Ascorbate transport and environmental regulation of ascorbate biosynthesis in plants and green algae

Project report

Ascorbate (Asc, also called vitamin C) is essential to the cellular functions in plants and animals with the most known role of being a scavenger of reactive oxygen species. In plants, it also participates in cell division, synthesis of the cell wall, the synthesis of several signaling molecules and plant hormones and it is also involved in stomatal movement (reviewed by Smirnoff, 2018). It was also shown to be required for the regulation of gene expression and more recently, for DNA methylation.

In the NN114524 project, we have addressed several open questions regarding the roles, the biosynthesis and the transport of Asc that are of high relevance for breeding plant varieties with elevated Asc content. Several members of the "Lendület" Laboratory for Molecular Photobioenergetics, both postdocs and PhD student participated in this work and several subprojects were carried out in collaboration with the Max Planck Institute of Molecular Plant Physiology (MPI-MP, Potsdam-Golm). André Vidal-Meireles, László Kovács, Dávid Tóth, Anikó Galambos and Szilvia Z. Tóth had the opportunity to spend several weeks to months at the MPI-MP and there are two more joint projects going on.

The experiments were carried out on Arabidopsis, serving as a model organism for crop plants, and on a green alga species, *Chlamydomonas reinhardtii* that has a high biotechnological application potential, for instance in biohydrogen production.

I. Regulation of ascorbate biosynthesis in higher plants

Several Asc biosynthetic pathways have been proposed in plants: The "Smirnoff-Wheeler" (L-galactose) pathway (Wheeler et al. 1998) involves the conversion of D-mannose into Asc via a series of L-galactose containing intermediates. Three other, alternative pathways have been suggested to occur in plants, including (i) the L-gulose pathway (Wolucka and Van Montagu 2003), (ii) the galacturonate ("pectin scavenging") pathway (Cruz-Rus et al. 2010), and (iii) the animal-like Asc biosynthetic (myo-inositol) pathway (Lorence et al., 2004).

In order to reveal the physiological significance of the alternative pathways, we studied the Asc production of mutants affected in the "Smirnoff-Wheeler" Asc biosynthesis pathway. A key Asc biosynthesis gene is *VTC2*, encoding GDP-L-galactose phosphorylase. In the first set of experiments, the widely used *vtc2-1* mutant was used; however, in 2016 it turned out that this mutant contains several cryptic mutations that are unrelated to Asc biosynthesis (Lim et al., 2016). For this reason, the experiments were repeated on another, more recently generated mutant, the *vtc2-4* knockout line. The experiments included various stress treatments. The high light treatment (about 500 µmol photons m^{-2} s^{-1}) lead to approx. 50% increase in Asc concentration of the Col-0 (wild type) plants; however, in spite of several attempts, we could not detect any significant and reproducible increase of Asc content in the *vtc2-4* mutant (Fig. 1). High temperature treatments

(34°C day temperature) led to minor increase in Asc concentration only in the wild type, whereas treatments with Rose Bengal (a singlet oxygen generating compound) and H_2O_2 did not induce any Asc accumulation.

Therefore, we concluded that the proposed alternative Asc biosynthesis pathways do not become induced upon stress treatments and their contribution to Asc production is minor or non-existing in Arabidopsis leaves. We also concluded that reactive oxygen species alone do not lead to Asc accumulation in vascular plants.

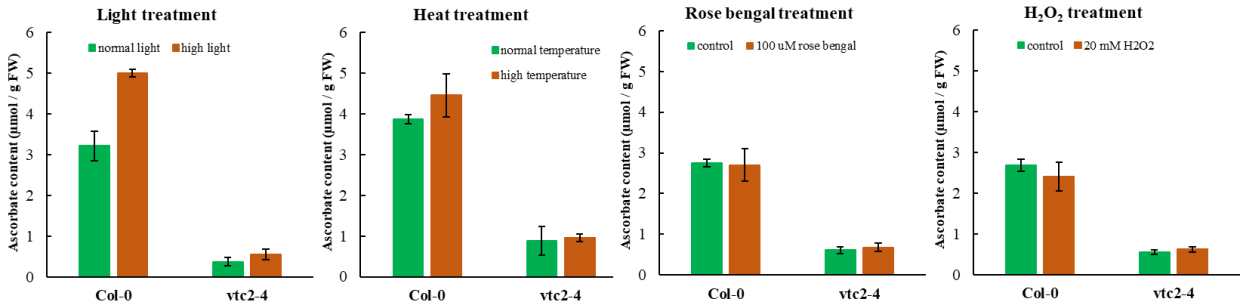

Fig. 1. Ascorbate levels upon high light (500 µmol photons m^{-2} s^{-1}), high temperature (34° during the day), Rose Bengal and H_2O_2 treatments in the Asc-deficient *vtc2-4* mutant and its wild type (Col-0).

Publications: Asc biosynthesis and its physiological roles have been summarized in a review article published in Antioxidants and Redox Signaling (IF: 5.828, Tóth et al., 2018, see also Fig. 2) and our article was also selected for a cover page of the 2018 October issue.

We plan to publish our experimental data as part of a metabolomics study on the compensation mechanisms of Asc deficiency (see also section III).

II. Ascorbate biosynthesis and its physiological roles in the green alga *Chlamydomonas reinhardtii*

II/1. The regulation of Asc biosynthesis in *C. reinhardtii*

C. reinhardtii is a useful model organism to answer a wide range of biological questions and for biotechnological applications. At the beginning of this project, a direct evidence for the operation of the Smirnoff-Wheeler pathway in green algae was lacking, and no mutants affected in Asc biosynthesis were available.

In collaboration with the MPI-MP (Potsdam-Golm) we have generated Asc-deficient *Chlamydomonas* transformants using the artificial microRNA (amiRNA) approach. *VTC2* was selected as a target gene, because gene expression studies in *C. reinhardtii* suggested that it is essential for Asc biosynthesis (Urzica et al., 2012), similarly to vascular plants.

Ascorbate concentrations in *VTC2* amiRNA lines were reduced to 10% showing that GDP-L-galactose phosphorylase plays a pivotal role in Asc biosynthesis in green algae. The *VTC2* amiRNA lines also grow more slowly, has lower chlorophyll content, and are more susceptible to stress than the control strains, as shown by metabolite profiling. We also demonstrate that the expression of the *VTC2* gene is rapidly induced by H_2O_2 and $^1O_2$, resulting in a manifold increase

in Asc content. In addition, and in contrast to plants, there is no circadian regulation of Asc biosynthesis in green algae and photosynthesis is not required per se for ascorbate biosynthesis. We have also shown that, unexpectedly, *C. reinhardtii VTC2* lacks negative feedback regulation by Asc in the physiological concentration range. Thus we demonstrated that Asc biosynthesis is also highly regulated in *C. reinhardtii* albeit via mechanisms distinct from those previously described in vascular plants.

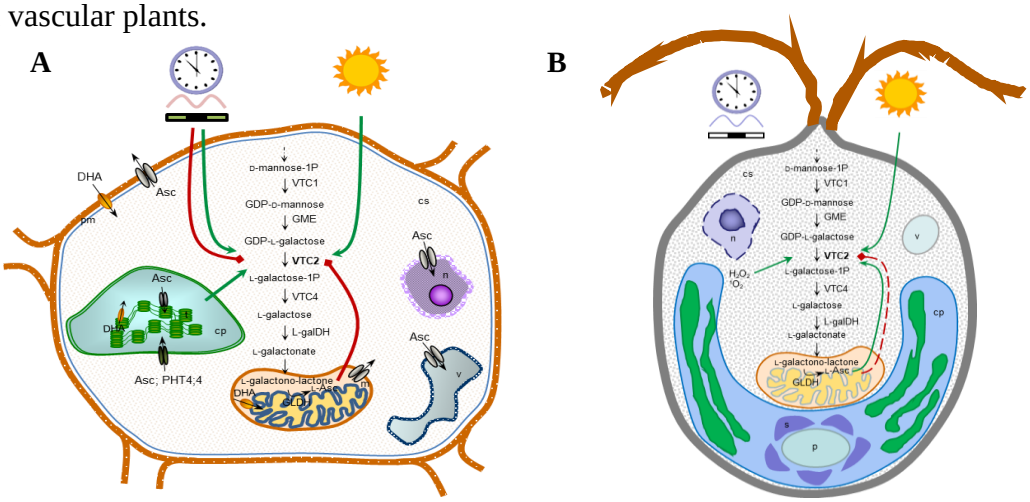

Fig. 2. Ascorbate biosynthesis and its regulation in seed plants (A) and green algae (B), and identified and putative Asc transporters in seed plants (A). Asc is synthesized mostly via the Smirnoff-Wheeler pathway both in seed plants and green algae. The *VTC2* gene, encoding GDP-L-galactose phosphorylase, plays a major role in the regulation of Asc biosynthesis. In higher plants, its expression is induced by light, regulated by photosynthetic reactions and by the circadian clock. The expression of *VTC2* and the translation of the enzyme are both feedback inhibited by Asc. In green algae, light and reactive oxygen species induce the expression of *VTC2* and Asc has a stimulatory effect on its expression in the low, physiological concentration range; however, a feedback inhibition in the mM range is also likely to take place. The Asc content does not depend on the circadian rhythm in green algae. In seed plants, AtPHT4;4 was identified as a chloroplast-localized Asc transporter; putative Asc and DHA transporters include Asc transporters responsible for the export of Asc out of the mitochondrium, into the thylakoid lumen and through the plasma membrane, transporters for ensuring the uptake of Asc into the vacuole and possibly into the nucleus. DHA also has to be transported out of the thylakoid lumen for regeneration. cs, cytosol; cp, chloroplast; m, mitochondrium; n, nucleus; p, pyrenoid; pm, plasma membrane; s, starch granules; t, thylakoid membrane; v, vacuole. Tóth et al., (2018) Antioxidants and Redox Signaling.

Publications: Our results on the regulation of Asc biosynthesis were published in a leading journal of plant science, New Phytologist (IF: 7.333, Vidal-Meireles et al., 2017) and served as a basis in the PhD thesis of André Vidal Meireles (defended in 2018).
A methodological paper on determination of the Asc content in green algae was published in the journal Bio-Protocol (Kovács et al., 2016).

II/2. Asc plays an important role in sulphur-deprivation induced biohydrogen production
In nature, H_2 production in *C. reinhardtii* serves as a safety valve during the induction of photosynthesis in anoxia and it prevents the over-reduction of the photosynthetic electron transport chain (Fig. 3). Sulphur limitation may restrain cell growth and viability and thereby it triggers a complex metabolic response resulting in the induction of oxidative stress-related genes,

downregulation of photosynthesis, the establishment of anaerobiosis and expression of active hydrogenase. Due to the relatively long H_2 production phase imposed by sulphur limitation, there has been a considerable effort by the scientific community to exploit this phenomenon for biohydrogen production. However, it turned out not to be possible, mostly due to the damage it exerts on the photosynthetic apparatus.

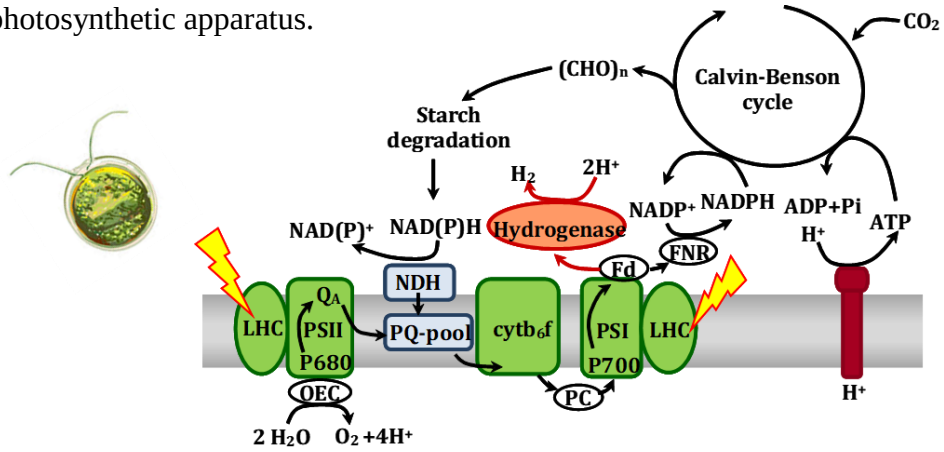

Fig. 4. H_2 production by green algae. The [Fe-Fe] hydrogenases of green algae are located directly at the acceptor side of photosystem I and they are highly efficient. Upon sulphur deprivation, the Calvin-Benson cycle and photosystem II become inactivated, resulting in the establishment of anoxia. Due to the severe stress effects imposed by sulphur limitation, alternative methods based on Calvin-Benson cycle limitation are preferred for future biotechnological applications. Published in: Tóth and Yacoby, 2019.

The inactivation of photosystem II (PSII) has been long assumed to be caused by the sulphur-limited turnover of its reaction center protein, PsbA. In this part of the project, we have reinvestigated this issue in detail and showed that

i) upon transferring *C. reinhardtii* cells to sulphur-free media, the amount of cellular sulphur content decreases only by about 25%;

ii) PsbA has a significant turnover and other photosynthetic subunits, namely RbcL and CP43, are degraded more rapidly than PsbA;

iii) sulphur limitation imposes oxidative stress early on, most probably involving the formation of singlet oxygen in PSII, which leads to an increase in the expression of GDP-L-galactose phosphorylase;

iv) the Asc content in *C. reinhardtii* increases about 50-fold, reaching the mM concentration range that may inactivate the oxygen-evolving complex and provide electrons to PSII albeit at a low rate. In the absence of a functional donor side and sufficient electron transport, PSII reaction centers get inactivated and degraded.

We have therefore demonstrated that the inactivation of PSII is a complex and multistep process, which may serve to mitigate the damaging effects of sulphur limitation.

Publications:

Our results were published in the prestigious journals of the field of plant physiology, Plant, Cell and Environment (IF. 6.173, Nagy et al., 2016) and Plant Journal (IF: 5.726, Nagy et al., 2018).

II/3. Effect of Asc-deficiency on the photosynthetic electron transport and non-photochemical quenching (NPQ)

In vascular plants, violaxanthin de-epoxidase (VDE) requires Asc as reductant, thereby it is required for the energy-dependent component of non-photochemical quenching (NPQ). To assess the role of Asc in NPQ in green algae, which contain low amounts of Asc, we searched for an insertional *C. reinhardtii* mutant affected in the *VTC2* gene. The Chlamydomonas Library Project, containing tens of thousands of random insertional mutants, became available at the end of 2016. The insertional mutants have the advantage compared to amiRNA constructs that they are more stable and knock out mutants for specific genes may be available.

The *Crvtc2-1* knockout mutant was viable and, depending on the growth conditions, contained 10 to 20% Asc relative to its wild type. When *C. reinhardtii* was grown photomixotrophically at moderate light, the zeaxanthin-dependent component of NPQ emerged upon strong red illumination both in the *Crvtc2-1* mutant and in its wild type. Deepoxidation was unaffected by Asc deficiency, demonstrating that the Chlorophycean VDE found in *C. reinhardtii* does not require Asc as a reductant. The rapidly induced, energy-dependent NPQ component characteristic of photoautotrophic *C. reinhardtii* cultures grown at high light was not limited by Asc deficiency either. On the other hand, a reactive oxygen species-induced photoinhibitory NPQ component was greatly enhanced upon Asc deficiency, both under photomixotrophic and photoautotrophic conditions.

Thus we have demonstrated that Asc has distinct roles in NPQ formation in *C. reinhardtii* than in vascular plants.

Publications: These results were published in Plant Physiology, one of the leading journal of plant sciences (IF: 6.305, Vidal-Meireles et al., 2019, in press).

III. Identification of Asc transporters in higher plants

Ascorbate biosynthesis occurs in the cytosol, with the exception of the terminal step that takes place on the inner mitochondrial membrane (Smirnoff, 2018, see also Fig. 2). From the mitochondria, Asc has to be transported to basically all the other cell compartments, necessitating specific transporters. Up to now, only one ascorbate transporter has been identified in seed plants, the chloroplastic AtPHT4;4 transporter in *Arabidopsis thaliana* (Miyaji et al., 2015).

III/1. The physiological role of AtPHT4;4

In this part of the project, we investigated the physiological role of AtPHT4;4 in detail, and compared *pht4;4-1* knockout and Asc-deficient *vtc2-4* mutant plants with their respective wild types. We found that the absence of AtPHT4;4 results in hardly discernible effects on photosynthesis and stress sensitivity. The metabolite phenotype (see section III/3) is very mildly affected by the absence of PHT4;4 in comparison with the effects of cellular Asc deficiency. The

absence of PHT4;4 did not affect the *in vivo* uptake of Asc into the chloroplast either, strongly suggesting that PHT4;4 is not the sole Asc transporter acting in the chloroplast envelope membrane.

Publication: We have published a brief overview on PHT4;4 and Asc transporters in Molecular Plant (IF: 7.142, Fernie and Tóth 2015).
There is a manuscript under revision in which the physiological importance of PHT4;4 was studied (Tóth D. et al.,).

III/2. Homologues of AtPHT4;4 in Chlamydomonas

In green algae, no Asc transporters have been identified so far. In Chlamydomonas, we have found three homologues of the AtPHT4;4 transporter, called CrPHT3, CrPHT4 and CrPHT7. In frame of a collaboration, the laboratory of Attila Molnar (University of Edinburgh), generated knockout lines for CrPHT3 and CrPHT7 for us, using their recently developed CRISPR/Cpf1 technique (Ferenczi et al., 2017). Both knockout mutants and the double mutants showed decreased growth rate, high light sensitivity, increased Asc content, and decreased growth rate under phosphate-limited conditions. In addition, their photosynthetic parameters were also largely altered. In the frame of a collaboration with the Max-Planck Institute of Molecular Plant Physiology, we have generated complementation lines, which showed a clear restoration of the phenotype.

In order to study the substrate specificity, we have expressed the CrPHT7 transporter in a phosphate transporter-deficient yeast strain (EY917). The CrPHT7-expressing yeast strains showed a very clear restoration of the growth rate compared to the EY917 mutant. As a next step, the Asc uptake will be studied in these yeast strains.

In addition, we have also generated YFP-PHT7 constructs to study the cellular localization of PHT7. In spite of the restoration of the phenotype of the *pht7* mutants to the wild-type level, we could not detect any YFP fluorescence, suggesting that CrPHT7 has a very low abundance or the YFP protein was inactivated and degraded in the cellular environment. Therefore, we have decided to generate HA-tagged constructs to study the cellular localization of the PHT7 transporter.

Publication: In this part of the project, several methodologies are used that were new to us (expressing transporters in yeast, generating constructs for cellular localization in Chlamydomonas, etc), that resulted in significant delays in the progress of this part of the project. We expect, however, that these efforts will manifest themselves in a publication in a highly ranked journal, within one year.

III/3. Identification of novel Asc transporters in vascular plants

Our approach to identify chloroplastic Asc transporters is based on co-expression analysis using the key Asc biosynthesis gene, *VTC2*, as a bite vs. the identified chloroplast-localized transporters. We have found approx. 30 putative Asc transporters and at least two Arabidopsis T-DNA lines for each transporter gene were selected. Altogether, we screened about 120 T-DNA lines for 30

transporter proteins. The screening method was based on NPQ and on the fast chl a fluorescence transient measured on heat-stressed leaves. Both methods are sensitive to chloroplastic Asc content; however, the fast chl a fluorescence transient is more specific, because under heat stress conditions, Asc becomes an alternative PSII electron donor and this results in a marked peak in the fluorescence transient (Tóth et al., 2009).

Based on these measurements, we decided to concentrate on 2 transporter proteins, namely on the bile acid transporter 2 and 3 (BAT2, BAT3) that are known to be located in the chloroplast envelope membrane whereas their substrates are unknown (Gigolashvili et al., 2009). These transporter proteins are homologous to each other, therefore double mutants were generated. However, the single and double mutant lines of these transporters showed no changes in the phenotype and only mild physiological alterations (in photosynthetic electron transport rates, xanthophyll cycle pigments, etc) both under control and light stress conditions. Due to the hardly discernible effects of the mutations, we have opted for another approach, namely metabolite profiling. For this purpose, a high resolution mass spectrometer, Thermo Q Exactive Focus was purchased by our laboratory, in collaboration with the research group of Dr. Balázs Papp (BRC Szeged). The method development includes the optimization of a suitable HPLC method, optimization of the MS parameters and building an in-house library. Currently, we are capable of separating approx. 1000 metabolites and identify approx. 400 of them based on their exact masses.

Our preliminary results revealed a strong similarity between the *BAT2* and *BAT3* mutants and marked differences between them and the wild-type plants. We expect that an in-depth metabolomics analyses will enable the identification of the metabolite pathways that are most affected by the *BAT2* and *BAT3* mutations. This may corroborate our hypothesis that these are Asc transporters or other hypothesis may be formulated and tested later.

IV. Experiments on the signaling role of Asc

The research group of our collaborator, Dr. Alisdair R. Fernie at the MPI-MP aimed at identifying putative Asc receptors in Arabidopsis plants. To this end, they overexpressed galactono-lactone dehydrogenase in Arabidopsis to generate lines with elevated Asc levels. As a next step, they planned to carry out a suppressor mutation screen on these lines (using EMS as a mutator) in order to identify the putative Asc receptors. Unfortunately, their approach was unsuccessful, because the increase in Asc level in the galactono-lactone dehydrogenase overexpressing lines were unexpectedly small and the obtained lines could not serve as a basis for the subsequent EMS mutagenesis. For this reason, the project was, unfortunately, discontinued.

Other achievements and scientific dissemination:

In 2017, I received the L'Oréal-UNESCO National Award For Women in Science. In relation to this, I had the opportunity to talk about our research and my scientific carrier to the public. Here are some examples (in Hungarian):

UNESCO.hu: Dr. Wohner Nikolett és Dr. Tóth Szilvia Zita a L'Oréal-UNESCO A nőkért és a tudományért magyar ösztöndíj pályázat idei nyertesei http://www.unesco.hu/termeszettudomany/dr-wohner-nikolett-dr

SzegedMa: Tóth Szilvia Zita: Falakat döntenek le a tudományban dolgozó nők https://szegedma.hu/2017/09/toth-szilvia-zita-falakat-dontenek-le-a-tudomanyosban-dolgozo-nok

Index: Csajoskodást otthon hagyni lányok, irány a labor! https://divany.hu/vilagom/2017/10/10/vilagom_kutatas_unesco_loreal/

Magyar Nemzet: „Egyáltalán nincs a fejekben, hogy nőket is érdemes mentorálni" https://mno.hu/tudomany/egyaltalan-nincs-a-fejekben-hogy-noket-is-erdemes-mentoralni-2425941

Kossuth Rádió, a Tudomány Hangjai: Magas C-vitamin tartalmú növények előállítása https://www.mediaklikk.hu/cikk/2018/03/14/a-tudomany-hangjai-magas-c-vitamin-tartalmu-novenyek-eloallitasa/

Szeged Televízió Kvantum szegedi tudósok magazinja, 2017. október 24-i hírműsor, portré: https://www.youtube.com/watch?v=jEsywl1ziXs&t=86s

NB: I was on maternity leave between 01 August 2017 and 19 February 2018. For this reason, the project was prolonged by 6 months.

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