

**ASCORBATE BIOSYNTHESIS AND ITS  
PHYSIOLOGICAL ROLES IN THE GREEN ALGA  
*CHLAMYDOMONAS REINHARDTII***

**Summary of the PhD Thesis**

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## **1.) Introduction**

*C. reinhardtii* has emerged as an excellent model system to study both photosynthesis and biogenesis of the chloroplast, mostly due to its capability for heterotrophic growth, using acetate as a carbon source; thanks to this property, mutants deficient in photosynthesis are viable, provided that acetate is added to the growth medium in the dark (Harris, 2001). Another advantage of using *Chlamydomonas* as a model system is that it represents the only photosynthetic organism that is suitable for transformation of all genetic compartments: nucleus, chloroplast, and mitochondria (Nickelsen, 2005). Recent development in genetic tools (amiRNA and CRISPR/Cpf1 techniques) make it also an ideal model organism.

Ascorbate (Asc) is of vital importance to the cellular functions of both animals and plants. It is an essential scavenger of reactive oxygen species (ROS) including singlet oxygen and superoxide and it is also a cofactor of several 2-oxoacid-dependent dioxygenase enzymes, which catalyze a large number of physiological processes in the cell. In higher plants, besides its role as an antioxidant, Asc also participates in cellular development and synthesis of the cell wall. Asc also modulates the synthesis of several signaling molecules such as abscisic acid, gibberellins, ethylene and salicylic acid. It also influences anthocyanin accumulation during high light

acclimation; it is involved in the regulation of stomatal movement and also modulates the expression of specific sets of photosynthesis and defense genes via poorly understood mechanisms. Asc also contributes to the non-photochemical quenching in higher plants because it is a cofactor of violaxanthin deepoxidase.

In higher plants, the main Asc biosynthesis pathway is the L-galactose pathway, also called the “Smirnoff-Wheeler” pathway; this is the main contributor of Asc synthesis in higher plants, shown by the observation that mutants targeting key enzymes of this pathway are unable to compensate for the loss of the Asc biosynthetic capacity. The rate of Asc biosynthesis is largely determined by the expression level of *VTC2*, encoding GDP-L-galactose phosphorylase, as demonstrated by the *vtc2-1* Arabidopsis mutant which contains approx. 80% less Asc relative to its control strain (Conklin et al., 2000; Müller-Moulé et al., 2002, 2004).

The genome of the model green alga *C. reinhardtii* encodes all the enzymes of the Smirnoff-Wheeler Asc biosynthesis pathway; based on gene expression analysis, it was suggested that the algal GDP-L-galactose phosphorylase is a highly regulated enzyme, similarly to higher plants (Urzica et al., 2012). Overall, Asc biosynthesis and its regulation are poorly studied in non-vascular plants and in green algae, organisms that possess low Asc contents (Gest et al., 2013; Wheeler et al., 2015).

We showed recently that Asc may have an important modulatory effect on the photobiological H<sub>2</sub> production of *C. reinhardtii* (Nagy et al., 2016). The oxidation of water, primary source of reducing equivalents for photosynthesis, is carried out by the oxygen evolving complex (OEC) of PSII. We have shown that upon sulphur deprivation, Asc accumulates dramatically and it inactivates the OEC due to its reducing property. Even though Asc can donate electrons to PSII, that happens at a slow rate and donor-side induced photoinhibition takes place, causing a loss of the charge separation activity of PSII; these events lead to the establishment of anaerobiosis, which is essential for H<sub>2</sub> production (Nagy et al., 2016). However, the exact mechanism by which Asc may inactivate PSII during sulphur deprivation-induced H<sub>2</sub> production in *C. reinhardtii* remained unclear.

For photosynthetic organisms, light absorption is required for growth, but too much light can be harmful. The photo-protective mechanisms responsible for dissipating the excess energy as heat are collectively called non-photochemical quenching (NPQ). NPQ includes short-term responses to rapid fluctuations in light, as well as responses that occur over longer periods allowing for acclimation to high light exposure. One of the key components of NPQ involves the conversion of violaxanthin to zeaxanthin, which is catalyzed by the enzyme violaxanthin de-epoxidase (VDE). The VDE enzyme of *C. reinhardtii* (CrVDE) differs from the VDE

enzyme found in plants and in most algae (Li et al., 2016b), and it was unknown whether Asc is required as a co-factor for the algal-type VDE, and thereby to qZ induction.

## 2.) Aims

- Studying the regulation of Asc biosynthesis and its physiological importance in *C. reinhardtii*
- Determination of the role of Asc in the inactivation of PSII during sulphur deprivation-induced H<sub>2</sub> production in *C. reinhardtii*
- Determination of whether Asc is required as a co-factor of CrVDE and investigating the role of Asc in NPQ induction.

### 3.) Applied methods and techniques

- amiRNA;
- *C. reinhardtii* nuclear transformation;
- DNA isolation;
- PCR;
- Protein isolation;
- Western blot;
- RNA isolation;
- cDNA synthesis;
- qRT-PCR;
- Chl *a* fluorescence;
- Northern blot;
- HPLC;
- GC-MS;
- ICP-OES;
- Enzymatic assays;
- Thermoluminescence;
- Thylakoid isolation.

#### 4.) Summary of findings

Ascorbate biosynthesis and its regulation are poorly studied in non-vascular plants, and even though it was known that the genome of the model green alga *C. reinhardtii* encodes all the enzymes of the Smirnoff-Wheeler Asc biosynthesis pathway, no direct evidence for its physiological function was available. In addition, in this thesis it was also demonstrated that the regulation of Asc biosynthesis has evolved markedly different mechanisms in *C. reinhardtii* than in higher plants:

- The expression of the *VTC2* gene of *C. reinhardtii* is not directly influenced by the photosynthetic electron transport chain;
- In contrast to higher plants, *VTC2* transcript abundance is not under circadian control in *C. reinhardtii*;
- Upon oxidative stress, *VTC2* is strongly upregulated, allowing a very rapid Asc accumulation;
- There is no negative feedback regulation in the physiological Asc concentration range.

This set of results was published in Vidal-Meireles et al (2017) *New Phytologist* 214: 668-681, doi: 10.1111/nph.14425.

Under stress conditions, such as sulphur deprivation, *C. reinhardtii* can increase their Asc concentration to levels that may lead to inactivation of the OEC, which may lead to

the establishment of hypoxia, followed by the initiation of H<sub>2</sub> production. Based on these earlier results (Nagy et al., 2016), we aimed to better understand the role of Asc in these processes and we found that:

- The moderate decrease in sulphur content is unlikely to be the main cause for substantial PsbA loss upon sulphur deprivation as it is generally assumed;
- PsbA has a discernible turnover during sulphur-deprivation;
- The transcript abundance of the *VTC2* gene increases upon sulphur deprivation, which leads to Asc accumulation up to the mM range;
- The decline of PSII activity may be caused by donor-side induced photoinhibition exerted by the strong Asc accumulation.

These results were published in Nagy et al. (2018) *The Plant Journal* 94: 548-561; doi: 10.1111/tpj.13878.

Ascorbate plays an important role in the photosynthesis of seed plants and acts as a cofactor of VDE (Müller-Moulé et al., 2002). This enzyme, activated upon thylakoid lumen acidification, is responsible for the conversion of violaxanthin to zeaxanthin and plays an essential role in the qE component of NPQ (Müller-Moulé et al., 2002).

*C. reinhardtii* has a VDE enzyme which is significantly different from that of seed plants: CrVDE is

related to a lycopene cyclase of photosynthetic bacteria, and it is located on the stromal side of the thylakoid membrane (Li et al., 2016b). Besides, it is unknown whether Asc is a cofactor of CrVDE and in general, the role of Asc in the NPQ of green algae is poorly studied. We have found that:

- As opposed to seed plants, in the green alga *C. reinhardtii* Asc is not required as a co-factor for VDE;
- Under photomixotrophic growth at normal light intensities, H<sub>2</sub>O<sub>2</sub> enhances the slow component of NPQ;
- If the cultures are kept under photoautotrophic conditions at high light intensities, the lack of Asc causes photoinhibition, which is probably triggered by H<sub>2</sub>O<sub>2</sub> accumulation.

Based on these results, a manuscript is in preparation for publication.

## 5.) List of Publications

*MTMT identification number: 10052738*

Nagy V, **Vidal-Meireles A**, Tengölics R, Rákhely G, Garab G, Kovács L, Tóth SZ (2016) Ascorbate accumulation during sulphur deprivation and its effects on photosystem II activity and H<sub>2</sub> production of the green alga *Chlamydomonas reinhardtii*. Plant Cell Environ 39(7): 1460-1472. doi: 10.1111/pce.12701

*Impact factor: 6.173*

Kovács L, **Vidal-Meireles A**, Nagy V, Tóth SZ (2016) Quantitative determination of ascorbate from the green alga *Chlamydomonas reinhardtii* by HPLC. Bio-protocol 6(24): BioProtoc. 2067. doi: 10.21769/BioProtoc.2067

*Impact factor: N.A.*

**Vidal-Meireles A**, Neupert J, Zsigmond L, Rosado-Souza L, Kovács L, Nagy V, Galambos A, Fernie AR, Bock R, Tóth SZ (2017) Regulation of ascorbate biosynthesis in green algae has evolved to enable rapid stress-induced response via the *VTC2* gene encoding GDP-L-galactose phosphorylase. New Phytologist 214(2): 668-681. doi: 10.1111/nph.14425.

*Impact factor: 7.330*

Nagy V, **Vidal-Meireles A**, Podmaniczki A, Szentmihályi K, Rákhely G, Zsigmond L, Kovács L, Tóth SZ (2018) The mechanism of photosystem II inactivation during sulphur deprivation-induced H<sub>2</sub> production in *Chlamydomonas reinhardtii*. The Plant Journal 94(3): 548-561. doi: 10.1111/tpj.13878.

*Impact factor: 5.901*

Nagy V, Podmaniczki A, **Vidal-Meireles A**, Tengölics R, Kovács L, Rákhely G, Scoma A, Tóth SZ (2018) Water-splitting-based, sustainable and efficient H<sub>2</sub> production in green algae as achieved by substrate limitation of the Calvin-Benson-Bassham cycle. Biotechnology for Biofuels 11:69. doi: 10.1186/s13068-018-1069-0.

*Impact factor: 5.203*

**Vidal-Meireles A**, Lambrev P, Galambos A, Kovács L, Tóth SZ (in preparation) Ascorbate is not required for zeaxanthin-dependent quenching in the green alga *Chlamydomonas reinhardtii*.

## 6.) Communications at International Conferences

1. **Vidal-Meireles A**, Nagy V, Neupert J, Tengölics R, Rákhely G, Bock R, Garab G, Kovács L, Tóth SZ. The effects of ascorbate on the O<sub>2</sub> evolution and H<sub>2</sub> production of sulphur-deprived *Chlamydomonas reinhardtii* cultures. 1<sup>st</sup> International Solar Fuels Conference, 26<sup>th</sup> April – 1<sup>st</sup> May 2015, Uppsala (Sweden) [Poster presentation and Flash talk]

2. Paneerselvam N, **Vidal-Meireles A**, Nagy V, Kovács L, **Tóth SZ**. The PSBO1 protein of photosystem II protects the oxygen-evolving complex from the reducing power of luminal ascorbate. ICAR 2015, 5<sup>th</sup> - 9<sup>th</sup> July 2015, Paris (France) [Poster presentation]

3. **Vidal-Meireles A**, Neupert J, Nagy V, Kovács L, Zsigmond L, Rosado-Souza L, Fernie AR, Bock R, **Tóth SZ**. Regulation of ascorbate biosynthesis in the green alga *Chlamydomonas reinhardtii*. ENCAPP 2016, 26<sup>th</sup> - 29<sup>th</sup> April 2016, Saint Paul's Bay (Malta) [Poster presentation]

4. **Nagy V**, **Vidal-Meireles A**, Tengölics R, Rákhely G, Garab G, Kovács L, Tóth SZ. Ascorbate accumulation during sulphur deprivation and its effects on photosystem II activity and H<sub>2</sub> production of the green alga *Chlamydomonas reinhardtii*. ENCAPP 2016. 26<sup>th</sup> - 29<sup>th</sup> April 2016, Saint Paul's Bay (Malta) [Poster presentation]

5. **Tóth SZ**. Regulation of ascorbate biosynthesis in the green alga *Chlamydomonas reinhardtii*. Photosynthetic

electron and proton transport in plants and algae (Satellite meeting of the 17<sup>th</sup> International Congress on Photosynthesis Research). 4<sup>th</sup> – 7<sup>th</sup> August 2016, Arnhem (The Netherlands) [Oral presentation]

6. **Vidal-Meireles A**, Neupert J, Zsigmond L, Kovács L, Nagy V, Rosado-Souza L, Fernie AR, Bock R, Tóth SZ. Ascorbate biosynthesis and its regulation by *VTC2* in the green alga *Chlamydomonas reinhardtii*. SEB Gothenburg 2017, 3<sup>rd</sup> - 6<sup>th</sup> July 2017, Gothenburg (Sweden) [Poster presentation]

7. **Tóth D**, Galambos A, Nagy V, **Vidal-Meireles A**, Podmaniczki A, Kovács L, Molnár A, Tóth SZ. Identification of ascorbate transporters in green algae. First European Congress on Photosynthesis Research, 25<sup>th</sup> – 28<sup>th</sup> June 2018, Uppsala (Sweden) [Poster presentation]

8. **Vidal-Meireles A**, Galambos A, Kovács L, Tóth SZ. In the green alga *Chlamydomonas reinhardtii* ascorbate is not required for the induction of non-photochemical quenching. Plant Biology 2018, 14<sup>th</sup> - 18<sup>th</sup> July 2018, Montreal (Canada) [Poster presentation]

9. **Vidal-Meireles A**, Galambos A, Kovács L, Tóth SZ. In the green alga *Chlamydomonas reinhardtii* ascorbate is not required for the induction of non-photochemical quenching. ISPR meeting *From Light to Life*, 17<sup>th</sup> - 20<sup>th</sup> July 2018, Montreal (Canada) [Poster and oral presentation]

## **7.) Awards and Fellowships**

1. Biological Research Centre of the Hungarian Academy of Sciences *PhD scholarship* (2013)
2. Campus Hungary *Conference travel grant* (2015)
3. Hungarian Academy of Sciences *Young Researcher Fellowship* (2016)
4. Szeged Biology PhD School *Best presentation award at the PhD Student Conference* (2016)
5. Company of Biologists *Travel award* (2018)
6. U.S. Department of Energy *Travel award* (2018)
7. ISPR / Plant Cell & Environment *Best poster by a graduate student at the ISPR Meeting "Photosynthesis: from Light to Life"* (2018)