Final report (NKFI PD-112047)

Chloroplast iron homeostasis: The iron uptake machinery of chloroplasts

Chloroplasts are the organelles of prime importance in the intracellular iron (Fe) metabolism of plants. Since only pieces of information were previously available on the Fe uptake and metabolism of chloroplasts that contain 90% of the shoot Fe, the closing project intended to shed more light on the mechanism and regulation of chloroplast Fe homeostasis. It aimed to reveal the mechanism of action of chloroplast Fe acquisition machinery and its alterations under Fe starvation. Our investigations focused on the chemical forms of Fe used as *in vivo* substrate in the uptake process, on the organisation and transcription level regulation of the Fe uptake machinery of chloroplast envelopes under Fe limitation and excess of Fe and the interaction of the chloroplast Fe uptake to the presence of other heavy metals.

Natural substrates of the chloroplast iron uptake machinery

Among the most important open questions, first we have investigated the $Fe^{(III)}$-complex preference of chloroplast Fe uptake system. Since neither there are literature information on the *in vivo* chemical from of iron present in the cytoplasm (the concentration of iron is lower than the threshold of techniques such as Mössbauer spectrometry that are sensitive to the microenvironment of iron), nor there can be found any methods of cytoplasm isolation that guarantee the unchanged iron microenvironment, the indirect (chloroplast iron form preference-based) determination of this *in vivo* form is the only way to get valid information.

Theoretically, ferric iron can be complexed by carbonic acids such as citrate or carbonic amino acids, such as nicotianamine in the cytoplasm. We have selected to test the uptake of $Fe^{(III)}$-citrate 1:1.1, $Fe^{(III)}$-citrate 1:10, $Fe^{(III)}$-citrate 1:100, $Fe^{(III)}$-malate 1:1.1 (carbonic acid complexes), $Fe^{(III)}$-nicotianamine (NA) 1:1.2 (carbonic amino acid complex), and $Fe^{(III)}$- *o-o'*-EDDHA (synthetic N-containing chelate). Since NA is also reported previously to form stable complexes with ferrous iron, $Fe^{(II)}$-NA 1:1.2 complexes were also included into the uptake assays. $Fe^{(III)}$-NA was prepared from a mixed 0.1 M $Fe(NO_3)_3$ - $FeCl_3$ solution by adding NA in small quantities together with the balance of the pH to 7.5 by KOH. $Fe^{(III)}$-NA solution was prepared on a similar way using $FeSO_4$ solution (containing 100% Fe(II) according to Mössbauer spectroscopy tests). The characteristics of iron-NA complexes were tested by

Mössbauer spectroscopy at 80 K to prove their purity. In a frame of a cooperation, the characteristics of the iron-NA complexes were also tested by helium temperature Mössbauer spectroscopy in a magnetic field by Krisztina Kovács (Institute of Chemistry, Eötvös Loránd University), Jiří Pechoušek, Libor Machala and Radek Zbořil (Department of Experimental Physics and Physical Chemistry, Palacký University, Czech Republic) to reveal its structure.

To test the iron substance preference of chloroplasts, intact chloroplasts were isolated. The integrity of chloroplasts was checked by an immunoblot-based measurement of RbcL (soluble protein) to apoLhcII (thylakoid membrane protein) ratio. We could certify that the intactness of chloroplasts was over 85% in all measurements: the purification of intact chloroplasts resulted in intactness of 96.8±8.3% (Class 1) and 91.3±6.8% (washed chloroplasts), where the incubation during the uptake assays did not result in the decrease of the intactness.

The uptake form ferric-carbonic acid complex sources at a ratio of low complexing compound to iron was significantly higher ($Fe^{(III)}$-citrate 1:1.1: 36.0±5.4 amol Fe min^{-1} $chloroplast^{-1}$; $Fe^{(III)}$-malate 1:1.1: 19.6±9.1 amol Fe min^{-1} $chloroplast^{-1}$) compared to that complexes and chelates where Fe also binds to N ligands ($Fe^{(III)}$-nicotianamine 1:1.2: 7.1±0.0 amol Fe min^{-1} $chloroplast^{-1}$; $Fe^{(III)}$- *o-o'*-EDDHA: 2.1±3.3 amol Fe min^{-1} $chloroplast^{-1}$), but also compared to that complexes, where the ratio of the complexing citrate was high to iron ($Fe^{(III)}$-citrate 1:10: 6.4±6.9 amol Fe min^{-1} $chloroplast^{-1}$). Fine-scale uptake measurements indicated, that there is possibly a double saturation for $Fe^{(III)}$-citrate uptake (first predicted saturation in the 25-40 µM external Fe range at 12.1±0.8 amol Fe min^{-1} $chloroplast^{-1}$ and second assumed saturation in the 100-250 µM external Fe range at 36.7±1.1 amol Fe min^{-1} $chloroplast^{-1}$), similarly as it was found previously for chloroplast ferric chelate oxidoreductase enzyme activity (Solti *et al.* 2014. *New Phytol. 202: 920*). Data were presented on the 18th International Symposium on Iron Nutrition and Interactions in Plants, Madrid, Spain (2016), and on the EPSO/FESPB 2016 Congress, Prague, Czech Republic (2016), on the 3rd Global Summit on Plant Science, Rome, Italy (2017), and on the 12th Congress of the Society for Hungarian plant Biology, Szeged, Hungary (2017). A manuscript, in writing stage: "Müller B, Kovács K, Pham HD, Halász K, Kavak Y, Pechoušek J, Machala L Zbořil R, Fodor F, Klencsár Z, Solti Á: Iron uptake of chloroplasts prefers ferric-citrate over iron-nicotianamine complexes in *Brassica napus*" is planned to submit to New Phytologist.

Natural and biodegradable iron complexes ($Fe^{(III)}$-lignosulphonate; $Fe^{(III)}$-EDDS; $Fe^{(III)}$-IDHA; $Fe^{(III)}$-gluconate) along with chelates of high stability ($Fe^{(III)}$- *o-o'*-EDDHA) were also

involved into utilisation assays in order to find cheap and biodegradable iron fertilisers. The reduction of $Fe^{(III)}$-EDDS and $Fe^{(III)}$-IDHA biodegradable chelates was effective, whereas natural complexes proved to be inert in a ferric chelate reductase assay. In contrast, iron deficiency alleviation experiment indicated that over the widely-used $Fe^{(III)}$- *o-o'*-EDDHA chelate, natural iron substances $Fe^{(III)}$-lignosulphonate and $Fe^{(III)}$-gluconate are also effective substrates of the iron uptake system. Data were published in: Martín-Fernández C, Solti Á, Czech V, Kovács K, Fodor F, Gárate A, Hernández-Apaolaza L, Lucena JJ (2017) Response of soybean plants to the application of synthetic and biodegradable Fe chelates and Fe complexes. *Plant Physiology and Biochemistry 118: 579-588.*

Chloroplast ferric chelate reductase (cFRO – identified as AtFRO7 in *Arabidopsis*) is an enzyme of essential function in the chloroplast iron uptake. Previously, the enzyme kinetics of cFRO was determined using a synthetic chelate, $Fe^{(III)}$-EDTA (Solti *et al.* 2014. *New Phytol. 202: 920*). Since $Fe^{(III)}$-citrate, and all other ferric-carbonic (amino-) acid complexes react with NADPH very fast at pH 7.0, the only way to measure the ferric chelate reductase (FCR) activity against natural complexes is the enclosing of NADPH into inner envelope vesicles. Nevertheless, the incorporation of both NADPH and FAD coenzymes into chloroplast inner envelope vesicles by freezing, thawing and washing/pelleting by ultracentrifugation at 25.00 rpm for 75 min resulted in a sharp decrease in the enzyme activity against $Fe^{(III)}$-citrate 1:1.1. This decrease prevented us to measure the FCR activity using potential natural substrates. Since the NADPH binding site of the enzyme locates in the stromatal surface of the protein, the reason for this decrease can be only explained by the inhibiting role of the removal of FAD from the external solution (equivalent to the intermembrane space of the chloroplasts). This finding let us hypothesize that a similar mechanism may operate at the cFRO level as it was shown by Sisó-Terraza *et al*. (2016, *New Phytol. 209: 733-745*) in relation with the root FCR enzyme: flavin derivatives may have a role in the reduction of ferric iron. To clarify this phenomenon we are continuing our investigations in a frame of our recently starting research grant.

Impact of the iron nutrition on the organisation and function of the chloroplast iron uptake machinery

The iron nutrition status of plants is well known to regulate the root iron uptake from the soil: in the reduction-based strategy-I iron uptake, iron deprivation induces a strong enhancement

on the iron uptake system. Such a regulation was not known at chloroplast level before. In the iron uptake of chloroplasts, the most important components known so far are the cFRO (identified at gene and transcript levels as *BnFro7* [*Bra037953*] in *Brassica napus*), and two transport-related membrane proteins, PIC1 (identified as *BnPic1* [*Bra036409*]) and NiCo (identified as *BnNico* [*Bra037287*]). Here, we studied (i) the changes in the expression of these genes during the development and aging of leaves, and (ii) the iron uptake as well as the (iii) inner envelope FCR activity of the chloroplasts isolated from leaves grown under iron deficiency (grown on Fe deficient nutrient solution from the 4-leaves stage in the presence of $CaCO_3$), optimal (grown on 20 μM $Fe^{(III)}$-citrate) and superoptimal (grown on non-toxic: 100 μM $Fe^{(III)}$-citrate from the 4-leaves stage) iron nutrition conditions.

In *Brassica napus*, *AtFro7* was determined to have a single ortholog copy (*BnFro7*) in the A genome parent *Brassica rapa* (*Bra037953*, EST sequence homology is >85%), whereas no close ortholog was found in the C genome parent (*Brassica oleracea*) sequences so far. To follow the changes in the expression of *BnFro7* gene, primers were designed based on the Brassica Date Base (brassicadb.org). To validate the quantitative real-time-PCR (qPCR) results, expression of *ß*-tubulin [accession number: *XM_009125342.1*] and 18S rRNA [accession number: *KT225373*] was also studied. Among these reference genes, 18S rRNA and *ß*-tubulin were found to be stable under changes in the iron nutrition level based on total quantification of copy ssDNA measurements, thus we used them for normalisation of the genes of interest.

Primers were designed and optimised for the expression analysis. The following primer sequences were found to be optimal for the expression analysis: *BnFro7*: forward: GGTGTTCGCTAAGAAGAAGATATCG, reverse: GTCAAGATCCCTCATGGTATATGC; *BnPic1*: forward: TGCGGTCACTACTCTTGC, reverse: GATGGTGGCTCTCCTCTTC; *BnNico*: forward: CTTCCGCCACAATCCTTC, reverse: CATAACTCCGCACGATCC. For 18S rRNA and *ß*-tubulin internal control gene, the following primers were used: forward: GCATTCGTATTTCATAGTCAGAGGTG, reverse: CGGAGTCCTAAAAGCAACATCC and forward: TCSATCCAGGARATGTTCAGG, reverse: ACTCTGCAACAAGATCATTCATG, respectively.

The expression of *BnFro7* was strongly dependent on the iron nutrition status: although the expression did not altered significantly under iron deficiency compared to that of in plants of optimal iron nutrition, the superoptimal iron nutrition decreased its expression significantly (on the 14th day of treatment, the relative expression of *BnFro7* was: 1.038±0.008;

1.000±0.163; 0.667±0.144 in leaves grown under iron deficiency, optimal iron nutrition and superoptimal iron nutrition, respectively). A peak expression was found in both leaves of iron deficient and control (optimal iron nutrition) plants at the 4th day of treatment. A strong correlation was found between the iron content of chloroplasts and the expression of *BnFro7*: whereas low chloroplast iron contents showed a positive correlation to the expression of *BnFro7* in leaves of iron deficient and control plants (a=1.79; R^2=0.53 and a=3.00; R^2=0.84 in leaves of iron deficient and control plants, respectively), it proved to be a negative regulator under the excess of iron (a= -3.23; R^2=0.68). In contrast to the changes in the expression pattern, ferric chelate reductase (FCR) activity of the isolated chloroplast envelope membranes was more sensitive to iron deficiency. FCR activity of membrane vesicles isolated from *Brassica napus* leaves grown under optimal iron nutrition showed a similar double saturation that was published before on *Beta vulgaris*: K_{M1}=39.4±1.5 µM external Fe; v_{sat1}=7.9±0.2 pmol Fe min^{-1} $protein^{-1}$ and K_{M2}=97±0,9 ; µM external Fe; v_{sat2}=10.9±1.3 pmol Fe min^{-1} $protein^{-1}$, whereas FCR activity of chloroplast inner envelope membrane vesicles isolated from iron deficient leaves showed a single saturation with K_M=26.9±6.9 µM external Fe and v_{sat}=4.8±0.2 pmol Fe min^{-1} $protein^{-1}$. Since the FCR activity decreased significantly but the *BnFro7* expression remained unchanged under iron deficiency, a post-translational level regulation is predicted for BnFRO7. Superoptimal iron nutrition also decreased the FCR activity (K_M=11.6±0.8 and µM external Fe and v_{sat}=5.2±1.3 pmol Fe min^{-1} $protein^{-1}$) in parallel to the decreased *BnFro7* expression that support the hypothesis that increasing chloroplast iron content is a feedback regulation factor of their iron uptake machinery.

In our previous studies we have pointed out that iron reduced in the chloroplast FCR activity does not accumulate between the two envelope membranes that suggests cooperation between FRO7 and the iron uptake transporters. Thus, we also studied the expression and amount of PIC1 and NiCo. In the expression profile analysis of *BnPic1* and *BnNico* genes, two storeys of leaves: 4th leaves (grown before the start of treatments) and 6th leaves (grown under the treatments) were involved. As for different iron nutritional level treatments, iron deficiency, optimal iron supply and excess of iron were applied. The detailed expression analysis of *BnPic1* and *BnNico* revealed that all of these genes change their expression in parallel to the development of the leaves. All of them reached their expression maxima when the leaves reached their full development (maximal area): around the 14th day of treatment for 4th leaves and around the 21st days of treatment for the 6th leaves. After these maxima, expression of all genes under all treatments decreased. Iron deficiency induced an enhancement in the expression of both genes genes, where this enhancement were more pronounced in 6th leaves

compared to the older 4th leaves. In contrast, the iron stress treatment caused no alteration in the peak expression, but the level of expression did not decreased during the aging of the leaves.

In parallel to the analysis of changes in the expression of *BnPic1* and *BnNico* during the leaf development and aging and under different iron nutrition status of the plants, the protein amount of BnPIC1 and BnNiCo were also studied. In collaboration with Prof. Katrin Philippar (Sarland University, Saarbrücken, Germany) we received antibodies against these target proteins. Total leaf proteins were solubilised and subjected to immunoblot against rabbit anti-PsPIC1 and anti-PsNiCo. As for normalisation, immunoblot against AtRbcL, and measurement of total protein content were used. Both in 4th and 6th leaves, the tendency of changes in the expression of *BnPic1* and the amount of BnPIC1 were similar that the expression/protein amount increased during the development of leaves but decreased after reaching their full size/during aging and by the increase of the iron nutrition status, the enhancement in the expression/protein accumulation decreased. Only we find differences in the timing of the peak expression/peak protein amount: in the 4th leaves, it was found at the 21-28th and 14th days of the treatments, respectively, and in the 6th leaves 14th-21st and 28th days of treatments, respectively. This pattern of changes also suggests post-translational level regulation of the accumulation of PIC1 proteins in the chloroplast inner envelope membranes. In contrast, in the expression of *BnNico* and the protein amount of BnNiCo we found different tendencies of changes in the 4th and 6th leaves. Changes in the iron nutrition levels did not altered the expression profile of *BnNico* among the treatments significantly: it increased more or less continuously during the time of treatments, whereas its protein level showed a clear sensitivity to the iron nutrition status and found to be the highest under iron deficiency. Moreover, in 6th leaves, the expression of *BnNico* showed sensitivity to the iron nutritional status and decreased by the increasing iron nutrition. It clearly showed an expressional peak during the 21st – 28th days of treatments. However, the amount of BnNiCo in the 6th leaves was the highest under superoptimal iron nutrition and decreased strongly by the decrease of the iron nutrition. These differences also support the theory that *BnNico* stays under a strong post-translational level regulation.

Iron deficiency resulted in a significant decrease in the iron uptake of chloroplasts (saturating iron uptake was 0.211±0.015 fmol Fe chloroplast^{-1} whereas 2.307+0.446 fmol Fe chloroplast^{-1} at 150 µM external Fe during 30 min in chloroplast of plants grown under iron deficient and optimal iron nutrition conditions). The detailed measurements indicated that the uptake of chloroplasts of iron deficient plants showed a double saturation with K_{M1}=23.0±0.9 µM

external iron with saturating uptake of 0.132±0.01 fmol Fe chloroplast^{-1} and K_{M2}=46.6±3.3 μM external iron with saturating uptake of 0.210±0.012 fmol Fe chloroplast^{-1} (K_M of the iron uptake of control chloroplasts was K_M=43.7±1.9 μM external iron). Under iron excess, the iron uptake of chloroplasts also decreased where the saturating chloroplast iron uptake was 0.960±0.010 fmol Fe chloroplast^{-1} at 150 μM external Fe during 30 min, K_M=29.2±0.6 μM external iron.

To address these changes in the expression of the chloroplast iron uptake related genes, the iron content of the chloroplasts and the excitation energy allocation of the leaves were measured. Iron deficiency treatment induced 40 and 50% decrease in the chloroplast iron content in the 4th and 6th leaves, respectively. Interestingly, the chloroplast Fe content also showed 7-8% decrease in both 4th and 6th leaves under superoptimal iron nutrition, indicating, that iron does not accumulate in the chloroplasts in the form of ferritin. By immunoblot against AtFER1-4 we also got negative results under iron excess treatment. Satellite immunoblot measurements for the detection of plant Ferritin in various plant tissues revealed that under non-toxic iron nutrition, Ferritin can be only detected from the root branching zone whereas no Ferritin apoprotein was found in root tip, seed and leaf homogenate samples according to the immunoblot data. Data were published in: Kovács K, Pechoušek J, Machala L, Zbořil R, Klencsár Z, Solti Á, Tóth B, Müller B, Pham HD, Kristóf Z, Fodor F (2016) *Planta 244: 167–179*.

The Fe content of the chloroplasts of control and iron deficient leaves (both in the 4th and the 6th leaves) showed decreasing iron content after the leaves reached their full development (14th and 21st days of treatment for 4th and 6th leaves, respectively). Since the chlorophyll content did not changed significantly in this period, it is unlikely that this decrease happened because of the multiplication of the chloroplasts. More likely, the iron content of the chloroplasts mobilised and re-translocated. However, under superoptimal iron nutrition treatment, a significantly smaller decrease was measured in the chloroplast Fe content. The actual quantum efficiency of the photosystem II reaction centres decreased in parallel to the Fe content of the leaves during the aging. Nevertheless, this was not observed under Fe surplus, and the leaves retained the photosystem II actual quantum efficiencies, together with the Fe content of the chloroplasts. Parallel changes in the amount of BnPIC1, in the chloroplast iron content and in the physiological activity indicate that there may be an iron release from the chloroplasts in connection with the senescence processes.

Data were presented on the 18th International Symposium on Iron Nutrition and Interactions in Plants, Madrid (2016), on the 8th Symposium on Microalgae and Seaweed Products in Plant/Soil Systems, Mosonmagyaróvár, Hungary (201) and on the 12th Congress of the Society for Hungarian plant Biology, Szeged, Hungary (2017). A manuscript: "Pham HD, Müller B, Szenthe K, Pólya S, Fodor F, Tamás L, Solti Á: Leaf age and iron nutrition level affects PIC1 and NiCo, members of the chloroplast iron uptake machinery in *Brassica napus*." is tended to be submitted to Functional Plant Biology. A second manuscript: "Müller B, Szenthe K, Gyuris B, Sági-Kazár M, Pham HD, Fodor F, Tamás L, Solti Á: The regulative role of the iron nutrition status on the expression and function of chloroplast ferric chelate oxidoreductase in *Brassica napus*" is also under preparation from these results.

Cadmium treatment is a good model system that induces iron deficiency in the shoot by blocking root-to-shoot iron translocation and thus induces an iron accumulation in the roots but the leaves remain iron deficient. By replacing plants from cadmium-free iron-enriched nutrient solution, a synchronised iron uptake and translocation occurs. To clarify the impact of the *in vivo* iron uptake of iron deficient chloroplast, plants were regenerated from induced iron deficiency and the chloroplast iron content was monitored together with the changes in the content of photosynthetic pigments, thylakoid pigment-protein complexes and the excitation energy allocation in the photosynthetic apparatus. Iron-enriched nutrient solution induced an intensive iron uptake, translocation of iron to the shoot with a short delay, and finally the accumulation of iron in the chloroplasts. The accumulation of complexes, particularly the Fe-containing photosystem I reaction centres began in parallel with the increase in the iron content of chloroplasts. In conclusion, iron was taken up and translocated to the leaves incorporates into the chloroplasts in a short time. The chloroplast iron uptake is directly proportional to the iron translocation to leaves. The accumulation of photosystem (PS) I reaction centres and the recovery of PSII function begin in parallel to the increase in the Fe content of chloroplasts. The initial reorganization of PSII is accompanied by a peak in the antennae-based non-photochemical quenching. In conclusion, Fe accumulation of the chloroplasts is a process of prime importance in the recovery of photosynthesis. Data were published in: Solti Á, Sárvári É, Tóth B, Mészáros I, Fodor F (2016) Incorporation of iron into chloroplasts triggers the restoration of cadmium induced inhibition of photosynthesis. *Journal of Plant Physiology 202: 97–106*.

Interaction of the chloroplast iron machinery to the presence of other heavy metals

Heavy metals are known to interact to the iron homeostasis of plants at various levels, including chloroplasts. Literature data on isolated chloroplast inner envelope membrane vesicles showed that divalent transition metal cations can competitively inhibit the translocation of iron across the membrane (Shingles *at al.*; 2002; *Plant Physiol 128: 1022*). Since isolated chloroplast inner envelope vesicles cannot model the whole iron acquisition machinery of the chloroplasts, we studied how the presence of these metals alters the chloroplast iron uptake. Isolated intact chloroplasts were incubated in the presence of Cd^{2+}, Zn^{2+} and Mn^{2+} in 200 or 500 μM concentration (together with either Cl^{-} or SO_4^{2-} counter ions). The presence of oxoanions (NO_3^{-}, SO_4^{2-}, and BO_3^{3-}) was also studied with K^{+} counter ion. K+ and Cl- did not affect the iron uptake of chloroplasts. Nevertheless, transition metal cations (Cd^{2+}, Zn^{2+}, and Mn^{2+}) enhanced, whereas oxoanions (NO_3^{-}, SO_4^{2-}, and BO_3^{3-}) reduced the chloroplast Fe uptake. The effect was insensitive to diuron (DCMU), an inhibitor of chloroplast inner envelope-associated Fe uptake but disrupted by ionophores that vanished all transmembrane ion and electrochemical gradient. The inorganic salts affected neither the microenvironment of iron in the uptake assay buffer nor those incorporated into the chloroplasts. The significantly lower Zn and Mn uptake compared to that of Fe indicates that different mechanisms/transporters are involved in their acquisition. The enhancing effect of transition metals on chloroplast Fe uptake is likely related to outer envelope-associated processes, since divalent metal cations are known to inhibit Fe^{2+} transport across the inner envelope. Thus, a voltage-dependent step is proposed to play a role in Fe uptake through the chloroplast outer envelope on the basis of the contrasting effects of transition metal cations and oxoaninons. Data were published in: Solti Á, Kovács K, Müller B, Vázquez S, Hamar É, Pham HD, Tóth B, Abadía J, Fodor F (2016) *Planta 244: 1303-1313.*

Since the presence of divalent heavy metals do not inhibits the chloroplast ion uptake *in vivo* but has an enhancing effect, we also studied the changes in the iron content, photosynthetic activity and antioxidative defence of chloroplast in the hardening phase of chronic cadmium stress. Satellite data on the role of the chloroplast iron accumulation in the hardening and restoration of chronic cadmium stress were published for invitation to Zeitschrift für Naturforschung C, Peter Böger memorial issue: Solti Á, Sárvári É, Szöllősi E, Tóth B, Mészáros I, Fodor F, Szigeti Z (2016) Stress hardening under long-term cadmium treatment is correlated with the activation of antioxidative defence and the iron acquisition of chloroplasts in *Populus*. *Zeitschrift für Naturforschung C 71: 323-334*. The results revealed that *Populus*

plants can acclimate to long-term cadmium stress, indicated by the decrease in the malondialdehyde content and the recovery of the photosynthethic activity. Re-distribution of the iron content of leaf mesophyll cells including increase in the iron content of the chloroplasts contributed to the biosynthesis of the photosynthetic apparatus and some antioxidative enzymes. Elimination of oxidative stress damage by acclimation mechanisms is required for the restoration of the photosynthetic apparatus.

Copper is also predicted to interact to the iron uptake machinery of the chloroplasts as competitor for the ferric chelate reductase enzyme. Bathocuproine disulfonic acid (BCDS) is an indicator of reduced cuprous ions but also gives positive sign in the presence of ferrous ions. Nevertheless, according to our tests, the absorption spectrum of $Cu^{(I)}$-BCDS complexes overlap to the spectrum of $Fe^{(II)}$-bathophenantroline disulfonic acid (BPDS) complexes, thus taken together this phenomenon and competition between the ions for the BCDS chelator, cupric and ferric chelate reductase assays are unable to be measured in a single assay on cIE membranes. Thus, the chloroplast copper and iron uptake was measured instead. Cooperation has been established with assoc. prof. Katalin Zichné Perényi (Department of Analytical Chemistry, Eötvös Loránd University) to use flame atomic absorption in the detection of copper taken up by chloroplasts in the presence of iron. In the presence of iron, chloroplasts took up a significant amount of copper, 17.7±4.0 amol Cu min^{-1} $chloroplast^{-1}$ at 150 µM external $Cu^{(II)}$-glycinate that is comparable to the metal uptake from other sources such as Cd, Zn and Mn (Solti *et al.,* 2016, *Planta 244: 1303-1313*), whereas the iron uptake of chloroplasts slightly decreased. In the presence of 50 µM external $Cu^{(II)}$-glycinate and 150 µM $Fe^{(III)}$-citrate, the iron uptake of chloroplasts decreased by 30.0±3.2%. Nevertheless, at low concentrations of $Fe^{(III)}$, the presence of $Cu^{(II)}$ induced a similar enhancement on the iron uptake of chloroplasts that was also found in the presence of other divalent metals. At 25 µM external $Fe^{(III)}$-citrate and 50 µM external $Cu^{(II)}$-glycinate, the iron uptake of chloroplasts was enhanced by 58.5±10.1%. Thus, the presence of $Cu^{(II)}$ regulates the chloroplast iron uptake, but over the predicted competition for the ferric chelate reductase enzyme, copper ions may also interact to the iron uptake on a similar way that was found for other divalent metal ions. Data were published as conference abstract in: Müller B, Horváth N, Zihné Perényi K, Tarı G, Kavak Y, Halász K, Sági-Kazár M, Gyuris B, Fodor F, Solti Á (2017) Copper interacts with the iron uptake mechanism of chloroplasts. *In: Ördög V, Molnár Z (eds.) Book of Abstracts of the 8th Symposium on Microalgae and Seaweed Products in Plant/Soil Systems, p. 77.*

Analysis of the composition of the chloroplast envelope membrane in *Brassica napus*

Pure winter chloroplast inner envelope (cIE) membranes were isolated from all leaves of four weeks old *Brassica napus* plants by gradient ultracentrifugation of ruptured purified chloroplasts in a ~1.5 mg dimension. Purity of intact cIE vesicles was tested based on the amount of triose-phosphate translocator (cIE marker) and main contaminant chloroplast membrane markers (outer envelope TOC75, thylakoid LHCII). The purity of the samples was also tested by AtOEP16.1 PsIE37 in collaboration with Prof. Katrin Philippar (Saarland University, Saarbrücken, Germany). cIE vesicles were subjected to two-dimensional (2D) BlueNative(BN)/SDS polyacrylamide gel electrophoresis (PAGE). Membrane solubilisation was found to be optimal by applying 2% *n*-dodecyl-maltoside. Complexes were separated in a 5-12% BN-PAGE gradient system.

BnFRO7 (BRA037953) protein is predicted to have a molecular weight of 83.310 kDa, thus 10-18% gradient SDS PAGE was applied to separate the larger molecular weight regions. By the 2D separation of the cIE membrane complexes, we obtained 33 major protein spots, 12 of that were selected for protein sequencing. Protein sequencing was performed in collaboration with Prof. Hong-Qing Ling (Chinese Academy of Sciences, Institute of Genetics and Developmental Biology). Among proteins, translocon at the inner envelope membrane of chloroplasts 55 (TIC55; AT2G24820.1), 54 kDa putative membrane protein (AT1G32080.1), 37 kDa S-adenosyl-L-methionine-dependent methyltransferases superfamily protein (AT3G63410.1), translocon at the outer envelope membrane of chloroplasts 75-III (TOC75; AT3G46740.1), glucose-6-phosphate/phosphate translocator-related transporter (AT5G46110.2), chloroplast J-like domain 1 protein (AT1G08640.1), plastidic GLC translocator (AT5G16150.1) and ferredoxin-NADP(+)-oxidoreductase 1 (AT5G66190.2) ortholog protein sequences were found.

Since we did not find BnFRO7 in the predicted region, we continued the work by the detailed in silico analysis of the *Brassica napus* chloroplast envelope membrane proteins to detect differences among *Arabidopsis* and *Brassica* proteins. The envelope membrane proteins of the *Brassica napus* chloroplasts were also subjected to *in silico* analysis according to the Brassica Date Base. Orthologs of the yet identified *Arabidopsis thaliana* chloroplast envelope membrane proteins were identified in the *Brassica napus* A and C parental genomes (*Brassica rapa* and *Brassica oleracea*, respectively). 186 of the 246 investigated *Arabidopsis thaliana* proteins (75.6%) have orthologs (≥80% sequence homology) in *Brassica rapa* and *Brassica*

oleracea. From these 186 sequences, 139 were found in both genome, 34 were found only in the A genome and 13 were found only in the C genome. Gauss component analysis of the sequence similarities showed that among A genome orthologs, the peak sequence similarity is at 89.9%, whereas a secondary peak was found at 79.6%. This latter group can be excluded from the homologs. Among the C genome orthologs, the peak was found at 88.4%. In the Brassica A genome, 111, 45 and 16 *Arabidopsis thaliana* chloroplast envelope membrane protein encoding genes have 1, 2 or 3 orthologs, respectively, whereas in the C genome, 104, 41 and 6 genes have, 2 or 3 orthologs. Concerning the chloroplast iron uptake related envelope membrane protein genes, except that of NiCo2, all genes have a sequence homology higher than 80% in the *Brassica* genome. Data were presented on the 18th International Symposium on Iron Nutrition and Interactions in Plants, Madrid (2016). A manuscript on these results is in preparation: “Müller B, Kotán B, Fodor F, Sárvári É, Solti Á: *In silico* proteome analysis of chloroplast inner envelope membrane in *Brassica* species”.

Cumulative IF of the published articles: **12.930**

Publications related to the finishing project are:

full papers:

Martín-Fernández C, **Solti Á**, Czech V, Kovács K, Fodor F, Gárate A, Hernández-Apaolaza L, Lucena JJ (2017) Response of soybean plants to the application of synthetic and biodegradable Fe chelates and Fe complexes. *Plant Physiology and Biochemistry 118: 579-588.;* link to Real repository: http://real.mtak.hu/id/eprint/63027 **IF 2.724**

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inner envelope membrane protein composition in *Brassica* species; in Hungarian, with English summary]

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