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Targets of nitric oxide (NO) during modulation of photosystems in pea mesophyll protoplasts: studies using chlorophyll *a* fluorescence

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Abstract

Plants adapt to the environmental stresses by using the signalling molecules, such as H_2O_2 and nitric oxide (NO). We have assessed the NO-induced changes by using sodium nitroprusside (SNP, a NO donor) in photosynthetic components in protoplasts of pea (*Pisum sativum*) by measuring chlorophyll (Chl) *a* fluorescence induction kinetics. On exposure to SNP during preillumination, their photochemical activities were severely affected. There was a decrease of 40% in O_2 evolution by 5 min and complete inhibition by 20 min. The patterns of photosynthesis in absence of NO did not vary much over 20 min. Chl *a* fluorescence transient analysis and the energy pipeline models demonstrated that NO decreased the rates and efficiency of photochemical reactions. Presence of NO lowered the photosynthetic performance index and increased the dissipation per reaction centre with the time. Our results suggest that NO inactivates the PSII by targeting both the donor and acceptor sides of PSII.

Additional key words: energy pipeline model; OJIP transients; PSII efficiency.

Introduction

Chl *a* fluorescence analysis is widely used technique for both basic and ecophysiological studies on 'health status' of the plant (Papageorgiou and Govindjee 2004). In particular, fluorescence emission gives valuable information about the extent of damage and the plants photosynthetic ability to adapt against the stress (Baker 2008). The fast transient analysis of Chl *a* fluorescence is a simple, sensitive, noninvasive, and highly reliable tool for rapid measurement of chloroplast functionality, particularly of PSII (Lazár 2006, Stirbet and Govindjee 2011, Kalaji *et al*. 2016).

The induction kinetics of dark-adapted photosynthetic cells demonstrate several inflection points (O, J, I, and P) after exposing to actinic light with time, which are popularly known as OJIP transients (Kautsky *et al.* 1960). These transients correspond to the redox state of the photosystems and reflect changes in photosynthetic electron transport chain. These parameters interpret the important indicators of PSII characteristics, such as energy trapping, electron transport kinetics (Strasser *et al*. 2004, Stirbet and Govindjee 2011). The OJIP transients can be analysed using JIP-test (Strasser and Strasser 1995), which allows us to conceive the PSII functionality by translating the fluorescence transient measurements into several phenomenological and biophysical expressions.

Being the key cellular sites of carbon and nitrogen metabolism, chloroplasts are also involved in production of not only reactive oxygen species (ROS), but also nitric oxide (NO). It has been reported earlier that the generation of not only ROS, but also NO affects the process of photosynthesis (Asada 2006, Jasid *et al.* 2006, Gas *et al*. 2009). Both ROS and NO play a major role in signalling, but they can also potentially affect and regulate cellular metabolism (Møller *et al*. 2007, Hayat *et al*. 2010).

NO is a free gaseous radical, involved in several physiological processes, including photosynthesis. In biological systems, NO is capable of target and modulate the activity of proteins by posttranslational modifications (PTM), such as nitrosylation (Besson-Bard *et al*. 2008, Astier and Lindermayr 2012). The light reactions and Calvin cycle enzymes of photosynthesis have transition metal containing complexes regulated by a number of PTMs driven by NO. This can affect the efficiency of chloroplast metabolism and thus photosynthesis (Foyer and Noctor

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Abbreviations: Chl – chlorophyll; F_v/F_m – maximum quantum yield of PSII; NPQ – nonphotochemical quenching; RC – reaction center; Q_A – primary quinone acceptor; Q_B – secondary quinone acceptor of PSII; PI_{ABS} – performance index based on absorption of light energy.
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2009, Pfannschmidt *et al*. 2009). Exposure to NO or SNP decreased the efficiency of PSII and related fluorescence parameters of thylakoid membranes (Vladkova *et al*. 2011) and pea leaves (Wodala *et al*. 2008). In contrast, exogenous application of SNP could alleviate the decrease of leaf photosynthesis caused by different stress factors, such as heat (Chen *et al*. 2013), salt (Jahan *et al*. 2019), water deficiency (Shao *et al*. 2018), and chromium (Huang *et al*. 2018). It is therefore necessary to re-examine the responses to NO of photosynthetic machinery.

Any disturbance in the environment causes an immediate perturbation of photosynthesis, along with alterations in the chloroplast structure (Eberhard *et al*. 2008, Takahashi and Murata 2008). There have been reports that NO modulates the photosynthetic process in plants and that NO can reversibly bind to PSII and inhibit electron transfer (Schansker *et al*. 2002, Wodala *et al*. 2008). However, such experiments on isolated chloroplasts or intact leaves using NO donors have been contradictory (Hayat *et al*. 2010). Further, the effects of SNP (a NO donor) in the mesophyll protoplasts have not been investigated yet.

In the present study, we attempted to identify possible targets of NO in protoplasts by studying changes in photosynthetic electron transport components using *HandyPEA*. We treated the protoplasts of pea with or without sodium nitroprusside (SNP) for specific period, up to 20 min, and their photosynthetic activities were monitored, by determining O_2 evolution, as well as chlorophyll *a* fluorescence transients. We tried to assess the impact of elevated NO on PSII photochemistry of pea protoplasts.

Materials and methods

Plant growth: Pea (*Pisum sativum* L., cv. Arkel) plants were raised from seeds (*Qualitas Crop Sciences*, Hyderabad). Plants were raised in plastic trays on soil supplemented with manure (3:1) and were kept in a greenhouse (12-h photoperiod, average day/night temperature of 30/25°C). Second to fourth pair of fully expanded leaves from top were excised from 2- to 3-week-old plants and used for protoplasts isolation (Devi *et al*. 1992).

Mesophyll cell protoplasts (MCP): The experimental material was mesophyll protoplasts isolated from leaves of pea plants. The abaxial epidermis of pea leaves was stripped-off and subjected to digestion with a mixture of 2% (w/v) *Cellulase Onozuka R-10* and 0.2% (w/v) *Macerozyme R-10* (*Yakult Pharmaceuticals*, Japan). Further details of protoplast isolation and resuspension were as already described by Saradadevi and Raghavendra (1992) and Sunil *et al*. (2008). The protoplasts in suspension medium were kept stored on ice. Chl was estimated by extracting the protoplasts into 80% acetone (Arnon 1949).

Photosynthetic O_2 evolution: The carbon fixation capacity of protoplasts was monitored as bicarbonate-dependent O_2 evolution. The photosynthetic rates were measured by Clark type O_2 electrode (*Hansatech Instruments*, King's Lynn, UK). An aliquot of mesophyll protoplasts [10 µg(Chl) ml^{-1}] was added to oxygraph chamber containing 1 ml of reaction medium (0.4 M sorbitol, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ in 10 mM Hepes-KOH, pH 7.5). Light [700 µmol(photon) m^{-2} s^{-1}] was provided by slide projector (*Philips* lamp, 24 V/150 W). Air-saturated water (at 25°C) was used to calibrate the O_2 content in the electrode chamber.

Treatment of protoplasts with SNP: Protoplasts were illuminated at two different light intensities (at 50 or 150 µmol(photon) m^{-2} s^{-1} for various time periods. Protoplasts incubated in darkness at 25°C for 5 min were taken as control samples. SNP (*Sisco Research Laboratories*, Mumbai, India) at 100 µM was added to the reaction medium during incubation in light or darkness. The concentration of SNP on photosynthesis of protoplasts was earlier standardized in our laboratory, to have the maximum effect on photosynthesis of protoplasts. After stipulated time points of illumination (ranging from 0–20 min), protoplasts were taken out and their photosynthetic rates were monitored.

Fast Chl *a* fluorescence induction kinetics: The effect of NO on the electron transport of PSII in mesophyll protoplast was determined by monitoring the polyphasic rise of Chl *a* fluorescence transients of SNP-treated protoplasts for different time points. Chl *a* fluorescence (OJIP) transients exhibited by protoplasts were monitored by plant efficiency analyzer (*Handy PEA* fluorimeter, *Hansatech Instruments*, UK). Protoplasts were preilluminated [50 µmol(photon) m^{-2} s^{-1}] with or without 100 µM SNP, on an orbital shaker (120 rpm) for 0 to 20 min. Protoplasts incubated in dark were considered as control samples. After specified time points, samples were dark-adapted for 5 min and then Chl *a* fluorescence transient patterns were examined. The fluorescence kinetics (F_O to F_m) were recorded from 10 µs to 1 s. The OJIP fast transients were analyzed according to the JIP-test (Strasser and Strasser 1995). From the OJIP values, we calculated various energy fluxes and their ratios, such as absorption per RC or CS and the performance index (PI_{ABS}). The flux parameters are described in Strasser *et al*. (2004) and Rapacz *et al*. (2019) and shown in Appendix. Quantum efficiency of PSII (F_v/F_m) was determined from the *Handy PEA* (Strasser *et al*. 2004).

Chemicals: All the other chemicals (analytical grade) were procured from *Sisco Research Laboratories*, *LobaChemie*, *Himedia Laboratories* or *Qualigens*, all from Mumbai, India.

Statistical analysis: The data presented are mean (± SE) of three experiments, on different days. The statistical significance was tested by one-way analysis of variance (*ANOVA*), using *SigmaPlot* software *version 11.0*, with a significance threshold (P≤0.05).

Results

The changes induced by SNP in the Chl fluorescence decay kinetics were studied to identify the targets of NO in the photochemical machinery.

Time course analysis of NO effect on photosynthetic oxygen evolution: The sensitivity of the photosynthesis in absence of NO did not vary much (≤ 10%) over the time period up to 20 min (Fig. 1). In contrast, protoplasts preilluminated with SNP showed severe inhibition of photosynthetic performance in a time-dependent manner. It could be observed that there was about 40% inhibition in rate of O_2 evolution with 5-min SNP treatment and was completely inhibited by 20 min. Protoplasts exposed to SNP in dark were similar to those without SNP. The varying light intensities [50 or 150 μmol(photon) m^{-2} s^{-1}] during preillumination did not much affect the photosynthetic inhibition pattern, indicating that NO release from SNP did not require high light intensity (Fig. 1). Further, preillumination experiments with SNP was restricted to a low light intensity of 50 μmol(photon) m^{-2} s^{-1}.

Chl *a* fluorescence transients of protoplasts treated with SNP: The fluorescence kinetics obtained with protoplasts (Fig. 2) was typical of higher plant leaves displaying three phases of the fluorescence rise from F_O to F_P (~ F_M). Elevated concentrations of NO in protoplasts showed significant quenching of fluorescence intensity in the OJIP transients, in a time-dependent manner when compared to control protoplasts. The fluorescence yield at J phase slightly decreased with increasing time of NO exposure, whereas points I and P showed considerable decrease in fluorescence yield (Fig. 3).

NO decreased the maximal quantum efficiency (F_v/F_m): In order to evaluate the effect of NO on the photochemical efficiency of protoplasts, the F_v/F_m values were monitored over time with SNP treatment under dark/light conditions. The F_v/F_m values indicate the photochemical efficiency of PSII and in turn photochemistry. The control protoplasts showed an F_v/F_m value of 0.755. The SNP-treated protoplasts in dark did not show any significant change in the F_v/F_m values with time (Fig. 4). However, in presence of 100 μM SNP, protoplasts that were preilluminated showed a significant decrease in the F_v/F_m values with increase in NO exposure time (0 to 20 min), when compared to SNP-dark-treated protoplasts.

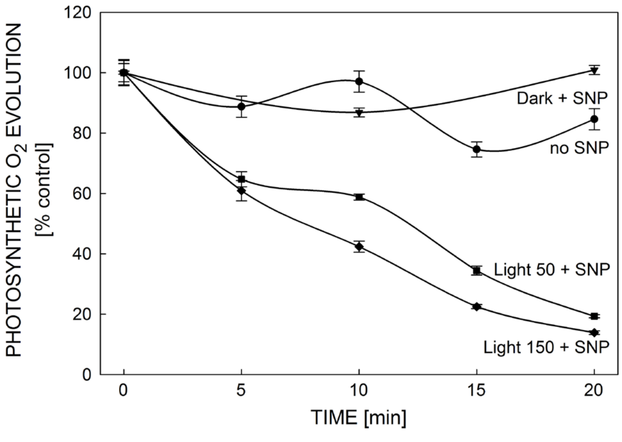

Fig. 1. Patterns of photosynthesis, with time in mesophyll protoplasts of pea, treated with or without 100 μM SNP. The protoplasts were preincubated with SNP either in dark or under a light of 50 (Light 50) or 150 (Light 150) μmol(photon) m^{-2} s^{-1}. Aliquots were examined at specified time points for their rate of O_2 evolution. The rate of photosynthesis in the absence of SNP (control) at the beginning, *i.e.*, zero time was 132 μmol(O_2) mg^{-1}(Chl) h^{-1}. The photosynthetic rates within 5 min of illumination were considered. Results correspond to the mean ± SE of at least three experiments.

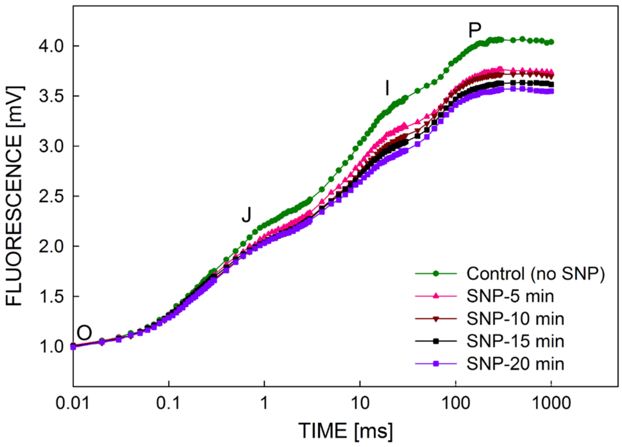

Fig. 2. Original traces of Chl *a* fluorescence (OJIP) recorded in mesophyll protoplasts of pea treated with 100 μM SNP under an illumination of 50 μmol(photon) m^{-2} s^{-1} at different time points. After a dark preadaptation (5 min), the OJIP transients were induced by a PPFD of 3,000 μmol(photon) m^{-2} s^{-1} with a *HandyPEA* (*Hansatech*). Fluorescence intensity is normalized to the value at the F_O. These readings are means from three independent experiments done on a particular day and experiments were repeated at least on two different days with similar results.

Effect of SNP on the parameters derived from JIP-test: We analyzed the fluorescence transients depicted in Fig. 2 using JIP-test to further evaluate the effect of NO on the structural and functional parameters quantifying the photosynthetic behavior of the pea protoplasts samples. Fig. 5 shows the radar plot of the parameters derived from JIP-test, depicting the specific (ABS/RC, TR_0/RC, ET_0/RC) and phenomenological fluxes (ABS/CS_0, TR_0/CS_0, ET_0/CS_0) in SNP-treated protoplasts with time; keeping the untreated protoplasts as control. For the control samples, a reference value (= 1) was given and the relative SNP-induced changes were plotted on the radar plot. These parameters are listed in Appendix.

Our results show that the parameters related to specific fluxes per active PSII reaction centre were unaltered, when the protoplasts were preilluminated with SNP. There was no significant change in trapping (TR_0/RC) and electron transport (ET_0/RC) with SNP treatment (Fig. 5). However, the absorption (ABS/RC) and dissipation (DI_0/RC) showed a marginal increase with SNP treatment. In contrast, the phenomenological fluxes, such as ABS/CS_m, ET_0/CS_m, TR_0/CS_m, besides density of the active PSII reaction centres (RC/CS), exhibited a steep decrease with NO exposure time (Fig. 5).

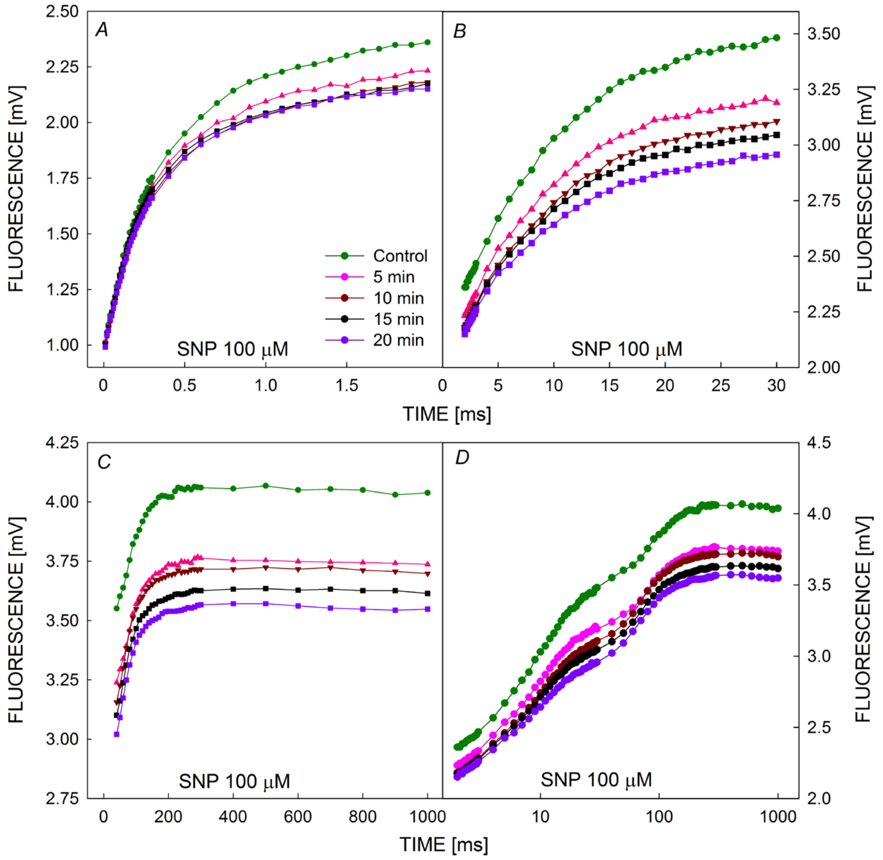

Fig. 3. The OJIP fluorescence kinetics of the mesophyll protoplasts of pea, preilluminated with SNP for different time points. (*A*) O–J phase, (*B*) J–I phase, (*C*) I–P phase, and (*D*) J–P phase. Normalization at F_O (stand for fluorescence yield at 20 µs) was done. These readings are means from three independent experiments done on a particular day and experiments were repeated at least on two different days with similar results.

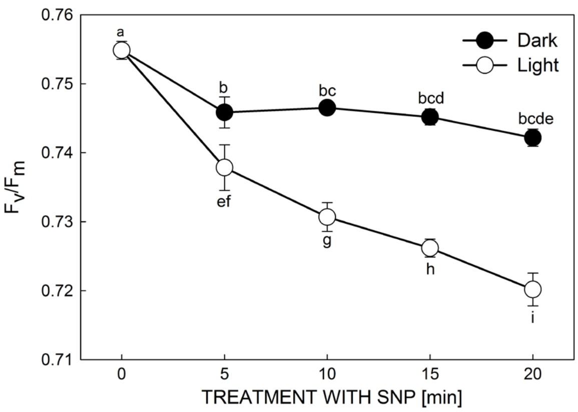

Fig. 4. Changes in the quantum yield of photosynthetic electron transport (F_v/F_m) in mesophyll protoplasts of pea treated with 100 µM SNP either in light [50 µmol(photon) m^{-2} s^{-1}] or dark for different time points. All the samples were dark adapted (5 min) before taking the readings. Each value is a mean ± SE of three independent experiments, done with at least two samples for each measurement on a particular day. *Different letters* indicate statistically significant difference as calculated by *ANOVA* test, $P<0.05$.

The performance index (PI) is a popular JIP-test parameter that provides quantitative information on the plant status under stress. PI_{ABS} and its component RC/ABS (efficiency of light absorption) decreased with exposure of protoplasts to SNP in light (Fig. 5). The PI_{CSM} (*i.e.*, performance index on cross section basis) also showed significant decrease with the NO treatment in time-dependent manner.

The phenomenological energy fluxes per cross section can be plotted as energy pipeline leaf model. SNP-treated protoplasts showed a decrease in absorption, trapping, and electron transport at cross section level when compared to control protoplasts (Fig. 6). On exposure to NO, the reaction centres were inactivated (indicated as *closed circles*). There was a decrease in ET_0/CS_m (*blue arrow*), ABS/CS_m (*yellow arrow*), and TR_0/CS_m (*cyan arrow*) with increase in NO exposure time (Fig. 6).

Discussion

The present work attempts to identify the target sites of NO in the photosynthetic electron transport in protoplasts using Chl *a* fluorescence. Experiments were carried out for time course analysis of photosynthetic performance after SNP treatment.

NO inhibits photosynthesis in a time-dependent manner: NO released by exogenous application of SNP during preillumination severely affected the photosynthetic performance of the protoplasts in a time-dependent manner (Fig. 1). The release of NO from SNP is light dependent but did not require high light intensity. Yet, the interpretation of SNP-mediated effects should be carefully considered, because there has been a caution that SNP may also release CN^-, besides NO and therefore SNP should be used with

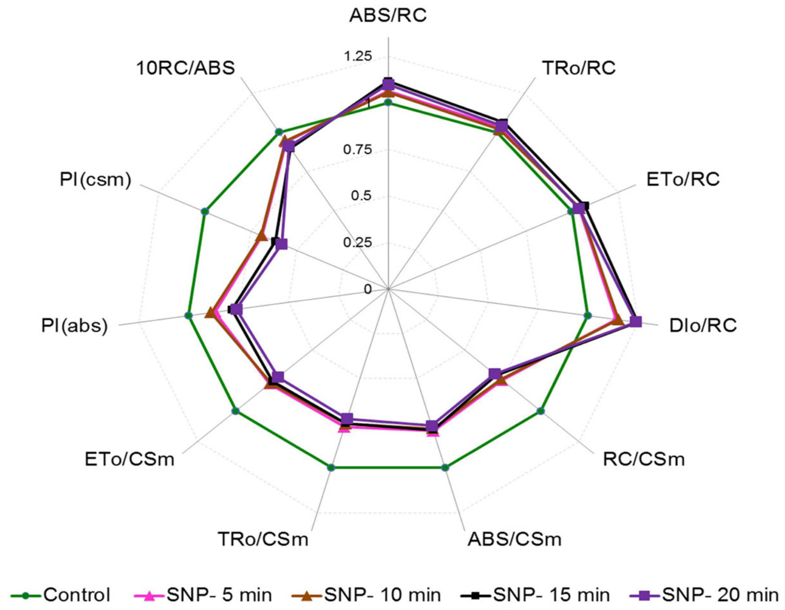

Fig. 5. Radar plot of specific fluxes (ABS/RC, TR_0/RC, ET_0/RC), phenomenological fluxes (ABS/CS, TR_0/CS, ET_0/CS) and the performance indices, derived by the JIP-test from OJIP transients of Fig. 2. The values are calculated relative to the control (without SNP), taken as 1. The flux parameters are described in Appendix.

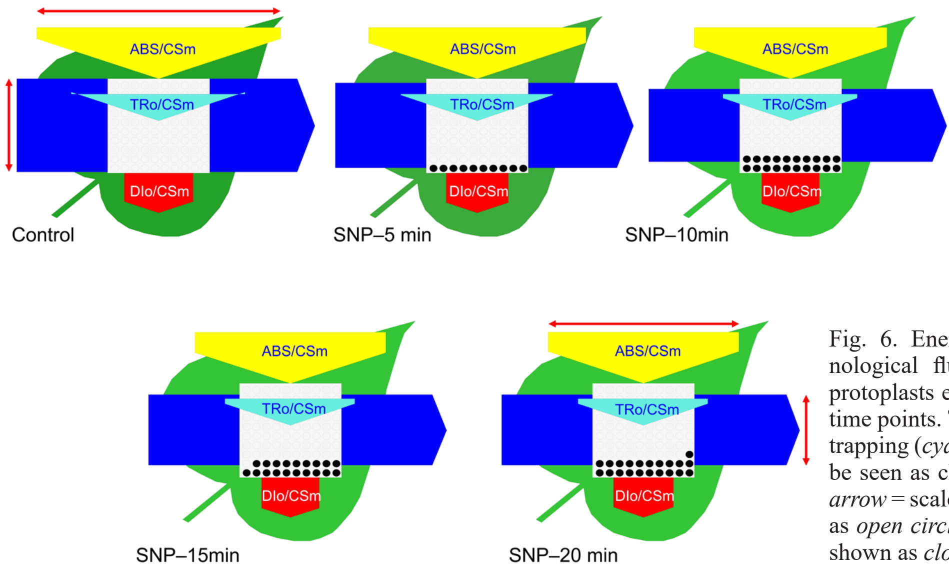

Fig. 6. Energy pipeline models of phenomenological fluxes (per cross section, CS_m) in protoplasts exposed to 100 µM SNP for various time points. The decrease in absorption (*yellow*), trapping (*cyan*) and electron transport (*blue*), can be seen as changes in width of each arrow (*red arrow* = scale). Active reaction centers are shown as *open circles* and inactive reactive centers are shown as *closed circles*.

diligence (Feelisch 1998, Lum *et al*. 2005). However, we are confident about the use of SNP, as in our lab, the SNP-induced stomatal closure can be reversed by specific NO scavenger 2-(4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) (Gayatri *et al.* 2017, Agurla *et al.* 2018).

Effect of NO on Chl fluorescence parameters: The Chl *a* fluorescence pattern with OJIP transients, seen in the control protoplasts, was similar to that described for plants, green algae, and cyanobacteria (Sunil *et al*. 2008, Stirbet and Govindjee 2011). The marked change in the shape of Chl *a* fluorescence induction curve with NO reflects alterations in PSII photochemistry (Fig. 2).

Fig. 3*A* shows a marginal decrease in fluorescence at O–J phase with SNP treatment, suggesting the over-reduction of Q_A to Q_A^- (Schansker *et al*. 2005). This reflects in the increase of dissipation due to decrease in e^- trapping efficiency. In J–I phase (representing reduction of PQ pool), the fluorescence was significantly quenched (Fig. 3*B*) indicating the reduction in flow of electrons from Q_A to Q_B. The decrease in the relative amplitude of the I–P phase, with increasing NO exposure (Fig. 3*C*) reflects the electron flow through cytochrome b_6f (Tsimilli-Michael and Strasser 2008, Yusuf *et al.* 2010).

Previous studies using isolated thylakoid membranes and leaves have demonstrated that the possible targets of NO are the nonheme iron (Diner and Petrouleas 1990), the Tyr residue of D2 (Sanakis *et al*. 1997), and manganese (Mn) cluster of PSII (Schansker *et al*. 2002). The decrease in fluorescence at J, I, and P phases (Figs. 2, 3) in the transient can be attributed to an inhibition of electron flow at donor side of PSII and it may also be due to a decrease in the pool size of Q_A.

NO affects the photochemical efficiency of protoplasts: Changes in F_v/F_m values indicate modulation of photosynthetic performance. There have been contradictory results with NO on Chl fluorescence studies. NO derived from SNAP did not affect F_v/F_m in isolated chloroplasts but reversibly inhibited the linear electron transport, light-induced ΔpH across thylakoid membrane, and decreased the rate of ATP synthesis (Takahashi and Yamasaki 2002). In contrast, Yang *et al*. (2004) showed that in intact leaves of potato, SNP reduced F_v/F_m but did not cause any change in ΔpH-dependent NPQ. In intact pea leaves, S-nitrosoglutathione (GSNO) caused a significant decrease in F_v/F_m value and decreased steady-state photochemical quenching, q_P (Wodala *et al*. 2008). These conflicting results are probably due to differences in the chemical properties of NO donor molecules besides experimental systems and conditions (Wodala *et al*. 2008). However, our results demonstrate that the exposure to NO (by SNP during preillumination) severely affected the efficiency of photochemical reactions (F_v/F_m) of protoplasts (Fig. 4).

NO affects the functional parameters of PSII as revealed by JIP-test analysis: The JIP-test translates the original OJIP transient data into biophysical parameters that quantify the stepwise flow of energy through PSII and performance index (Tsimilli-Michael and Strasser 2008). The significant increase in the energy dissipation (DI_0/RC) in SNP-treated protoplasts (Fig. 5) indicated an impairment of electron transport (Strasser *et al*. 2004). The decrease in absorption (ABS), trapping (TR), and electron transport per excited cross section (ET_0/CS_m) in SNP-treated protoplasts as observed in dynamic leaf models (Fig. 6) suggests that the elevated NO concentrations decreased the electron transport possibly due to increased numbers of inactive RCs in PSII of pea protoplasts. Performance index (PI) is considered to be a good indicator of stress, which is the combined measurement of the density of RC, maximum energy flux reaching PSII reaction centres and the electron transport (Oukarroum *et al*. 2009). The significant decreases in PI in SNP-treated protoplasts (Fig. 5) reflect an inefficient performance of PSII.

Comments on the use of SNP: A major comment can be that SNP can release CN^- ion as well as NO, particularly on exposure to light and the resulting CN^- can also affect mitochondrial respiration (Leavesley *et al*. 2008). In fact, CN^- can elevate further NO production. Fortunately, the extent of CN^- released from SNP is only a fraction of the amount of NO released into the medium (Arnold *et al*. 1984). Further, such doubt about exclusive role of NO can be resolved to a reasonable extent by the use of NO specific scavengers, such as cPTIO (Wodala *et al*. 2008, 2010).

In our experiments, SNP had negligible effect on respiration by mesophyll protoplasts. We ensured that the incubation times were short and did not exceed 20 min during our experiments. In addition, the effects of SNP on photosynthesis were almost completely reversed by cPTIO (Table 1S, *supplement*) confirming that the effects were mostly due to NO, being released.

Concluding remarks: In conclusion, NO decreased the fluorescence yield of OJIP transients due to restriction of electron flow at oxidizing side of PSII. Also, elevated concentrations of NO decreased the photochemical efficiency (F_v/F_m), photosynthetic performance index (PI_{ABS}), and increased the dissipation per RC (DI_0/RC) (Fig. 5). We therefore suggest that NO targets the components of PSII and affects the photochemical reactions of protoplasts.

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Appendix. Selected JIP-test components during OJIP transients.

Extracted/derived parameters	
F_O, F_J, F_I, and F_P	Fluorescence yield at O- (at ~ 50 µs), J- (at ~ 2 ms), I- (~ 30 ms), and P-step (~ 1,000 ms)
F_M	Maximal fluorescence intensity
F_t	Fluorescence at a given time
RC/ABS	Density of reaction centers (RC) per PSII antenna Chl
Specific fluxes or activities	
ABS/RC	Absorption flux per RC
TR_0/RC	Trapped energy flux per RC at t = 0
DI_0/RC	Dissipated energy flux per RC at t = 0
ET_0/RC	Electron transport flux per RC at t = 0
Phenomenological fluxes	
ABS/CS	Absorption flux per excited cross section (CS)
TR_0/CS	Trapping flux per excited CS
ET_0/CS	Electron transport flux per excited CS
DI_0/CS	Dissipated energy flux per excited CS
RC/CS	Density of active reaction centers per CS
Performance index	
PI_{ABS}	Performance index (PI) on absorption basis
PI_{CSM}	Performance index on cross section basis