

Thesis of the PhD dissertation

**Ascorbate-dependent alternative electron transport of higher plants and
the role of ascorbate in the regulation of hydrogen production
in *Chlamydomonas reinhardtii***

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Introduction

During photosynthesis, via using light energy and water as electron donor, plants and cyanobacteria produce molecular oxygen and organic compounds by reducing CO₂. Photosynthesis is one of the most important processes on Earth, which determines the composition and ensures the stability of the atmosphere and provides organic compounds virtually for the entire biosphere. In addition, photosynthesis-based systems are becoming more and more important as renewable energy sources.

Oxygenic photosynthesis of eukaryotic organisms takes place in the chloroplast, and consists of photophysical and photochemical processes and light-independent reactions, i.e. the Calvin-Benson cycle. The chloroplasts contain light-absorbing chlorophyll molecules, and other auxiliary pigments bound to protein complexes, which are embedded in the thylakoid membrane. The photosynthetic electron transport is performed by photosystems I and II (PSI and PSII), the cytochrome b₆f complex, the ATP synthase and several mobile components (plastoquinone, plastocyanin, ferredoxin).

The oxygen-evolving complex (OEC), which is located on the donor side of PSII, is one of the most vulnerable electron transport components and is the primary target of heat stress, UV radiation and donor-side photoinhibition. Upon heat stress the extrinsic proteins (primarily PsbO) are released. The release of PsbO is followed by a loss of at least two manganese ions, Cl⁻ and Ca²⁺, functionally inactivating the OEC. The donor-side photoinhibition is triggered by the accumulation of P₆₈₀⁺ and results in the inactivation of PSII.

By the aid of its Fe-Fe type hydrogenase enzyme, located on the PSI acceptor side, unicellular green algae are capable to produce H₂, linked to the photosynthetic electron transport. This hydrogenase enzyme is very sensitive to O₂. To avoid the inhibitory effect of O₂, a method was developed, which is based on sulphur deprivation resulting in a gradual inactivation of PSII reaction centres.

In *Chlamydomonas* cells, the electrons arrive to the hydrogenase via three different pathways:

- 1.) PSII-dependent pathway, which directly links water-splitting activity to H₂ evolution via the linear electron transport;
- 2.) PSII-independent pathway, where electrons derived from starch breakdown are transferred to the Fe-Fe hydrogenase via the PQ-pool and the PSI-mediated electron flow;
- 3.) dark fermentative pathway, where pyruvate is converted into acetyl-Coenzyme-A by a pyruvate ferredoxin oxidoreductase resulting in a reduced ferredoxin which, in the absence of light, may act as an electron donor to hydrogenase.

Ascorbate (vitamin C, Asc) is a metabolite with multifunctional roles in plants and can be found in all cell compartments. It plays roles in the elimination of reactive oxygen species, redox signalling, cell division, cell wall biosynthesis, regulation of gene expression and activity of certain enzymes and it also serves as a cofactor for violaxanthin deepoxidase. In my PhD work, we investigated a novel role of ascorbate in *Arabidopsis thaliana* plants and in the green algae *Chlamydomonas reinhardtii* cells.

Aims of the study

At the beginning of my PhD studies (2009), it was known that instead of water molecules, alternative electron donors provide electrons to PSII *in vivo* if the OEC is damaged by heat stress. Based on earlier data obtained *in vitro* we assumed that ascorbate is the alternative electron donor. Our aims were:

- to identify this alternative electron donor
- to investigate the physiological role of this alternative electron transport pathway

It is also known that oxygen production become partially inactivated upon sulphur deprivation in *C. reinhardtii* cells. The electrons derived from the

residual PSII activity and from degradation of endogenous substrates are channelled to the hydrogenase along the electron transport chain. It was our assumption that alternative electron donors may enhance the hydrogen production via stimulating the linear electron transport without oxygen production. To test this hypothesis we also aimed:

- to study the effect of ascorbate on electron transport and hydrogen production in *C. reinhardtii* cells

Materials and methods

Plant material and growth conditions

Wild-type (Columbia-0), Asc-deficient (*vtc2-1* and *vtc2-3*), and Asc-overproducing (*miox4*) *Arabidopsis thaliana* plants were grown in a growth chamber under short-day conditions (8-h light, 16-h dark), at approximately 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ in the light period. The temperature was kept between 18 °C and 24 °C. For the experiments 6-8 week old plants were used.

Point mutations in the *VTC2* gene encoding GDP-l-galactose phosphorylase enzyme drastically decrease the cellular ascorbate level in *A. thaliana* plants. In comparison to the wild-type, the ascorbate content decreased by approx. 85% and 50-70% in the *vtc2-1* and *vtc2-3* mutants, respectively. The leaves of *miox4*, a constitutive transgenic line of *A. thaliana* contain ascorbate at a 2-3 times higher concentration than the wild-type. The *MIOX4* gene encodes the myoinositol-oxygenase enzyme.

Chlamydomonas culture' growth conditions

We studied the involvement of ascorbate-dependent electron transport in the H₂ production of *Chlamydomonas reinhardtii*. The cells were maintained on Tris-acetate-phosphate (TAP) agar plates at a photon flux density of 40 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ and 24 °C in an algal growth chamber. For experiments *C. reinhardtii* cultures were grown in TAP media for 3 days at 80-100 $\mu\text{mol m}^{-2} \text{ s}^{-1}$

photon flux density at 24 °C under continuous shaking at 100 rpm. For sulphur deprivation, the cells were transferred to sulphur-free media by multiple centrifugations. The flasks, where hydrogen gas took place, contained 30 ml liquid culture with a chlorophyll concentration usually set to 8 µg/ml. The atmosphere in the flask was changed daily to nitrogen gas. The cultures were kept under the same conditions as the cultures grown in the presence of sulphur.

Applied treatments

- Heat and light treatments
- Chemical treatments

Applied methods

- Spectroscopic determination of chlorophyll content
- Spectroscopic and HPLC-based determination of ascorbate
- Starch content determination
- Fast chlorophyll-a fluorescence transient (OJIP) measurements
- Determination of ETR, NPQ, and qE fluorescence parameters
- Measurements of the oxidation-reduction kinetics of P₇₀₀
- Thermoluminescence measurements
- Measurements of flash-induced electrochromic absorbance transients (ΔA_{515})
- Determination of H₂ and O₂ production by gas chromatography
- *In vitro* hydrogenase activity measurements
- Western blot analysis

Results and discussion

Based on fast chlorophyll-a fluorescence transient measurements performed on wide-type and ascorbate-deficient (*vtc2-1*) *A. thaliana* leaves we concluded that if the OEC is inactivated, ascorbate can function as an alternative electron

donor to PSII *in vivo*. The rate of electron transfer to PSII depends on the ascorbate content of the leaves: The half-time of electron donation is approx. 25 ms in wild-type and 55 ms in ascorbate-deficient (*vtc2-1*) mutant plants. The rate of electron donation can be increased by incubating detached leaves in ascorbate solution. Thermoluminescence measurements revealed that ascorbate donates electrons to PSII via Tyr_Z⁺ either directly or indirectly. The ascorbate-dependent electron transport is also manifested in the 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-sensitive re-reduction kinetics of P₇₀₀. These observations prove that ascorbate donates electrons to PSII *in vivo* and is capable to maintain a continuous electron transport.

In order to completely inactivate the OEC, we applied a short heat pulse (48-50 °C, 40 s), but we could not detect ascorbate-dependent electron transport after moderate (39-40 °C, 15 min), physiologically relevant heat treatments, as well.

We have investigated the possible role of ascorbate in the protection against donor-side induced photoinhibition. Wild-type, ascorbate-deficient (*vtc2-3*) and ascorbate-overproducing (*miox4*) *A. thaliana* plants were exposed to 15 min 40 °C heat treatment followed by illumination at a photon flux density of 300 μmol photons m⁻² s⁻¹ and the extents of PSII inactivation were compared. We have shown that under these stress conditions the Tyr_Z-P₆₈₀⁺ electron transfer is decelerated in a few minutes, which is followed by the complete inactivation of PSII (approx. in one hour). Western blot analysis demonstrated that along with the D1 protein, the inner antenna protein complex of PSII, CP43 and the PsbO protein of OEC were also degraded at a similar rate. When *vtc2* leaves were incubated in the presence of diphenylcarbazide (DPC), an artificial PSII donor, the inactivation of PSII reaction centres was decelerated, corroborating our conclusion that alternative electron donors of PSII play an important role in photoprotection.

We have demonstrated that in *C. reinhardtii*, similarly to higher plants, ascorbate can support a linear electron transport in the presence of inactive OECs.

The fact that ascorbate can maintain electron transport without O₂ production raised the possibility of a potential biotechnological application.

Under normal physiological conditions the ascorbate content of *C. reinhardtii* cells is approx. 100 times lower than in plant cells – although it strongly increases under oxidative stress conditions. We have observed that upon sulphur deprivation the ascorbate content of the two algal cell lines examined (CC-124 and S-01) has dramatically increased. Furthermore, addition of exogenous ascorbate at a concentration of 10 mM during sulphur deprivation accelerated the rise of ascorbate content to its maximum, which was as high as in the absence of exogenous ascorbate, indicating that the ascorbate treatment we applied is physiologically relevant. Thermoluminescence measurements revealed that in addition to the role of ascorbate as an alternative electron donor, it also facilitates the development of anaerobiosis in the culture, most probably via the reduction of the Mn-complex of OEC.

The yields of photobiological H₂ production are different in the two examined *C. reinhardtii* cell lines, which may be explained by their different hydrogenase and respiration activities. Gas chromatography measurements have shown that in the absence of exogenous ascorbate the CC124 and S-01 cultures became anaerobic in 2 and 4 days, respectively, after the initiation of sulphur deprivation. Sulphur deprivation induced a more efficient H₂ production in CC124 than in S-01. The addition of ascorbate during sulphur deprivation stimulated the H₂ production in S-01 by suppressing the OEC activity and eliminating the inhibitory effect of O₂ and by donating electrons to PSII. In contrast, in CC124 cultures ascorbate decreases the yield of H₂ production, because ascorbate is a less efficient electron donor than water splitting.

Our results show that ascorbate may have an important regulatory effect on photobiological H₂ production; this raises the possibility of enhancing the H₂ production by regulating the activity of OEC and PSII via modulating the ascorbate biosynthetic pathways in *C. reinhardtii*.

Conclusions

Based on the experiments performed to identify the alternative electron donor we can conclude that:

- If the OEC is inactive, ascorbate provides electrons to PSII both in higher plants and *C. reinhardtii* green algae *in vivo*.
- Ascorbate donates electrons to PSII via Tyr_Z⁺.
- Ascorbate can maintain linear electron transport at a moderate rate.

Experiments performed to clarify the physiological role of ascorbate revealed that:

- As an alternative electron donor of PSII, ascorbate partially protects PSII against donor-side photoinhibition.

We have established that ascorbate plays a central role in hydrogen production of *C. reinhardtii*:

- In *C. reinhardtii* cells upon sulphur deprivation, the cellular ascorbate level increases significantly. Furthermore, the addition of 10 mM ascorbate during sulphur deprivation rises the ascorbate content to its maximum, which was as high as in the absence of exogenous ascorbate.
- Ascorbate induces anaerobiosis in the culture by reducing the Mn complex of OEC, while ascorbate also serves as an alternative electron donor of PSII.
- The difference between the photobiological hydrogen production of the two strains is attributed to their different hydrogenase and respiratory activities.

Publications

Articles which serve the basis for the thesis

Tóth SZ, Puthur JT, Nagy V, Garab G (2009) Experimental evidence for ascorbate-dependent electron transport in leaves with inactive oxygen-evolving complexes. *Plant Physiol.* 149: 1568–1578.
IF: 6.235

Tóth SZ, Nagy V, Puthur JT, Kovács L, Garab G (2011) The physiological role of ascorbate as photosystem II electron donor: protection against photoinactivation in heat-stressed leaves. *Plant Physiol.* 156: 382–392.
IF: 6.535

Nagy V, Tengölics R, Schansker G, Rákhely G, Kovács L K, Garab G, Tóth SZ (2012) Stimulatory effect of ascorbate, the alternative electron donor of photosystem II, on the hydrogen production of *Chlamydomonas reinhardtii*. *Int. J. Hydrogen Energy* 37: 8864–8871.
IF: 3.548

Nagy V, Vidal-Meireles A, Tengölics R, Rákhely G, Garab G, Kovács L, Tóth SZ Ascorbate accumulation during sulphur deprivation of the green alga *Chlamydomonas reinhardtii* and its effects on photosystem II activity and H₂ photoproduction (in preparation).

Oral presentations

Nagy V: Improvement of biohydrogen production in *Chlamydomonas reinhardtii* by ascorbate as an alternative electron donor of PSII. Environmental Effect on Photosynthesis, International Workshop, Nyíregyháza, 2010

Nagy V: A második fotokémiai rendszer alternatív elektrondonora és lehetséges alkalmazása zöldalgák hidrogéntermelésének serkentésére. Szegedi Tudományegyetem Sófi József a Szegedi Tehetségekért Alapítvány Ösztöndíj Konferencia, Szeged, 2011

Nagy V: Aszkorbát-függő alternatív elektrontranszport biotechnológiai alkalmazása a hidrogéntermelésben. Szegedi Tudományegyetem Sófi József a Szegedi Tehetségekért Alapítvány Ösztöndíj Konferencia, Szeged, 2012

Nagy V: Aszkorbát- függő alternatív elektrontranszport és biotechnológiai alkalmazása a hidrogéntermelésben. Szegedi Biológus Doktorandusz Konferencia, Szeged, 2012

Conference posters

Tóth SZ, Nagy V, Puthur JT, Kovács L, Garab G: The physiological role of ascorbate as photosystem II electron donor: protection against photoinactivation in heat-stressed leaves. 4th International Symposium of the SFB 429, Potsdam, 2010

Nagy V, Puthur JT, Kovács L, Garab G, Tóth SZ: Az aszkorbát élettani szerepe a második fotokémiai rendszer alternatív elektrondonoraként. 40. Membrán-Transzport Konferencia, Sümeg, 2010

Nagy V, Tóth SZ, Latinovics D, Kovács LK, Garab G: A második fotokémiai rendszer alternatív elektrondonorainak hatása a hidrogéntermelésre *Chlamydomonas reinhardtii* sejtekben. A Magyar Növénybiológiai Társaság X. Kongresszusa, Szeged, 2011

Nagy V, Tengölics R, Schansker G, Rákhely G, Kovács LK, Garab G, Tóth SZ: Az aszkorbát a második fotokémiai rendszer alternatív elektrondonora és lehetséges alkalmazása zöldalgák hidrogéntermelésének serkentésére. 42. Membrán-Transzport Konferencia, Sümeg, 2012

Tóth SZ, Nagy V, Tengölics R, Schansker G, Rákhely G, Kovács LK, Garab G: Novel role of ascorbate in the photosynthetic electrontransport. Physiological significance and potential biotechnological application. 75th Anniversary of Albert Szent-Györgyi's Nobel Prize Award, Szeged, 2012

Nagy V, Tóth SZ, Tengölics R, Schansker G, Rákhely G, Kovács LK, Garab G: Ascorbate, as alternative electron donor to photosystem II, protects plants against photoinhibition and stimulates the photoproduction of hydrogen in green algae. „Our future”, Keszthely, 2013

Nagy V, Tengölics R, Schansker G, Rákhely G, Kovács LK, Garab G, Tóth SZ: Ascorbate, as alternative electron donor to photosystem II, protects plants against photoinhibition and stimulates the photoproduction of hydrogen in green algae. 10th International Hydrogenase Conference, Szeged, 2013