Summary of the PhD Thesis

THE EFFECT OF NITRIC OXIDE ON THE PHOTOSYNTHETIC ELECTRON TRANSPORT OF PEA LEAVES

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INTRODUCTION AND AIMS OF THE STUDY

Plants, as well as animals, respond to ambient levels of nitric oxide (NO), and also generate NO themselves via various enzymatic and non-enzymatic pathways. Indeed, in the past years, a growing body of research has provided evidence for the multiple physiological roles of this gaseous free radical in plants including its involvement in metabolic and developmental processes as well as responses to various biotic and abiotic stressors.

Its small size and rapid turnover make NO an ideal signalling molecule. A key reason for its versatility is its interaction with calcium as well as other intracellular signalling molecules: NO readily reacts with molecular oxygen, hydrogen peroxide, superoxide and also thiol- and metal-containing proteins. Photosynthetic and mitochondrial electron transport chains are abundant in transition metal-containing complexes and previous *in vitro* and *in vivo* experiments suggest that NO modulates the photosynthetic processes and apparatus of plants. Electron paramagnetic resonance and chlorophyll fluorescence measurements on isolated thylakoid membrane complexes treated with NO gas have clearly demonstrated that NO can reversibly bind to several sites in PSII and inhibit electron transfer. However, experiments carried out on isolated chloroplasts and intact leaves using different NO donor molecules report contradicting effects on fluorescence induction parameters.

NO donors are widely implemented tools for investigating the biological roles of NO, but the diverse chemical properties of donors potentially lead to differences in NO yield and the release of other reactive agents. These factors, together with difficulties of direct measurement of NO especially in an *in vivo* setting, may well account for these conflicting results.

Using three NO donor molecules (SNAP, SNP and GSNO) and two scavenger chemicals (PTIO and Hb), the purpose of this work is to examine the effect of exogenously applied NO on the photosynthetic electron transport of pea leaf disks. Our aim was to resolve previous contradictory results concerning the effect of NO on photosynthetic electron transport and to identify *in vivo* target sites of NO by chlorophyll fluorescence. The additional goal was to investigate the potential effect of NO on PSI photochemistry. The particular goals of this work are summarized by the following points:

1, NO donors applied in this work are characterised by diverse chemical properties. Are there differences in the NO yield of these donor molecules under our experimental settings? Do other photolytical degradation products or the oxidative degradation products of NO itself (nitrite and nitrate ions) influence our measurements by interfering with specific NO effects?

2, EPR measurements on isolated thylakoid membrane complexes have shown that NO inhibits electron transfer processes in PSII and have identified its target sites, such as the nonheme iron between the Q_A and Q_B binding sites, the $Y_{D\cdot}$ tyrosine residue of the D2 protein, and the Mn cluster of the water-oxidizing complex. Can these effects be reproduced and the target sites identified *in vivo*?

3, Previous work on the effect of exogenous NO on photosynthetic parameters carried out on isolated chloroplasts and intact leaves using different NO donors has yielded conflicting results. In isolated thylakoid membranes, SNAP does not modify Fv/Fm values, but inhibits ΔpH formation across the thylakoid membrane. In intact leaves, however, SNP decreases Fv/Fm values, but does not modify ΔpH-dependent (NPQ). To what extent are these differences due to NO donors and differences in experimental settings? With that resolved what are the direct effects of exogenously applied NO on photosynthetic parameters?

4, The effect of NO on the electron transfer processes of PSI has not yet been addressed. Does NO modify PSI photochemistry?

MATERIALS AND METHODS

Plant material: 15 mm leaf disks of the youngest fully expanded leaves of two-week-old *Pisum sativum* L. cv Petit Provençal plants.

Treatment: prior to measurements, leaf disks were individually incubated for 2 h under 150 µmol m^{-2} s^{-1} PFD white light in covered Petri dishes containing 4 ml distilled water as control, or 4 ml of different dilutions of NO donor molecules and scavenger chemicals in aqueous solution. The leaf disks were then dark adapted for 15 min.

The concentration of NO was measured amperometrically using a NO electrode (ISO-NOP, World Precision Instruments Inc., Sarasota, FL, USA).

Q_A^- reoxidation was determined from flash-induced decay of chlorophyll fluorescence yield monitored by a double-modulation fluorometer (PSI, Brno, Czech Republic).

Fluorescence induction parameters were obtained from chlorophyll fluorescence of pea leaves monitored by a PAM fluorometer (PAM-2000, Heinz Walz GmbH, Effeltrich, Germany).

PSI photochemistry was measured by monitoring P700 absorbance using a Dual-PAM-100 P700 and chlorophyll fluorescence measuring system (Walz, Effeltrich, Germany) and a PAM fluorometer (PAM-101, Walz, Effeltrich, Germany) equipped with a dual-wavelength emitter-detector unit (ED-P700DW, Walz).

RESULTS AND DISCUSSION

1. NO donor molecules release different amounts of NO

Prior to examining the effect of our three NO donor molecules on photosynthetic electron transport we studied the NO emission characteristics of the NO donors under our experimental settings and checked whether their degradation products, or the oxidative degradation products of NO influence the photosynthetic parameters of the leaf disks.

The SNAP solution produced the least amount of NO throughout the two-hour incubation and, accordingly, failed to cause significant changes in the photosynthetic parameters of leaf disks, therefore SNAP is an unsuitable donor under our conditions.

The concentration of NO in the solutions of the other two donors – SNP and GSNO – is significant even after two hours of illumination and this NO yield is effectively quenched by both NO scavengers. In order to prove the NO specificity in the effects caused by these donors we examined the potential effects of nitrite and nitrate, and GSSG formed during the degradation of GSNO. Neither the nitrite and nitrate containing solutions, nor GSSG influenced the F_v/F_m, qP, and NPQ parameters of the leaf disks, but the SNP-induced changes are partly due to cyanide released simultaneously with NO during the photolysis of SNP. The effects of GSNO, on the other hand, are mediated exclusively by NO.

These findings highlight the importance of the correct choice of NO donor and justify the use of GSNO to study the effects of NO on photosynthetic electron transport *in vivo*.

2. NO causes donor and acceptor side inhibition of PSII electron transport *in vivo*

We identified the target sites of NO in PSII by measuring Q_A^- reoxidation kinetics of leaf disks treated with GSNO. GSNO treatment increased the amplitude and the time constant of the middle phase of the fluorescence decay curve, which indicates that NO reduces the rate of electron transfer between Q_A és Q_B providing circumstantial evidence in support for the competitive binding of NO to the non-heme iron (II) *in vivo*. In the presence of DCMU, GSNO prevents the total decay of fluorescence, which indicates that Q_A^- is unable to recombine with the S_2 state, possibly because NO inactivates or reduces either the Mn cluster, or the $Y_D^{\cdot}$ residue. These findings suggest that NO inhibits electron transfer on both donor and acceptor sides in PSII in intact leaves.

Kautsky-curve kinetics also confirm significant donor and acceptor side inhibition of electron transport: GSNO-treatment causes an F_I increase and a significant decrease in F_P and the maximal slope of és F_I-F_P section of the Kautsky-curve.

3. NO modifies chlorophyll fluorescence parameters

Chlorophyll fluorescence measurements reveal that GSNO treatment decreases the F_v/F_m and qP values. These results are in good correlation with fluorescence decay kinetics measurements and demonstrate that NO increases the proportion of closed PSII reaction centers through the accumulation of Q_A^- molecules. NO also reduces NPQ values indicating a reduced flow of electrons in the intersystem chain. Taken together, these data provide strong *in vivo* evidence that a partial inhibition of PSII by NO is indeed the cause of impaired steady-state electron transport *in vivo*. Besides direct inhibition, indirect effects cannot be ruled out: NO may inhibit Rubisco activation and activity; in addition, the reaction of NO with superoxide leads to peroxynitrite, which may also impair photosynthetic electron transport as well as damaging tylakoid membranes.

In addition, GSNO changed the length and amplitude of an NPQ transient, which arises upon the onset of illumination of dark-adapted leaves at low light intensities, and it is relaxed rapidly after a few minutes of illumination. NO might bring about such an increase through stimulating cyclic electron transport or delaying the activation of the Calvin cycle.

4. NO moderately enhances PSI photochemistry

Although NO failed to modify Φ_{PSI} values, it slightly increased the number of electrons in the intersystem chain probably by stimulating PSI cyclic electron transport.

In summary, data gathered in this work provide *in vivo* confirmation of the existence of target sites of NO in PSII and further evidence on the inhibitory effect of NO on PSII electron transport in intact leaves. We trust that this work inspires new questions and ignites further research to explore the perspective of NO as a potential regulator of photosynthetic electron transport yet to be discovered.

LIST OF PUBLICATIONS

(* publications directly related to the subject of the thesis)

Research articles published in peer-reviewed journals:

Hertel B, Horváth F, **Wodala B,** Hurst A, Moroni A, Thiel G. (2005) KAT1 inactivates at sub-millimolar concentrations of external potassium. *Journal of Experimental Botany*. **56:** 3103-10. IF: 3,336

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***Wodala B,** Deák Z, Vass I, Erdei L, Altorjay I, Horváth F. (2008) Involvement of NO in the regulation of photosynthetic electron transport *in vivo* studied by chlorophyll fluorescence measurements. *Plant Physiology*. **146:** 1920-7. IF: 6,367

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Horváth F, **Wodala B,** Erdei L, Moroni A, Van Etten J, Thiel G. (2002) pH-dependent regulation of a potassium channel protein encoded by a *Chlorella* virus PBCV-1. *Acta Biologica Szegediensis.* **46:** 21-2.

Ördög A, **Wodala B,** Horváth F. (2008) Investigating the role of potassium channel KAT1 in NO mediated stomatal closure. *Acta Biologica Szegediensis.* **52:** 163-5.

***Wodala B,** Deák Z, Vass I, Erdei L, Horváth F. (2005) Nitric oxide modifies photosynthetic electron transport in pea leaves. *Acta Biologica Szegediensis.* **49:** 7-8.

***Wodala B,** Horváth F. (2008) The effect of exogenous NO on PSI photochemistry in inact pea leaves. *Acta Biologica Szegediensis.* **52:** 243-5.

Posters presented at international conferences

***Wodala B,** Deák Z, Vass I, Erdei L, Horváth F. (2005) Nitric oxide targets photosynthetic electron transport in pea leaves. "Photosynthesis and Stress" Central European Conference on Biophysical and Biochemical Methods in Photosyntehsis Research, 2005. szeptember 15-16., Brno, Csehország.

***Wodala B,** Horváth F, Deák Z, Vass I, Erdei L. (2006) Nitric oxide hinders photosynthetic electron transport in pea leaves. XV. FESPB Congress, 2006. július 17-21., Lyon, Franciaország.

Lectures presented at international conferences

Wodala B, Horváth F, Erdei L, Thiel G. (2005) pH-dependent regulation of the virus-encoded miniature potassium channel, Kcv. 5th International Conference of PhD Students, 2005. augusztus 14-20., Miskolc, Magyarország

Wodala B, Horváth F, Moroni A, Thiel G. (2003) pH-dependent regulation of potassium channel protein encoded by Chlorella virus, PBCV-1. XXXIII. Membrán-transzport konferencia, 2003. május 20-23., Sümeg, Magyarország.