PhD thesis

Cd-Fe interference in iron homeostasis and in photosynthesis

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Introduction

Plants require numerous transition metals for their life. Among these essential transition metals, iron is highly important because it can take part in redox reactions as well as electron transport chains due to its redox characteristics. The majority of iron is found in heme groups and Fe-S clusters coordinated as prosthetic groups of many enzymes. As free coordinated ion, it is also bound in photosystem II (**PSII**) and Fe superoxide dismutase (**FeSOD**).

Many non-essential transitional heavy metals, such as cadmium, are poisonous for plants. Under natural conditions, cadmium can found as free Cd^{2+}. Taken up by plants, it disturbs the uptake and translocation of other metals, thus it interferes to the homeostasis of Ca^{2+}, $Fe^{2+/3+}$, Mn^{2+} and Zn^{2+}. Most of the land plants reduce ferri-compounds in the soil for an effective uptake of free ferrous ions across the root plasma membrane. Iron is found as ferri-citrate complexes in the xylem sap when it is translocated to the shoot. One of the most important symptoms of the cadmium toxicity is the induced iron deficiency in the shoot. Cd causes a so-called iron chlorosis due to the decreased level of iron translocation. Despite cadmium-caused iron deficiency is a well known feature, the reasons of the symptoms are poorly understood.

Q1: What is the role of cadmium creating an unbalanced iron homeostasis?

The homeostasis of iron is strongly regulated not only on the level of the whole organism but also on cellular level. Most of of the iron content (80-90%) of mesophyll cells can be found in the chloroplasts. In the absence of iron, the biosynthesis of both the chlorophyll molecules (Chl) and pigment-protein complexes are strongly inhibited thus the biogenesis of thylakoid membranes is damaged. In the symplast of the cells, Cd^{2+} can affect the photosynthesis strongly. Induced iron deficiency has, however, also a negative impact on the development of the photosynthetic apparatus. Nevertheless, the mechanisms tolerating cadmium in a sub-lethal concentration as well as the role of the change of iron homeostasis during the recovery of cadmium induced damages are not well known. The iron metabolism of chloroplasts seems to be the key point in the photosynthetic activity.

Q2: **Does the chronic cadmium-stress induce any changes in the localisation of iron within the leaves?**

PSII is the most sensitive component of the photosynthetic electron transport chain in the case of Cd-stress. To develop such a symptom Cd stress sensitive or Cd binding structures

may play a role during the development of this symptom, both on donor and acceptor side. Iron deficiency is the strongest limitation factor in the inhibited synthesis of iron-containing PSI complexes. As a consequence of all these effects, the photosynthetic activity drops substantially under Cd-stress. Although many of the above mentioned effects of Cd-stress are widely studied, the molecular mechanism of it has not been fully characterised yet. In our previous work (Solti, 2008) it has been reported that the developed symptoms of Cd-stress can be reversed by elevated iron supply.

Q3: **What is the time course of the recovery of the different physiological functions during the regeneration process?**

Oxidative stress is one of the main reasons why Cd is considered being toxic. Because Cd^{2+} is unable to change its valence under natural conditions, the reasons for oxidative stress should originate by indirect mechanisms. The disturbed iron homeostasis may cause deliberation of free iron in the cells. As free iron has redox activity, it can generate reactive oxygen species (**ROS**) in Fenton-reactions. Nevertheless, the increased concentration of ROS also inhibits the photosynthetic electron transport and the decreases the activity of protective mechanisms. Thus the tolerance of Cd-stress depends strongly on the activity of mechanisms. Cadmium as an abiotic stressor, elicits physiological and gene expressional answers depending on its concentration. In a non-lethal concentration cadmium can be tolerated by many plants, despite its strong acute damages.

Q4: **What kind of protective mechanisms are involved in the tolerance against longer-term Cd exposition? What are the reasons and what the results among the metabolic changes?**

In order to answer these questions a Cd-sensitive woody model plant was used and investigated its leaves developed under Cd-stress for a longer-term period. The effect of elevated iron supply on plants showing acute Cd-stress syndromes have also been studied.

Materials and methods

Plant material was micropropagated poplar plants (*Populus jacquemontiana var. glauca* (Haines) Kimura, 1982, cv. 'Kopeczkii'). Plants were grown in climatic chamber on ¼ strength Hoangland's solution supplemented with 10 μM ferric-citrate as iron source. Plants was treated with 10 μM $Cd(NO_3)_2$ (**Cad**) for four weeks. Artificial recovery was monitored

after one week of Cd treatment where iron supply was elevated from 10 to 50 μM of iron (**Cad/Ko50**). Plants without any treatments were also investigated as control (**Ko**). To study the possible effects of the relative light stress, data of Cad treated plants were compared to those plants which were grown on 250 μE m^{-2} s^{-1} light intensity (**L250**). Leaves developed under cadmium treatments were the object of the study.

Intact chloroplasts were isolated in 50 mM HEPES-KOH, pH 7.0, 330 mM szorbitol, 2 mM EDTA, 2 mM $MgCl_2$, 0.1% (w/v) BSA, 0.1% (w/v) Na-ascorbate. Plastids were purified on discontinuous sucrose gradient (20|45|60% sucrose) and chloroplast concentration was determined in Bürker haematocytometer.

Ion concentrations were measured using ICP-MS (Perkin-Elmer, USA) by Brigitta Tóth and László Lévai (Debrecen University) using acidic digestion.

Iron content of chloroplasts was measured as $[Fe(BPDS)_3]^{4-}$ complexes after addition of 100 μM ascorbic acid and 300 μM BPDS disodium salt to the solubilised chloroplast suspensions. Absorbances were measured at 535 nm (ε=22.14 mM^{-1} cm^{-1}) by UV-VIS spectrophotometer (Shimadzu, Japan). A similar method was applied to measure the iron concentration of the nutrient solutions.

Pigments were extracted in 80% (v/v) acetone/water solution. Chl contents were measured by the method of Porra *et al*. (1989). Carotenoids were separated using HPLC technique (column: Nucleosil C18, detector: UV/VIS [JASCO Int. Co., Japan]) by Erzsébet Szöllősi and Ilona Mészáros (Debrecen University).

Thylakoid membranes were isolated according to Jansson *et al*. (1997). **Chl-protein complexes** were solubilised by mild detergents and separated by Deriphat-PAGE according to Sárvári and Nyirtai (1994). **Separation of proteins** was performed on SDS-PAGE (Laemmli, 1970). Phoretix software (Newcastle, UK) was used to evaluate gel densities.

Leaf fluorescence spectra were measured between 400 and 700 nm (λ_{exc}=365 nm) by Fluoromax-2 (Jobin Yvonne, France). Native fluorescence micrographs were taken by Olympus BH-2 (Olympus Co., Japan) using UG2 excitation filter (λ_{exc}=360-370 nm) and a λ = 510-525 emission filter.

Chl *a* fluorescence was measured by PAM 101-102-103 Chlorophyll Fluorometer (Walz, Effeltrich, Germany) on intact leaves.

Lipid peroxidation was followed by measuring MDA content (Heath and Packer, 1968).

Activity of ascorbate peroxidise (PX) was measured according to the method of Nakano and Asada (1981), based on the H_2O_2-dependent oxidation of ascorbate. Absorbances

were measured at 290 nm by Shimadzu spectrophotometer (Japan).

Superoxid dismutase (SOD) isoforms were separated by native PAGE and the negative staining activity measurements were performed according to Smeets et al. (2005). Lane density was evaluated using Phoretix software.

All measurements were performed in 2×2 or 3×3 replicates. Student's t-test and ANOVA were used in significance tests.

Results and Discussions

1. Cad treatment (10 μM $Cd(NO_3)_2$) caused significant changes in the element composition. The cadmium content increased continuously reaching a relative high concentration in leaves by the end of the third week. In the presence of Cd, the concentration of iron decreased drastically in leaves even in the developmental stage and remained unchanged under the spontaneous recovery. Plants performed light-cycle dependent iron uptake from the nutrient solution during the artificial recovery process from the acute phase of the Cd stress (**Cad/Ko50**). The reason for light-dependent iron uptake could be explained by the activity of FRO2 a ferric chelate reductase enzyme in Strategy-I plants. This process requires NADH, which is produced in catabolic reactions. Citrate is also required for effective translocation of the iron in xylem elements, Citrate and reducing equivalents are, however, available in a high concentration only during photosynthetic activity. Thus the reason for decreased iron uptake and translocation was the stagnating of assimilation under dark periods. *Based on our measurements we found that in roots, the decrease of Cd and the increase of Fe concentration (increasing Fe/Cd ratio) was the reason for the recovery under artificial regeneration. Therefore, the increase of FRD3 (root xylem parenchyma citrate efflux transporter) expression is also predicted*.

2. Acute Cd stress led to a significant decrease in chloroplast iron content which increased, however, under longer-term Cd treatment. Chloroplast iron content strongly increased under artificial regeneration from the second photoperiod. In case of both spontaneous and artificial regeneration, the increase in chloroplast iron content preceded the recovery of all photosynthesis-related parameters (pigment composition, PSII activity, thylakoid membrane composition). Because of the Cd content in leaves of artificially

regenerated plants was unchanged, *the regeneration force restricted only to the increase of iron content in chloroplasts*. Under spontaneous regeneration, the leaf iron content remained unchanged. We confirmed that *the origin for increased iron content came from the reorganisation of iron content inside the mesophyll cells.*

3. Under acute Cd stress, both the Chl content and Chl *a/b* ratio were decreased remarkably and the composition of thylakoid complexes was perturbed. The content of PSI core and LHCII complexes (antennae of PSII) also decreased whereas the amount of PSII core, PSII connecting antennae and LHCI showed only minor changes. In Cad/Ko50 leaves, Chl *a/b* ratio started to increase after about half a day lag-time and reached a maximum level in the second photoperiod. The similar shape of the changes measured in Chl *a/b* ratio and *β*-carotene indicates that the recovery started with the synthesis of Chl *a* binding pigment-protein complexes, especially PSI core centres. *The increase of PSI content was similar to the increase in chloroplast iron content. This indicates that iron taken up by the chloroplasts incorporates into the PSI preferentially. The decrease of Chl a/b ratio in the second part of the regeneration process was due to the beginning of the synthesis of Chl b binding antennae. DDEEPS – the difference of light and dark-adapted de-epoxidation state of xanthophylls – showed parallel changes to Chl a/b ratio.* Similar changes have been observed under spontaneous recovery, but the amount of PSI and PSII reaction centres showed fluctuation in the chronic phase of the stress. The amount of LHCII antennae increased continuously.

4. The concentration of MDA increased significantly, as an acute Cd stress syndrome, whereas under chronic phase of the Cd stress a continuous decrease was observed. Ascorbate peroxidase (APX) activity showed opposite changes to MDA content. *The increase of APX activity preceded the decrease of MDA content.* Amongst the identified isoforms of SOD, the MnSOD and a Cu/ZnSOD isoform were the most sensitive for Cd stress, increased and decreased, respectively. Periodic changes have been detected in the activity of FeSOD. *A maximum in its activity was measured in the same time when APX activity reached the level of control leaves. The FeSOD activity and the amount of PSI core complexes showed parallel fluctuation under spontaneous recovery, supporting the theory of in-cell iron content reorganisation.*

Four form of the excitation energy allocation can be measured in leaves: i) photochemical quenching of PSII reaction centres (Φ_{PSII}), ii) thermal dissipation of inactive reaction centres (Φ_{NF}), iii) non-photochemical quenching of antennae (Φ_{NPQ}) and iv)

constitutive thermal dissipation and fluorescence ($\Phi_{f,D}$). *The increase of Φ_{NF} in the acute phase of Cd stress confirms that under acute Cd stress the dissipation of oligomerised, inactive PSII reaction centres is the main excitation quenching route*. In leaves, Cd content did not decrease under any type of recovery and the lipid peroxidation showed a parallel decrease to PSII inactivation. Thus, damages in PSII reaction centres were caused by ROS accumulation. *Our result showed that PSII inactivation together with the diminished accumulation of PSI reaction centres caused an inhibition in photosynthetic electron flow*. In the chronic phase of Cd stress, the elevated Φ_{NF} relaxed totally. Thus regeneration of PSII activity plays an important role in spontaneous recovery. The short increase in Φ_{NPQ} showed that *the restoration of PSII reaction centre-antennae connections is essential for the recovery of photosynthetic electron transport*.

Chronic Cd-stress, similarly to the relatively increased light intensity (L250) led to an increase of the concentration of compounds perform green fluorescence. The parallel recovery of Φ_{PSII} and accumulation of green emitting flavonoids hypothesize a connection between these two processes. In the green-yellow region of spectra, four peaks were identified showing elevation under Cd/excess light treatments. A F_{510} compound, proved to be a flavonol, accumulated only under Cd stress whereas other yellow-emitting compounds also responded to excess light. *F_{510} flavonol, accumulated in chloroplasts and/or vacuoles may have role in both the detoxification of ROS and the binding of free $Fe^{2+/3+}$ or Cd^{2+} in the symplast.*

Conclusions

i., Iron deficiency is the most important inhibiting factor of cadmium in plants. The reduced photosynthetic activity can be explained by the low iron content of the chloroplasts. The most important point of the Cd-Fe interference is the inhibited Fe translocation to the shoot which is restored with the decreasing Cd and increasing Fe concentration (increasing Fe/Cd concentration) in the roots.

ii., It is essential to the development of long term Cd tolerance that iron content is reorganised in the mesophyll cells and the iron content of chloroplast increases, which lead to the restoration of photosynthetic structures.

iii., During the artificial regeneration the acute stress symptoms of Cd stress were found to be recovered following the translocation of iron. The first indication of recovery was the

increase of iron content in the chloroplast which was followed by the rise of Chl *a/b* ratio and the accumulation of PSI reaction centres. The increase of chloroplast iron content precedes all the other recovery processes.

iv., Not only the remodelling of the iron content of mesophyll but the activation of antioxidative protection (enzyme activities: APX, MnSOD, FeSOD and protective compounds: flavonols) are also involved in the spontaneous regeneration. These processes lead to the recovery of the photosynthetic electron transport activity, especially the PSII function. It has been demonstrated that both processes strengthen each other

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List of publications

This thesis is based on the following publications

A. International journals

1. **Solti Á**, Szegi P, Basa B, Mészáros I, Sárvári É (2008): Alleviation of Cd induced inhibition of photosynthesis under long-term Cd treatment in poplar. *Cereal Research Communications 36(Suppl.): 239-242*.
2. **Solti Á**, Gáspár L, Szigeti Z, Mészáros I, Sárvári É (2008): F690-F740 is more suitable than F690/F740 for mapping the regeneration of Cd-induced chlorosis in poplar leaves by fluorescence imaging. *Acta Biologica Szegediensis 52:191-194.*

3. **Solti Á**, Gáspár L, Mészáros I, Szigeti Z, Lévai L, Sárvári É (2008): Impact of iron supply on the kinetics of recovery of photosynthesis in Cd-stressed poplar (*Populus glauca*). *Annals of Botany 102:771-782.* **IF 2.939**
4. **Solti Á**, Szűcs J, Basa B, Sárvári É (2009): Functional and organisational change of photosystem II in poplar thylakoids under Cd stress. *Cereal Research Communications 37(Suppl. 4): 525-528.*
5. **Solti Á**, Sárvári É, Tóth B, Basa B, Lévai L, Fodor F (2011): Cd affects the translocation of some metals either Fe-like or Ca-like way in poplar. *Plant Physiology and Biochemistry. 49: 494-498.* **IF 2.402**
6. **Solti Á**, Gáspár L, Vági P, Záray G, Fodor F, Sárvári É (2011): Cd, Fe and light sensitivity: interrelationships in Cd treated *Populus*. *OMICS - A Journal of Integrative Biology. 15: 811-818.* **IF 1.944**

B. Abstracts in journals

1. Szegi P, **Solti Á**, Gáspár L, Mészáros I, Sárvári É (2007): Kinetics of photosynthetic responses and development of protective mechanisms during Cd stress in poplar. *Cell Stress & Chaperones 12: 4G_06_P, doi: 10.1379/1466-1268(2007)12*
2. **Solti Á**, Szegi P, Gáspár L, Lévai L, Szigeti Z, Sárvári É (2007): Cd-induced inhibition of photosynthesis can be recovered by elevated Fe supply. *Cell Stress & Chaperones 12: 4G_07_P, doi: 10.1379/1466-1268(2007)12*

C. Conference abstracts

1. **Solti Á**, Szegi P, Szőllősi E, Mészáros I, Sárvári É (2009): Alleviation of PSII photoinhibition in hardening phase of Cd treated poplar. *Program and Abstracts, Plant Abiotic Stress Tolerance International Conference, Austria, Vienna, 2009: p.111.*
2. **Solti Á**, Basa B, Lévai L, Sárvári É, Fodor F (2010): Cd affects the translocation of some metals in poplar either Fe-like or Ca-like way. *Program and abstracts of the 15th International Symposion on Iron nutrition and Interactions in Plants p. 143. S7P5.*
3. **Solti Á**, Gáspár L, Vági P, Záray G, Gémesné Juhász A, Sárvári É (2011): A nyárfanövények Cd fitoremediáció szempontjából hasznos fiziológiai sajátságai. *Összefoglalók. XVII. Növénynemesítési Tudományos Napok, Budapest, p. 119*
4. **Solti Á**, Szabó K, Ross K, Fodor F, Sárvári É (2011): Changes in the pattern of the activity of some antioxidative defence enzymes under long-term Cd stress. *Scientific Programme, Abstracts, and List of Participants of the Conference 'Molecular Basis of Plant Stress', Varna, 21-23 September, 2011, p 93. (P53)*