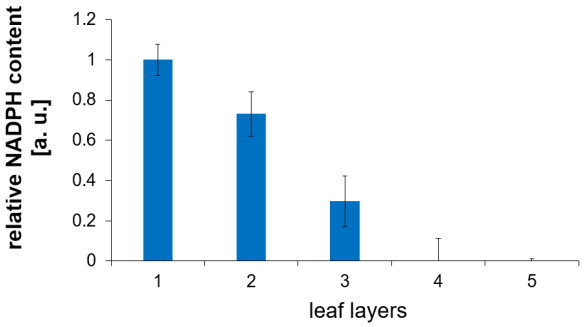

Figure 14. NADPH content of the plastids of the leaf layers in the kale head where storey 1 represents the outermost leaf layer of the kale's head and layer 5 is the innermost one.

Although we measured a four-fold increase in the relative transcript amount of *FRO7* in parallel to the de-etiolation status of leaves, it was clearly detected in etiolated leaves. Thus, FCR activity and *FRO7* expression together support the presence of the reduction based Fe uptake route in etioplasts. Except that of *YSL4*, expression all investigated members of the plastidial Fe homeostasis showed a correlation to that of *FRO7* according to Spearman correlation analysis. To give a positive answer for our question, one measurement is still pending. Using FRO7-GFP positive *Brassica oleracea* cv. *alboglabra* DH1012 T_2 generation plants established in Topic 7 we induced artificial etiolation of developing plant by a complete covering the shoot apex of the plants but leaving already developed leaved intact on the plants. We aimed to detect FRO7 at protein level in the etioplasts of the artificially etiolated leaves using the GFP tag. By proving the presence of FRO7 in the target, results will be ready for publication.

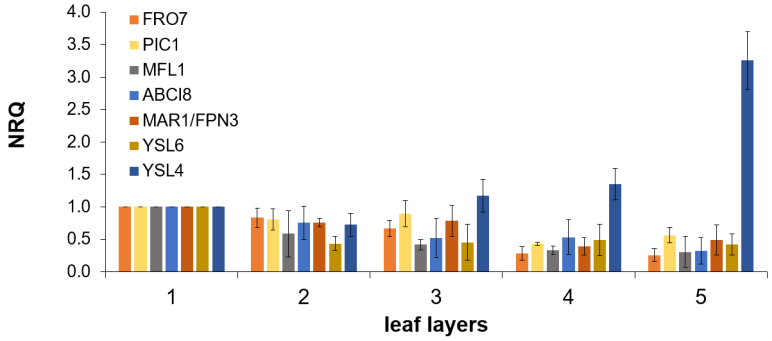

Figure 15. Normalised relative expression (NRQ) of chloroplast Fe homeostasis elements in leaf layers of the kale head. Layer 1 represents the outermost leaf layer of the kale's head and layer 5 is the innermost one.

→ In conclusion, we answered our initial question that reduction based Fe uptake mechanism already operational in the etioplasts, but in the lack of effective NADPH source, its effectivity is limited. Nevertheless, the reduction based Fe uptake becomes prominent in parallel with the de-etiolation processes. The high expression of *YSL4* but also the location of Fe along the leaf veins makes the etiolated leaves similar to *Arabidopsis* embryo, where *YSL4* was already

indicated to be involved into the plastidial Fe homeostasis. Preliminary results were presented on conference [15]. A manuscript of Sági-Kazár *et al*. [9] based on the data will be submitted.

As for a holistic conclusion of the research project K-124159, we summarize our findings as follows:

→ Flavin biosynthesis is an inclusive Fe deficiency response that inherently involved in the reduction based Fe uptake strategy of flavin secreting dicots
→ Riboflavin does not have an inherent ability to complex or reduce ferric Fe compounds.
→ Flavins operate a redox shuttle between plasma membrane localised ferric chelate reductase enzymes and (insoluble) ferric Fe compounds that mechanism enable a remote reduction of ferric Fe.
→ In turn, coumarin derivative fraxetin maintain a redox equilibrium between ferric and ferrous Fe by interacting with ferric Fe compounds. Since plants remove ferrous Fe from the equilibrium, fraxetin contributes to the reduction based Fe uptake on a ferric chelate reductase activity independent way.
→ In the light exposed plant tissues, photoreduction of ferric Fe complexes contributes to the reduction based Fe transport.
→ Natural substrate of chloroplast ferric chelate reductase enzyme is Fe(III)-citrate.
→ Chloroplast ferrous Fe transporter PIC1 does not collaborate to NiCo on an exclusive way
→ Expression and post-translational regulations of the chloroplast ferric chelate reductase are responsible for the balanced Fe uptake of chloroplasts
→ Reduction based Fe uptake is already operational in etoplasts. Nevertheless, all stress situation that compete for NADPH decrease the reduction based Fe uptake of chloroplasts.

Results were published in 5 peer-reviewed publications. One publication is still under review at peer-review journal. Based on the initial results of the research project we started additional investigations over the core research project that resulted in 4 additional manuscripts, will be published in the close future.

Along with the peer-reviewed publications, 5 BSc theses [25–29]; 4 institutional and National student competition theses [30–33]; 5 MSc theses [34–38]; and 2 PhD theses [39–40] were completed during the 4.5 years duration of the project. Results obtained in the frame of the project were presented 4 international [11–13; 15; 18; 20; 22; 24] and 3 inland congress [14; 16; 17; 19; 21; 23] in 14 presentations. Indeed, a few previously planned conferences were cancelled due to the COVID-19 pandemics.

Publications:

Cumulative IF of the already published articles: **21.301**

(Members of the research group are highlighted in all publications)

Full peer-reviewed papers (5):

[1] **Gracheva M**, Homonnay Z, **Singh A**, **Fodor F**, Marosi VB, **Solti** Á, **Kovács K** (2022) New aspects of the photodegradation of iron(III) citrate: Spectroscopic studies and plant related factors. *Photochemical & Photobiological Sciences DOI: 10.1007/s43630-022-00188-1* **IF 3.982***

[2] Sági-Kazár M, **Zelenyánszki H**, Müller B, Cseh B, Gyuris B, Farkas SZ, **Fodor F**, Tóth B, Kovács B, Koncz A, Visnovitz T, Buzás EI, Bánkúti B, Bánáti F, Szenthe K, **Solti Á** (2021) Supraoptimal iron nutrition of *Brassica napus* plants suppresses the iron uptake of chloroplasts by down-regulating chloroplast ferric chelate reductase. *Frontiers in Plant Science, 12, 748* **IF 5.753**

[3] Pham HD, Pólya S, Müller B, Szenthe K, Sági-Kazár M, Bánkúti B, Bánáti F, Sárvári É, **Fodor F**, **Tamás L**, Philippar, K, **Solti Á** (2020) The developmental and iron nutritional pattern of PIC1 and NiCo does not support their interdependent and exclusive collaboration in chloroplast iron transport in *Brassica napus*. *Planta 251: 96.* **IF 4.116**

[4] Vigani G, **Solti Á**, Thomine S, Philippar K (2019) Essential and detrimental – An update on intracellular iron trafficking and homeostasis. *Plant and Cell Physiology 60: 1420-1439.* **IF 4.062**

[5] Müller B, **Kovács K**, Pham H-D, Kavak Y, Pechoušek J, Machala L, Zbořil R, Szenthe K, Abadía J, **Fodor F**, Klencsár Z, **Solti Á** (2019). Iron uptake machinery of chloroplasts utilise ferric-citrate but not iron-nicotianamine complexes in *Brassica napus*. *Planta 249: 751-763.* **IF 3.390**

Paper submitted for publication / under review (1):

[6] **Singh A**, **Gracheva M**, Kovács Kis V, Keresztes Á, Sági-Kazár M, Müller B, Pankaczi F, Ahmad W, **Kovács K**, May Z, Tolnai G, Homonnay Z, **Fodor F**, Klencsár Z, **Solti Á**. Apoplast utilisation of nanohaematite initiates parallel suppression of *RIBA1* and *FRO1&3* in *Cucumis sativus*. Under review at *Journal of Experimental Botany; MS ID#: JEXBOT/2022/307620*

Manuscripts in preparation (4):

[7] **Zelenyászki H**, Sági-Kazár M, **Kovács K**, Tóth BS, Sagardoy R, Tóth B, Abadía J, **Fodor F**, **Solti Á**. Decreased production and enhanced need for reduction power in chloroplasts effectively compete to reduction based iron uptake of chloroplasts. *In preparation – will be submitted until June 2022*

[8] **Gracheva M**, Ahmad W, **Singh A**, Bányai A, Homonnay Z, **Solti Á**, **Kovács K**. Interaction of fraxetin with stable iron complexes – A Mössbauer spectroscopy study. *In preparation – will be submitted until June 2022*

[9] Sági-Kazár M, Lenk S, Cseh B, Müller B, Illés L, May Z, Barócsi A, Sárvári É, Solymosi K, **Solti Á**. Revisiting the reduction based iron uptake in plastids with undeveloped photosynthetic machinery. *In preparation – will be submitted until June 2022*

[10] **Singh A**, Bóta B, Hernández-Gamero R, Ahmad W, **Gracheva M**, Bányai A, **Boldizsár I**, **Fodor F**, **Kovács K**, **Solti Á**. Root exudate flavin compounds support ferric chelate

reduction by delivering reduction power between the membrane bound enzymes and the ferric compounds in the rhizosphere/apoplast. *In preparation – will be submitted until September 2022*

Conference abstracts (14):

[11] **Gracheva M**, Homonnay Z, Klencsár Z, **Kovács K** (2021) Spectroscopic studies of iron(III) citrate complexes in aqueous solution. *In: Greculeasa S, Locovei C, Kuncser V (eds.) International Conference on the Applications of the Mössbauer Effect - Book of Abstracts ICAME 2021, ISBN: 978-606-94603-3-7, p. 232*

[12] **Gracheva M**, Homonnay Z, **Kovács K**, Klencsár Z, Lengyel A, Németh S, Szalay R, Kis Kovács V, **Singh A**, **Solti Á**, **Fodor F**, Music S, Kuzmann E (2021) Ferrihydrite formed from co-precipitated nanomagnetites in citric acid solution for plant nutrition. *In: Greculeasa S, Locovei C, Kuncser V (eds.) International Conference on the Applications of the Mössbauer Effect - Book of Abstracts ICAME 2021, ISBN: 978-606-94603-3-7, p. 237*

[13] **Gracheva M**, **Singh A**, Kovács Kis V, Keresztes Á, May Z, Sági-Kazár M, Ahmad W, Pankaczi F, Müller B, Tolnai G, Homonnay Z, **Fodor F**, Klencsár Z, **Solti Á**, **Kovács K** (2021) Iron nanooxides applied as iron fertilizers: Transformation and utilization. *In: Greculeasa S, Locovei C, Kuncser V (eds.) International Conference on the Applications of the Mössbauer Effect - Book of Abstracts ICAME 2021, ISBN: 978-606-94603-3-7, p. 231*

[14] Sági-Kazár M, Lenk S, Cseh B, Illés L, Barócsi A, Sárvári É, Solymosi K, **Solti Á** (2021) A redukción alapuló plasztidiális vasfelvétel mintázata és szabályozása. *In: Györgyey J (ed.) XIII. Magyar Növénybiológiai Kongresszus - Összefoglaló Kötet. ISBN 978-615-01-2350-9. E2-07*

[15] Sági-Kazár M, Lenk S, Sárvári É, Hegedűs C, Barócsi A, Solymosi K, **Solti Á** (2021) Molecular background of iron uptake mechanisms of non-photosynthetic plastids. *19th Congress of the European Society for Photobiology - Book of Abstracts, ISBN 978-3-200-07802-4, SP-8.4.6*

[16] Sági-Kazár M, **Singh A**, **Zelenyánszki H**, Ahmad W, Hernández-Gamero R, Cseh B, Bóta B, Müller B, **Gracheva M**, **Boldizsár I**, **Fodor F**, **Kovács K**, **Solti Á** (2021) Ferric chelate reductases, key enzymes in ferric iron reduction control iron homeostasis of plants at effector level. *In: Györgyey J (ed.) XIII. Magyar Növénybiológiai Kongresszus - Összefoglaló Kötet. ISBN 978-615-01-2350-9. E1-11*

[17] **Singh A**, Hernández-Gamero R, Bóta B, **Gracheva M**, Ahmad W, Pankaczi F, Kovács Kis V, Keresztes Á, **Boldizsár I, Fodor F**, Klencsár Z, **Kovács K**, **Solti Á** (2021) Riboflavin derivatives represent reducing power deposits and shuttles between plasma membrane ferric chelate reductases and apoplast iron compounds in the Strategy I plant *Cucumis sativus*. *In: Györgyey J (ed.) XIII. Magyar Növénybiológiai Kongresszus - Összefoglaló Kötet. ISBN 978-615-01-2350-9. P35*

[18] **Singh A**, Kovács Kis V, Keresztes Á, Pankaczi F, Müller B, **Fodor F**, Klencsár Z, **Solti Á** (2021) Diurnal rhythmicity of cucumber root iron deficiency response eliminates rapidly upon nanohaematite iron resupply. *19th Congress of the European Society for Photobiology - Book of Abstracts, ISBN 978-3-200-07802-4, OC-7.4.4*

[19] **Solti Á**, Sági-Kazár M, Pham H-D, **Zelenyánszki H**, Ahmad W, Szenthe K (2019) The role of ferric chelate reductase enzyme in the iron transportation processes across the chloroplast inner envelope membrane – in Hungarian. *In: Tóth Sz. (ed.) Előadás Absztraktok, 49. Membrán-transzport Konferencia, Sümeg, p. 2.*

[20] Pham H-D, Sági-Kazár M, Szenthe K, Tóth I, **Zelenyánszki H**, Ahmad W, **Fodor F**, Philippar K, **Solti Á** (2019) Interaction of leaf aging to iron nutrition in the iron

homeostasis of chloroplasts. *In: Balazadeh S, Mueller-Roeber B (eds.) Program & Abstracts, 9th International Symposium on Plant Senescence. p. 92.*

[21] Halasy V, **Kovács K**, Klencsár Z, Homonnay Z, **Fodor F**, **Tamás L**, **Solti Á**, Pólya S (2018) Quantitative real-time PCR analysis of the utilization of an iron-containing nanomaterial by a dicot model plant. *In: Tamás L, Zelenyánszky Z (eds.) Fiatal Biotechnológusok Országos Konferenciája „FIBOK 2018" Abstract Book, JATE Press, Hungary, ISBN 978-963-315-370-3, p. 119.*

[22] Müller B, Pham HD, **Kovács K**, Kavak Y, Gyuris B, Sági-Kazár M, Soós V, Ahmad W, **Zelenyánszki H**, Szenthe K, **Fodor F**, **Solti Á** (2018) Iron uptake machinery of chloroplasts tends to utilise stoichiometric ferric-citrate complexes in *Brassica napus*. *In: Schmidt W et al. (eds.) 19th International Symposium on Iron Nutrition and Interactions in Plants. Academia Sinica, Taipei, Taiwan. p. 40.*

[23] Müller B, Pham HD, **Kovács K**, **Zelenyánszki H**, Ahmad W, Sárvári É, **Fodor F**, **Solti Á** (2018) The connection between photosynthetic and iron uptake processes in chloroplasts – in Hungarian. *In: Garab G, Janda T, Solymosi K, Darkó É, Pál M (eds.) Hazai Fotoszintézis-kutatók Találkozója - Program és Összefoglalók, Fotoszintézis - Élet a Fényből Alapítvány, ISBN 978-615-00-3750-9 p. 16.*

[24] Pankaczi F, Farkas Z, Halasy V, Larsson S, Pólya S, Müller B, **Kovács K**, Klencsár Z, May Z, Sándor Z, Szabó GE, Bódis E, Szabó L, Kuzmann E, Homonnay Z, Tolnai G, **Solti Á**, **Fodor F** (2018) Manufactured nanomaterials: new iron based fertilizers or potentially toxic agents? *In: Schmidt W et al. (eds.) 19th International Symposium on Iron Nutrition and Interactions in Plants. Academia Sinica, Taipei, Taiwan. p. 35.*

<u>BSc theses related to the project (5):</u>

[25] Bóta BK (2020) The iron homeostasis of the mesophyll cells– in Hungarian with English abstract. BSc thesis, Eötvös Loránd University; supervisor: **Solti Á**

[26] Tóth BS (2020) Copper homeostasis and copper reducing FRO enzymes of plants – in Hungarian with English abstract. BSc thesis, Eötvös Loránd University; supervisor: **Solti Á**

[27] Farkas S (2019) Mechanism of iron sensing and signalling in plants – in Hungarian with English abstract. BSc thesis, Eötvös Loránd University; supervisor: **Solti Á**

[28] Sági-Kazár M (2018) A növényi vas-kelát-oxidoreduktáz enzimek [Ferric-chelate reductase enzymes in plants – in Hungarian with English abstract]. BSc thesis, Eötvös Loránd University; supervisor: **Solti Á**

[29] Serdült A (2018) Növénytranszformálási módszerek a Brassicaceae családban [Methods of genetic modification in the family Brassicaceae – in Hungarian with English abstract]. BSc thesis, Eötvös Loránd University; supervisor: **Zelenyánszki H**

<u>National & institutional student competition theses related to the project (1):</u>

[30] Hegedűs Cs (2021) Investigating the reduction based iron uptake mechanism in non-photosynthetic plastids – in Hungarian with English abstract; Institutional student competition in Biology; Eötvös Loránd University; supervisor: Sági-Kazár M & **Solti Á**

[31] Nagy-Herczeg BK (2021) Riboflavin contributes to ferric chelate reduction by increasing ferric chelate reductase activity of the root plasma membrane. Institutional student competition in Biology; Eötvös Loránd University; supervisor: **Singh A** & **Solti Á**

[32] Bóta BK (2020) *In vitro* activity measurements of ferric chelate reductase activity on plasma membranes isolated from different plant organs – in Hungarian with English abstract; Institutional student competition in Biology; Eötvös Loránd University; supervisor: **Solti Á**

[33] Sági-Kazár M (2019) Regulative role of iron nutrition on the expression and activity of chloroplast ferric-chelate reductase enzyme – in Hungarian with English abstract. 34th National Student Competition in Biology, thesis (1st place in Plant Biology); supervisor: **Solti Á**

MSc theses related to the project (5):

[34] Serdült A (2020) Production of a marker tagged ferric chelate reductase chloroplast envelope protein in transgenic broccoli model. – in Hungarian with English abstract. MSc thesis, Eötvös Loránd University; supervisor: **Zelenyánszki H**

[35] Sági-Kazár M (2020) The regulative role of the iron nutrition on the gene expression of chloroplast ferric chelate reductase – in Hungarian with English abstract. MSc thesis, Eötvös Loránd University; supervisor: **Solti Á**

[36] Hernández Gamero R (2020) The effect of flavin and coumarin derivatives on the ferric chelate reductase activity. MSc thesis, Eötvös Loránd University; supervisor: **Solti Á**

[37] Soós V (2019) Functional analysis of the rott ferric chelate oxidoreductase enzyme – in Hungarian with English abstract. MSc thesis, Eötvös Loránd University; supervisor: **Solti Á**

[38] Halasy V (2018) A vas-tartalmú nanoanyagok hatása a gyökér vas-kelát-reduktáz génexpressziójára és enzimaktivitására [The role of iron containing nanoparticles on the expression and enzyme activity of root ferric chelate reductase – in Hungarian with English abstract]. MSc thesis, Eötvös Loránd University; supervisor: **Solti Á** & **Tamás L**

PhD theses related to the project (2):

[39] Lantos B [author name: Müller B] (2019) A kloroplasztiszok vasfelvételi mechanizmusának vizsgálata [The mechanism of iron uptake in chloroplasts]. Eötvös Loránd University; supervisors: **Fodor F**; **Solti Á**; https://doktori.hu/index.php?menuid=193&vid=21111&lang=EN

[40] Pham HD (2020) Leaf development and the plants iron nutrition status impact chloroplast physioloy as well as the iron uptake machinery components PIC1, NiCo, MAR1 and YSL4. Eötvös Loránd University; supervisors: **Fodor F**; **Solti Á**; https://doktori.hu/index.php?menuid=193&vid=22314&lang=EN