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RESEARCH PAPER

# Regulation of guard cell photosynthetic electron transport by nitric oxide

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## Abstract

**Nitric oxide (NO) is one of the key elements in the complex signalling pathway leading to stomatal closure by inducing reversible protein phosphorylation and $Ca^{2+}$ release from intracellular stores. As photosynthesis in guard cells also contributes to stomatal function, the aim of this study was to explore the potential role of NO as a photosynthetic regulator. This work provides the first description of the reversible inhibition of the effect of NO on guard cell photosynthetic electron transport. Pulse amplitude modulation (PAM) chlorophyll fluorescence measurements on individual stomata of peeled abaxial epidermal strips indicated that exogenously applied 450 nM NO rapidly increases the relative fluorescence yield, followed by a slow and constant decline. It was found that NO instantly decreases photochemical fluorescence quenching coefficients (*q*P and *q*L), the operating quantum efficiency of photosystem II ($\Phi_{PSII}$), and non-photochemical quenching (NPQ) to close to zero with different kinetics. NO caused a decrease in NPQ, which is followed by a slow and continuous rise. The removal of NO from the medium surrounding the epidermal strips using a rapid liquid perfusion system showed that the effect of NO on *q*P and $\Phi_{PSII}$, and thus on the linear electron transport rate through PSII (ETR), is reversible, and the constant rise in NPQ disappears, resulting in a near steady-state value. The reversible inhibition by NO of the ETR could be restored by bicarbonate, a compound known to compete with NO for one of the two coordination sites of the non-haem iron (II) in the $Q_AFe^{2+}Q_B$ complex.**

**Key words:** Chlorophyll *a* fluorescence, GSNO, guard cell, Microscopy-PAM, nitric oxide, non-photochemical quenching, photochemical quenching, *Vicia faba*.

## Introduction

The fact that the exogenous nitric oxide (NO) radical induces stomatal closure was discovered over a decade ago (Garcia-Mata and Lamattina, 2001). Later it was found that the plant hormone abscisic acid (ABA) induces NO production in guard cells, which is part of a $Ca^{2+}$-dependent signalling pathway leading to stomatal closure (Garcia-Mata and Lamattina, 2002; Neill *et al*., 2002; Garcia-Mata *et al*., 2003). Besides internal hormonal signals, NO levels may also rise in response to biotic and abiotic environmental factors, many of which lead to stomatal closure (Neill *et al*., 2008). NO synthesis and accumulation have been shown during light to dark transition- (She *et al*., 2004), water stress- (Zhang *et al*., 2007; Ribeiro *et al*., 2009), and bacterial infection-induced stomatal closure (Melotto *et al*., 2006).

Abbreviations: AL, actinic light; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-l-oxyl-3-oxide; ETR, apparent relative electron transport rate through PSII; $F_o$, minimal fluorescence level in dark-adapted leaves; $F_o'$, minimal fluorescence level in light-adapted leaves; $F_m$, maximal fluorescence level in dark-adapted leaves; $F_m'$, maximal fluorescence level in light-adapted leaves; $F_v$, maximum variable fluorescence level in dark-adapted leaves; $F_v'$, maximum variable fluorescence level in light-adapted leaves; $F_v/F_m$, maximal efficiency of PSII photochemistry; $F_v'/F_m'$, efficiency of excitation energy capture by open PSII reaction centres; $\Phi_{PSII}$, photochemical efficiency of PSII measured in the light; GSNO, *S*-nitrosoglutathione; GSSG, glutathione disulphide; NO, nitric oxide; NPQ, non-photochemical quenching; PAM, pulse amplitude modulation; PPFD, photosynthetically active photon flux density; PSII, photosystem II; *q*E, energy-dependent quenching component; *q*I, photoinhibitory quenching component; *q*L, coefficient of photochemical fluorescence quenching assuming interconnected PSII antennae; *q*P, coefficient of photochemical fluorescence quenching assuming non-interconnected PSII antennae; Rubisco, ribulose-1,5-bisphosphate carboxylase⁄oxygenase.

Besides its role as a signal molecule in stomatal closure, NO is also known to reversibly inhibit the photosynthetic electron transport chain, light-induced ΔpH formation across the thylakoid membrane, and photophosphorylation (Takahashi and Yamasaki, 2002). Experiments using isolated thylakoid membranes (Diner and Petrouleas, 1990; Schansker *et al*., 2002) and intact pea leaves (Wodala *et al*., 2008) have shown that NO slows down electron transfer between the $Q_A$ and $Q_B$ binding sites and inhibits charge recombination between $Q_A^-$ and the $S_{-2}$ state of the water-oxidizing complex in photosystem II (PSII). NO also decreases steady-state non-photochemical quenching (NPQ) and energy-dependent quenching (*q*E) in a concentration-dependent manner (Wodala *et al*., 2008). In addition, NO may slow photosynthesis by inactivating thiol group-containing enzymes in the Calvin–Benson cycle, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which is inhibited by *S*-nitrosylation (Abat *et al*., 2008; Abat and Deswal, 2009). Vladkova *et al*. (2011) found that the NO donor sodium nitroprusside (SNP) is the only NO donor which stimulates electron transport through PSII. According to their hypothesis, NO interacts with the tyrosine residue of the D2 protein, $Y_D$ (Sanakis *et al*., 1997), and the resulting $Y_D$·–NO couple has a decreased redox potential low enough to become a more efficient electron donor in isolated thylakoid membranes than the immediate redox-active tyrosine residue, $Y_Z$, located on the D1 protein. Booij-James *et al*. (2009) have shown that NO inhibits the phosphorylation of the D1 protein in *Spirodela in vivo*. The repair cycle of PSII includes the rapid turnover of D1 protein, which undergoes redox-dependent phosphorylation prior to its degradation. However, they also found that NO does not alter the degradation of D1 *in vivo*, and thus the repair mechanism and stability of PSII.

Chloroplasts are not only targets of NO, but also produce NO in a number of ways including an arginine/NADPH-dependent pathway (Guo *et al*., 2003), a nitrite-dependent pathway (Desikan *et al*., 2002), and through the decomposition of endogenous *S*-nitrosoglutathione (GSNO) (Jasid *et al*., 2006). NO levels in chloroplasts also depend on decomposition, and there are two major pathways for the elimination of NO in chloroplasts. The main one is the reaction of NO with the superoxide radical ($O_2^{-}$·), which yields the highly reactive peroxynitrite ($ONOO^-$) (Blough and Zafiriou, 1985; Yamasaki and Sakihama, 2000). The other important pathway is the reaction with glutathione (GSH) that is present in concentrations up to 3.2 mM in chloroplasts (Krueger *et al*., 2009).

Guard cell chloroplasts may contribute to stomatal function in at least four possible ways, by (i) producing ATP and reductants used in osmoregulation; (2) storing starch, which is an important source of $malate^{2-}$ and sucrose synthesis; (iii) producing osmotically active sugars via the Calvin–Benson cycle; and (iv) participating in blue light signalling and response (Lawson, 2009). Most of these findings originate from experiments that were carried out on epidermal strips from *Vicia faba*. One such study by Poffenroth *et al*. (1992) showed that red light increases the size of stomatal openings compared with a dark control in parallel with the sugar content of guard cells. In their experiments, treatment of epidermal strips with the inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) inhibited red light-induced sugar production. Tominaga *et al*. (2001) showed that red light-induced stomatal closure is strongly inhibited by DCMU. In guard cells treated with oligomycin, which inhibits oxidative phosphorylation, red light induced a 2-fold increase in ATP content, and this induction was eliminated by DCMU. A close connection between ATP content and fusicoccin-induced $H^+$ pumping was also demonstrated.

It is hypothesized that besides its role in signalling, NO in guard cells may also function as a reversible inhibitor of photosynthesis. In this work, this hypothesis is tested at a cellular level, using chlorophyll *a* fluorescence measurements in abaxial epidermal strips of *V. faba*. The results show that exogenously applied NO reversibly inhibits photosynthetic electron transport, which can be restored by bicarbonate, a compound known to compete with NO for one of the two coordination sites on the non-haem iron (II) in the $Q_AFe^{2+}Q_B$ complex in PSII.

## Materials and methods

### *Plant material and growth conditionsx*

Seeds of broad bean (*V. faba* L. cv. Mirna) were germinated at 26 °C for 3 d in the dark, and seedlings were subsequently transferred to hydroponic culture containing 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM KCl, 0.5 mM $KH_2PO_4$, and 0.5 mM $Na_2HPO_4$, pH 6.0. The concentrations of micronutrients were 0.001 mM $MnSO_4$, 0.005 mM $ZnSO_4$, 0.0001 mM $(NH_4)_6Mo_7O_{24}$, 0.01 mM $H_3BO_4$, and 0.02 mM Fe(III)-EDTA. The plants were grown in a controlled environmental chamber (Fitoclima S 600 PLH, Aralab, Portugal) under a 12 h day/12 h night cycle, at 25/20 °C day/night temperature, 150 µmol $m^{-2}$ $s^{-1}$ light intensity, and 55–60% relative humidity. Fully expanded leaves from 3- to 4-week-old plants were collected and used for each experiment.

### *NO measurements and chemicals*

NO was released from GSNO (Sigma-Aldrich, Budapest, Hungary) in the incubation medium (10 mM KCl, 100 µM $CaCl_2$, and 10 mM MES/TRIS, pH 6.15) by white light of 500 µmol photons $m^{-2}$ $s^{-1}$ in a separate reaction vessel. The solution was introduced into the experimental chamber placed on the stage of a Zeiss Axiovert 40 inverted epifluorescence microscope (Zeiss GmbH, Germany) by a rapid perfusion system (ALA Science, USA). The NO concentration in the experimental chamber was measured by an NO electrode (ISO-NOP; World Precision Instruments Inc., USA) dipped in the stirred aqueous phase. The NO electrode was calibrated by adding different volumes of SNAP solution to a copper (II) sulphate solution set to pH 4 by addition of sulphuric acid following the manufacturer's instructions.

All other chemicals were purchased from Sigma (Sigma-Aldrich, Budapest, Hungary).

### *Epidermal strip bioassay*

Experiments in this study were carried out using epidermal strips, which eliminated the influence of mesophyll cells on stomatal function. In order to ensure a controlled and balanced water and nutrient supply, and also to reduce stomatal heterogeneity, plants were cultivated hydroponically and measurements were performed between 10:00 h and 15:00 h. Prior to each experiment, the abaxial epidermis was peeled carefully from the third to fourth completely unfolded

leaves submerged in the incubation medium. The strips contained only small regions contaminated with mesophyll cells—mainly around the major veins—and these regions were excised with a razor blade. The strips were transferred and washed for 5 min in the hypoosmotic incubation medium in order to remove any remaining mesophyll cell debris and mesophyll chloroplasts by severe osmotic shock.

The cleaned strips were then submerged in fresh incubation medium and clamped to the bottom of the experimental chamber by a fine wire mesh before the dark adaptation. The GSNO-containing incubation medium was introduced onto the strips by a rapid perfusion system.

Cell viability tests carried out following the preparation of epidermal strips showed that guard cells remained intact after floating in the incubation medium. Microscopy-pulse amplitude modulation (PAM) measurements confirmed that this treatment did not influence the chlorophyll fluorescence parameters in guard cells. In addition, no correlation was found between the photosynthetic performance of epidermal strips and the age of the plant (3–4 weeks), or the position of the leaf (third or fourth) they were collected from.

### *Chlorophyll a fluorescence measurements*

The chlorophyll *a* fluorescence yield of 2–3 stomata was monitored with a Microscopy-PAM chlorophyll fluorometer (Heinz Walz GmbH, Germany) mounted on the microscope. The actinic light (AL), the 0.8 s wide single pulse (SP), and measuring light (ML) were provided by a single blue light-emitting diode (470 nm). Light intensities were measured with a Micro Quantum Sensor (MC-MQS, Heinz Walz GmbH, Germany). Peels were never used for more than 1 h.

Epidermal strips were dark adapted for at least 15 min for a precise determination of minimal and maximal fluorescence yields in the dark ($F_o$ and $F_m$, respectively). $F_m$ was obtained by exposing stomata to an SP. Maximal quantum efficiency was calculated as $F_v/F_m$ according to Butler (1978), where $F_v=F_m-F_o$.

Rapid light–response curves were obtained using a light curve protocol, consisting of eight consecutive 30 s steps with an increasing AL intensity, and an SP was applied at each AL intensity to obtain the maximal fluorescence yield of a light-adapted sample ($F_m$'). Due to the short time of light adaptation, the photosynthesis of guard cells was not in the steady state at any of the light intensities. The PSII efficiency factor ($q$P) was obtained as $q\text{P}=(F_m'-F')/(F_m'-F_o')$, where $F'$ is the fluorescence yield when the sample is exposed to AL and $F_o$' is the minimum chlorophyll fluorescence yield in the presence of open PSII reaction centres in the light (Schreiber *et al.*, 1986). Since $F_o$' cannot be determined by the Microscopy-PAM fluorometer, it was estimated as $F_o'=1/(1/F_o-1/F_m+1/F_m')$ according to Oxborough and Baker (1997). The coefficient of photochemical fluorescence quenching assuming interconnected PSII antennae ($q$L) was calculated as $q\text{L}=q\text{P}\times(F_o'/F')$ (Kramer *et al.*, 2004). The maximal quantum efficiency of light-adapted PSII photochemistry was obtained as $F_v'/F_m'$, where $F_v'=F_m'-F_o'$. The effective quantum efficiency of PSII photochemistry ($\Phi_{PSII}$) was calculated as $\Phi_{PSII}=q\text{P}\times(F_v'/F_m')$ according to Genty *et al.* (1989). The Stern–Volmer type NPQ was determined as $\text{NPQ}=(F_m-F_m')/F_m'$ (Bilger and Björkman, 1990).

In other experiments, the fluorescence yield of guard cells was monitored under constant AL intensity in the course of a rapid solution exchange above the epidermal peels. The $q$P, $q$L, $F_v'/F_m'$, $\Phi_{PSII}$, and NPQ of 2–3 stomata were determined every 30 s by the application of an SP.

## Results

### *Specificity, side effects, and the amount of NO released from GSNO*

Before investigating the effect of NO on the photosynthetic activity of guard cells, the specificity and possible side effects of the NO donor GSNO were examined. A freshly peeled abaxial epidermal strip from *V. faba* was securely anchored under a fine wire mesh on the bottom of the experimental chamber, which enabled the fluorescence emission of the same guard cell pair to be monitored before and after adding GSNO. The sample was dark adapted prior to measurements and, after a 15 min actinic illumination of 500 µmol photons $m^{-2}$ $s^{-1}$ photosynthetically active photon flux density (PPFD) AL, it was found that 10 µM GSNO caused a significant decrease in the steady-state $\Phi_{PSII}$ (Fig. 1). No such effect was observed using 10 µM GSNO solution inactivated in an open chamber by 24 h illumination with 500 µmol photons $m^{-2}$ $s^{-1}$ PPFD white light (iGSNO). Photolysis of GSNO yields glutathione disulphide (GSSG), which may interfere with the redox signalling processes in the chloroplast (Michelet *et al.*, 2005), but GSSG applied at a concentration of 5 µM did not modify $\Phi_{PSII}$ values. The simplest way to test the specificity of the NO donor GSNO is to use an NO scavenger chemical. This test was carried out by adding 40 µM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-l-oxyl-3-oxide (cPTIO) to 10 µM GSNO, which completely prevented the GSNO-induced decrease in $\Phi_{PSII}$. It is important to note that 40 µM cPTIO itself caused no changes, and 20 µM cPTIO failed to prevent the decrease in $\Phi_{PSII}$ induced by 10 µM GSNO (Fig. 1). In aqueous solutions, NO may rapidly react with water and oxygen to form $NO_2^-$ and $NO_3^-$, but neither of these anions modified photosynthetic parameters (data not shown).

In addition, the amount of NO released from the GSNO solution used in all subsequent experiments was determined. The incubation medium containing 50 µM GSNO was illuminated by 500 µmol photons $m^{-2}$ $s^{-1}$ PPFD white light in a separate reaction vessel. The vessel was connected to an

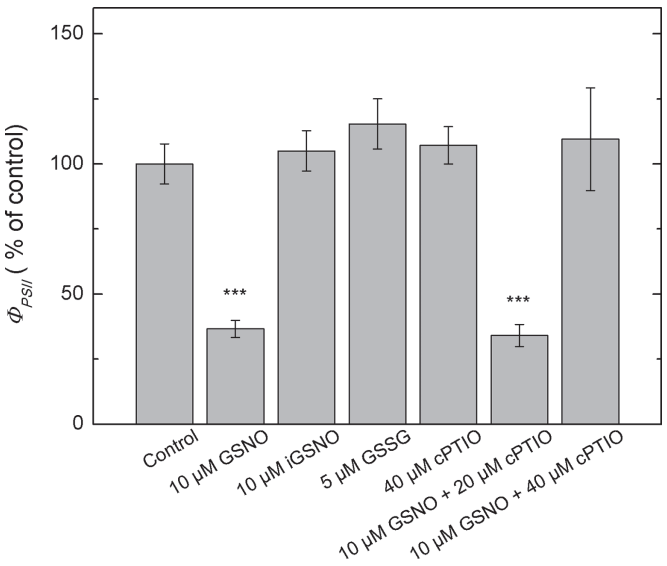


**Fig. 1.** Steady-state photochemical efficiency of PSII measured in the light ($\Phi_{PSII}$) in guard cells following a 15 min illumination with 500 µmol photons $m^{-2}$ $s^{-1}$ PPFD AL. Values are expressed as a percentage of the control. Bars represent means ±SEM ($n$=5). Asterisks indicate the level of statistical significance calculated by Student's $t$-test: ***$P < 0.001$.

experimental chamber of the microscope by the tubing of a perfusion system, which enabled rapid yet smooth solution exchange in the experimental chamber during experiments. The NO concentration of the solution was measured using an NO electrode dipped into the solution in the experimental chamber. After a 1 min lag phase following the activation of the perfusion system, the concentration of NO in the chamber rose to >450 nM in 3–4 min, and was more or less constant for 10 min after perfusion (Fig. 2, black line). Wash out of NO started ~2 min after perfusing the chamber with NO-free incubation medium (Fig, 2, grey line) and took ~5 min to complete.

### *NO-induced changes in the light–response curves of stomata are reversible*

In the following experiments, a field diaphragm, which narrowed the field of view to allow fluorescence emitted by 2–3 stomata to enter the detector of the Microscopy-PAM, was used. Figure 3 shows a typical light–response curve experiment, which was performed after the determination of maximal quantum efficiency ($F_v/F_m$) of the sample following a 15 min dark adaptation in incubating media. Figure 3A shows the relative fluorescence yield during measurement (Control), and the corresponding fluorescence parameters are shown in Fig. 3C–G (Control).

The sample was then perfused with 450 nM NO in the dark and $F_v/F_m$ was determined, but its value showed no significant difference from the control (Fig. 3B, grey column). Running the next light–response curve protocol on the same sample, it was found that the exogenous NO treatment caused a rise in the relative fluorescence yield (Fig. 3A, +NO), and a large reduction in $\Phi_{PSII}$ (Fig. 3C) at every AL intensity. $\Phi_{PSII}$ is given by ($q$P×$F_v$'/$F_m$'), where $q$P is the coefficient of photochemical fluorescence quenching assuming non-interconnected PSII antennae and $F_v$'/$F_m$' is the maximum quantum efficiency of PSII photochemistry, which reflects the probability for the energy of an absorbed photon to reach the reaction centre and drive photochemistry. $F_v$'/$F_m$' also indicates the extent to which photochemistry at PSII is limited by competition with thermal decay processes. It was found that the NO-induced decrease of $\Phi_{PSII}$ is caused by a reduction in $q$P (Fig. 3E), but not in $F_v$'/$F_m$' (Fig. 3D). $F_v$'/$F_m$' was even improved by NO treatment, implying a reduction in thermal decay processes, which is also shown by the decreased NPQ at every AL intensity (Fig. 3F). The coefficient of photochemical fluorescence quenching assuming interconnected PSII antennae ($q$L) is linearly related to the fraction of open PSII centres, and this relationship is not modified by changes in light-induced NPQ. It was found that $q$L, as well as $q$P, was reduced after the NO treatment (Fig. 3G).

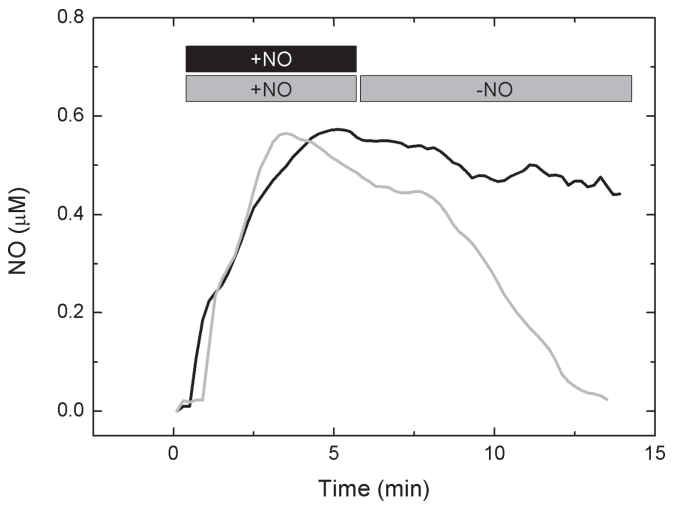


**Fig. 2.** Concentration of NO in the experimental chamber released by incubation medium containing 50 µM GSNO. GSNO photolysis was induced in a separate vessel by illumination with 500 µmol photons $m^{-2}$ $s^{-1}$ PPFD white light. The NO-containing solution was then introduced into the experimental chamber (+NO) filled with incubation medium by a solution exchange system for 6 min (black line) or, after 6 min, the NO-containing solution was changed to GSNO-free incubation medium (–NO, grey line). The speed of perfusion was 4.2 ml $min^{-1}$ and the volume of solution in the experimental chamber was set to 2 ml throughout the experiment.

The NO-containing medium was then washed out by perfusing the sample with NO-free incubation medium throughout the next dark adaptation period, and $F_v/F_m$ was determined again, showing a significant reduction compared with the control (Fig. 3B, white column). Running the next light–response curve protocol, it was found that the wash out of NO fully ($\Phi_{PSII}$, $q$P, $q$L) or partially ($F_v$'/$F_m$', NPQ) restored the parameters and the relative fluorescence yield (Fig. 3A, Recovery).

It should be noted that NO decreased $F_m$ and $F_v/F_m$ only if the sample was treated with NO and illuminated before the dark adaptation. $F_v/F_m$ values remained low even after washing out NO and showed no recovery in the following period of a couple of minutes (data not shown). If the sample was only treated with NO without illumination, $F_m$ and $F_v/F_m$ values did not change significantly.

### *Kinetic features of the NO-induced changes in photosynthetic parameters*

To the authors knowledge, no study has yet addressed the reversibility and kinetics of NO-induced changes in plant cells. Changes in fluorescence parameters of guard cells following the addition or removal of NO took place within minutes and, to study the kinetics of these changes, the relative fluorescence yield induced by a weak 30 µmol photons $m^{-2}$ $s^{-1}$ AL illumination was monitored while perfusing the epidermal strips with different incubation media. Relative fluorescence yield, $\Phi_{PSII}$, $F_v$'/$F_m$', $q$P, NPQ, and $q$L values were close to their steady state 7 min after the onset of AL illumination, as shown by a typical recording in Fig. 4A and B. Perfusion with medium containing 450 nM NO resulted in a steep rise in fluorescence yield, reaching its maximum in ~3 min, followed by a slow decrease. The steep rise in fluorescence yield is caused by the rapid decrease of $\Phi_{PSII}$, $q$P, $q$L, and NPQ with different kinetics (Fig. 4A, B). NPQ reached its minimum in ~1 min, while $\Phi_{PSII}$, $q$P, and $q$L became close to zero

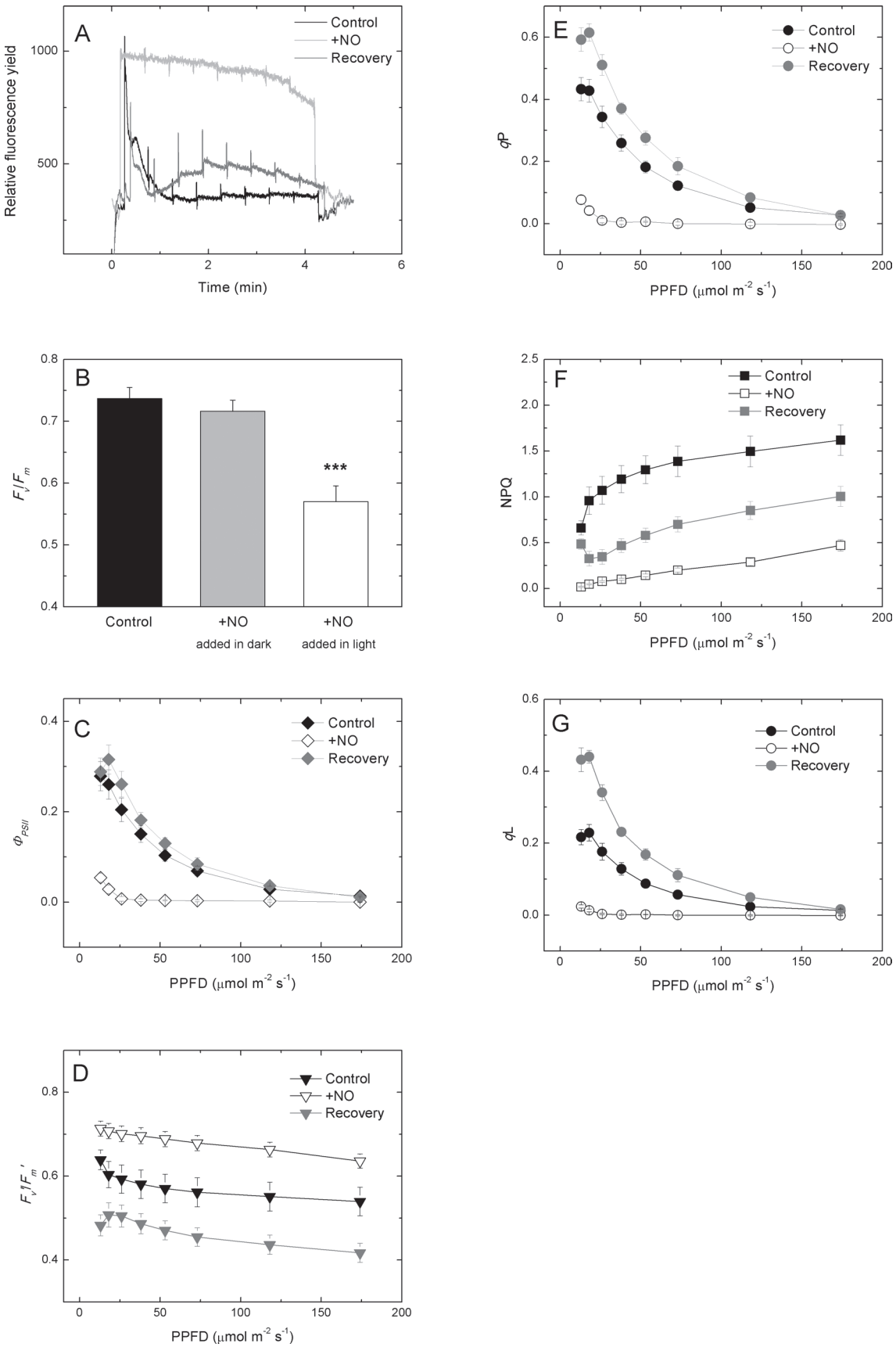


**Fig. 3.** The effect of NO on the light–response curves of guard cells. Adding 450 nM NO led to a rise in relative fluorescence yield at each light intensity, which proved to be reversible after removing NO (A). $F_v/F_m$ values were only lowered significantly if the sample was treated with NO and illuminated before the dark adaptation (B). NO reversibly reduces $\Phi_{PSII}$, *q*P, NPQ, and *q*L (C, E, F, G), and reversibly increases $F_v'/F_m'$ (D). Asterisks indicate the level of statistical significance calculated by Student's *t*-test: ***$P < 0.001$. ($n = 6$, ±SEM).

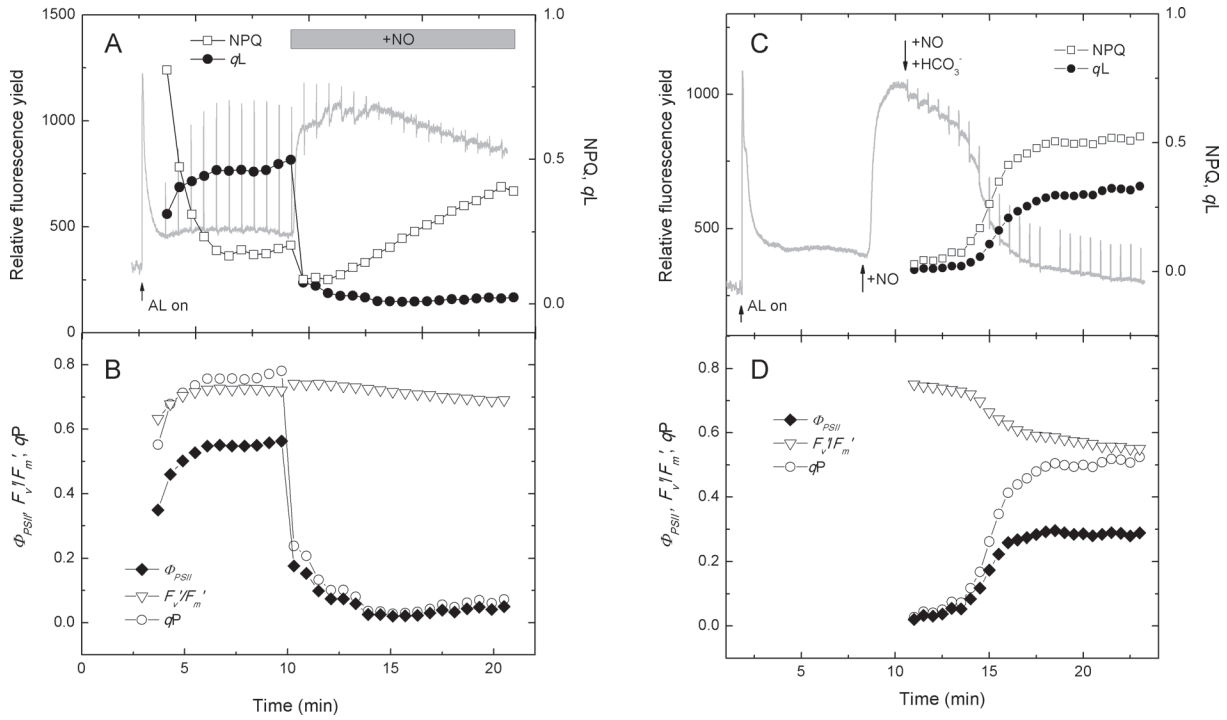


**Fig. 4.** Two typical measurements of kinetics of relative fluorescence yield, NPQ, *q*L, $\Phi_{PSII}$, $F_v'/F_m'$, and *q*P following the constant perfusion of NO- or NO- and bicarbonate-containing medium. (A) In the first experiment, NO was added after the photosynthetic parameters reached steady state in the presence of 30 µmol photons $m^{-2}$ $s^{-1}$ AL. The concentration of NO around guard cells resulting from the photolytic decomposition of GSNO amounted to >450 nM 2–3 min after perfusion. The steep rise in fluorescence yield and the rapid decrease in NPQ and *q*L occurs simultaneously. (B) Kinetics of $\Phi_{PSII}$, $F_v'/F_m'$, and *q*P in the same experiment. (C) The second experiment shows that bicarbonate added to NO reduces the relative fluorescence yield induced previously by NO and increases NPQ and *q*L. Epidermal strips were first placed in incubation medium and illuminated using 30 µmol photons $m^{-2}$ $s^{-1}$ PPFD AL. When the fluorescence yield approached the steady state, the sample was constantly perfused with an incubation medium containing 450 nM NO, which was replaced with an incubation medium containing 450 nM NO and 100 mM $KHCO_3$. (D) Kinetics of $\Phi_{PSII}$, $F_v'/F_m'$, and *q*P in the same experiment. The photosynthetic parameters were determined every 30 s during perfusion with bicarbonate.

in 3–5 min and $F_v'/F_m'$ did not change or even increased. Unlike *q*P, the rapid drop in NPQ was followed by a slow increase, which is in good agreement with the slow reduction of fluorescence yield 3–5 min after the addition of NO. The same change was found in the average of multiple measurements shown in Fig. 5 (black symbols), where photosynthetic parameters are expressed as a percentage of their steady-state values measured before the treatments.

### *Bicarbonate in the presence of NO restores qP and enhances the NPQ increase*

Previous results have shown that NO competes with bicarbonate in binding to the non-haem iron (II) in the $Q_AFe^{2+}Q_B$ complex and slows down the electron transfer rate (ETR) between $Q_A$ and $Q_B$ by displacing bicarbonate (Diner and Petrouleas, 1990; Goussias *et al*., 2002). In the experiments conducted here involving various concentrations of potassium bicarbonate added to 450 nM NO in the incubation medium, it was shown that bicarbonate applied at 10 mM had no effect on the NO-induced changes; only at 100 mM was it able largely to suppress the relative fluorescence yield (Fig. 4C) and also partly restore $\Phi_{PSII}$, *q*P, and *q*L (Figs 4D, 5A, C, E, grey symbols). In addition, bicarbonate enhanced the NO-induced slight NPQ increase, which is in good agreement with the continuous decrease in the relative fluorescence yield (Figs 4C, 5D).

### *Reversibility of photosynthetic parameters after removal of NO*

As a next step following the rapid perfusion with NO, the effect of a fast removal of NO on fluorescence parameters was examined. Rapid wash out of NO restored $\Phi_{PSII}$, *q*P, and *q*L to their original steady-state values within 5 min, but NPQ increased to values above its original steady-state value prior to the NO treatment (Fig. 5, black symbols). However, the NPQ increase was almost halted after the wash out of NO and returned to its near steady-state value. The higher value of $F_v'/F_m'$ caused by the NO treatment was decreased by the wash out and stabilized at ~97% of its original level (Fig. 5B, black symbols).

Washing out NO and bicarbonate using an NO- and bicarbonate-free incubation medium slowed the rate of increase of $\Phi_{PSII}$, *q*P, NPQ, and *q*L, and they returned to near steady-state values (Fig. 5, grey symbols). The rate of

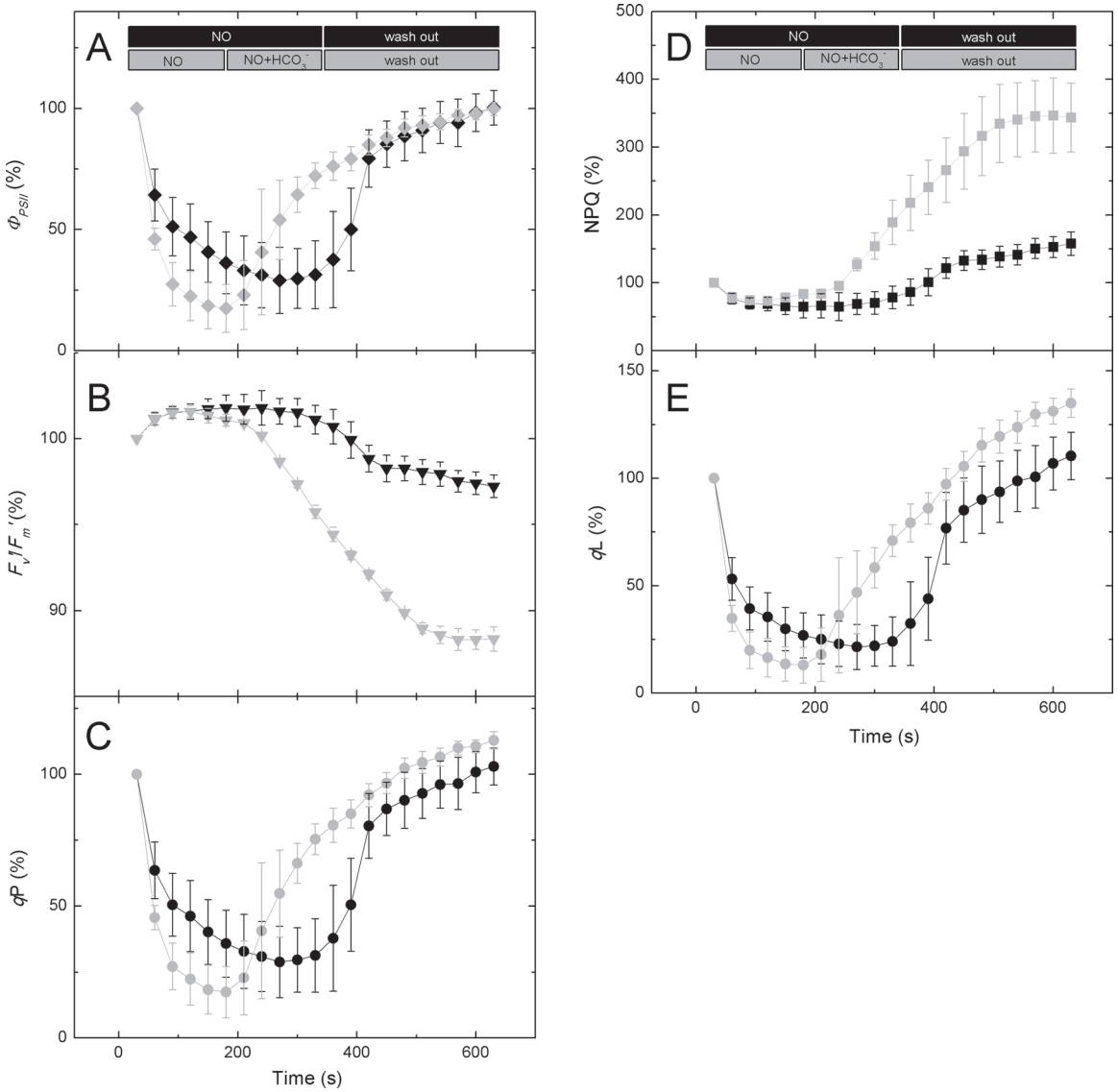


**Fig. 5.** Changes in $\Phi_{PSII}$, $F_v'/F_m'$, *q*P, NPQ, and *q*L of guard cells in the presence of NO with or without bicarbonate. Epidermal strips were placed in incubation medium and illuminated using 30 µmol photons $m^{-2}$ $s^{-1}$ AL. When values reached roughly the steady state, the incubation medium was exchanged with either an incubation medium containing 450 nM NO (black symbols), or an incubation medium containing 450 nM NO, then another containing 450 nM NO and 100 mM $KHCO_3$ in rapid succession (grey symbols). At the end of these experiments, NO and bicarbonate were washed out with incubation medium. Photosynthetic parameters are expressed as percentage of their steady-state values (*n*=4, ±SEM).

$F_v'/F_m'$ reduction decreased and stabilized at ~88% of its original value measured before treatments (Fig. 5B, grey symbols).

## Discussion

The effect of guard cell photosynthetic electron transport on stomatal function is supported by a number of experiments, as reviewed by Lawson (2009). The rapid light–response curves recorded in guard cells clearly show that NO, a signalling molecule for stomatal closure (Desikan *et al.*, 2004; Bright *et al.*, 2006; Neill *et al.*, 2008), reversibly decreases *q*P, NPQ, *q*L, and $\Phi_{PSII}$, resulting in a higher chlorophyll fluorescence yield. As the relationship between $\Phi_{PSII}$ and the apparent relative photosynthetic ETR at a given AL intensity is linear (Schreiber, 2004), it can be concluded that 450 nM NO used in the present experiment reversibly inhibits the linear electron transport chain, and therefore reduces the amount of ATP and NADPH available for osmoregulation.

Vladkova *et al.* (2011) found that the NO donor SNP is the only donor which stimulates the ETR, probably through electron donation to the reaction centre chlorophyll of PSII from the $Y_D$·–NO couple in isolated thylakoid membranes. The GSNO donor used in the present measurements did not stimulate, but inhibited the ETR in guard cells. These conflicting findings may be explained by the different NO donor and/or biological sample used in the experiments.

Triose phosphates produced by guard cell carbon fixation are used for starch synthesis in the chloroplast, while in the cytosol these compounds are also used for the synthesis of malate$^{2-}$ and sucrose, which are important osmolytes involved in stomatal opening and turgor maintenance, respectively (Shimazaki *et al*., 2007). The NO-dependent decrease in ATP and NADPH supplies for the Calvin–Benson cycle may lead to reduced sucrose and malate$^{2-}$ synthesis. Starch hydrolysis in guard cells (Vavasseur and Raghavendra, 2005), an alternative pathway for malate$^{2-}$ and sucrose synthesis, is also limited by reduced amounts of ATP and reducing power. Inhibition of photophosphorylation has only a limited effect on the plasma membrane $H^+$-ATPase involved in blue light-induced stomatal opening, as its ATP demand is largely supplied by mitochondria (Parvathi and Raghavendra, 1995) with little contribution from chloroplasts (Mawson, 1993).

Therefore, NO may influence the turgor of guard cells not only as a signalling molecule mediating stomatal closure. The present results clearly indicate that NO reversibly inhibits photosynthetic electron transport in guard cells, which reduces the amount of osmolytes for stomatal opening and turgor maintenance, and may even hamper the activity of the plasma membrane $H^+$-ATPase.

The liquid perfusion system used in this study enabled monitoring of the kinetics of changes in the fluorescence parameters of guard cells measured during rapid addition and removal of NO. The fluorescence yield rose immediately after the NO concentration increased in the Petri dish (Figs 2, 4A). It is likely that the rapid increase in the fluorescence yield was caused directly by NO itself, rather than trans-nitrosylation mediated by the NO donor GSNO, because the diffusion of GSNO into the chloroplast is considerably more limited than that of NO gas, which diffuses freely in solutions and across membranes. The sharp increase in fluorescence yield is explained by the rapid and robust drop in $q$P and $q$L, and by a less potent decrease in NPQ. The subsequent slow decrease in relative fluorescence yield is in agreement with the continuous increase in NPQ (Fig. 4A).

The rapid decreases of NPQ and $\Phi_{PSII}$ upon NO addition correlate well with each other. A reduction in $\Phi_{PSII}$ is predicted to result in a decreased proton accumulation in the lumen, which down-regulates $q$E, the fastest NPQ component, and thus explains the initial drop in NPQ. However, if the NO concentration of the incubation medium is kept over 450 nM for a prolonged time, NPQ may increase well above its steady-state value without any change in the intensity of AL (Figs 4A, 5D). What explains the continuous rise in the NPQ?

NPQ increased when $q$P and $\Phi_{PSII}$ values are low. The $q$E component of NPQ rises with proton accumulation in the lumen (Muller *et al*., 2001), which may be caused by an increasing ETR, or a reduced activity of chloroplast ATP synthase. The present results indicate that until its removal, NO supresses the ETR, so a potential rise in $q$E may be due to inhibition of ATP-demanding processes—which entail the decrease of ADP and inorganic phosphate, the substrates of ATP synthase—or to the direct inactivation of chloroplast ATP synthase. In *Arabidopsis*, the small and large subunit of the Calvin–Benson cycle enzyme Rubisco and the CF1 subunit of ATP synthase were found to be *S*-nitrosylated by NO gas or GSNO (Lindermayr *et al*., 2005). In *Kalanchoe pinnata*, it was shown that Rubisco activity is inhibited by *S*-nitrosylation *in vitro* and *in vivo* in a dose-dependent manner (Abat *et al*., 2008). NO-dependent inhibition of the Calvin–Benson cycle leads to reduced ATP hydrolysis and chloroplast ATP synthase activity, which would increase $q$E.

This assumption is in line with the present experiment in which bicarbonate was added to NO (Fig. 4C). In sufficiently high concentrations, bicarbonate displaces NO from one of the two the coordination sites of the non-haem iron (II) in the $Q_AFe^{2+}Q_B$ complex, which may restore the rate of electron transfer between the two quinones (Diner and Petrouleas, 1990). Adding bicarbonate in the presence of NO resulted in a rapid increase in $\Phi_{PSII}$, $q$P, and $q$L (Fig. 5A, C, E, grey symbols), and thus the ETR, with a simultaneous increase in NPQ and a decrease in $F_v'/F_m'$ (Fig. 5B, D, grey symbols), suggesting a sustained inhibition of the Calvin–Benson cycle by NO. The high concentration of bicarbonate almost completely restored $q$P and $\Phi_{PSII}$ parameters in the presence of NO, which indicates that the NO-induced inhibition of ETR is primarily due to the competition of these two substances for the non-haem iron.

Besides competing with NO in PSII, bicarbonate may also increase the rate of the Calvin–Benson cycle, as it is converted to $CO_2$ by carbonic anhydrase (Hu *et al*., 2010) in guard cell chloroplasts. However, the strong increase in NPQ induced by the simultaneous presence of NO and bicarbonate indicates that the inhibitory effect of 450 nM NO on the Calvin–Benson cycle is stronger than the enhancing effect of $CO_2$ derived from bicarbonate. In addition, bicarbonate has been reported to induce $H_2O_2$ and NO synthesis in guard cells, leading to stomatal closure (Kolla and Raghavendra, 2007). Potential induction of NO synthesis by bicarbonate does not interfere with the experimental design as bicarbonate was added together with NO.

NO applied in the dark did not modify the $F_v/F_m$ (Fig. 3B), but decreased the $F_v/F_m$ value of illuminated samples. This finding is in contrast to the results of Takahashi and Yamasaki (2002) found in thylakoid membranes, but in good agreement with experiments using intact leaves (Yang *et al*., 2004; Wodala *et al*., 2008). A decrease in $F_v/F_m$ is an indication of the non-relaxed $q$E component or an appearance of photoinhibitory quenching ($q$I).

Jasid and co-workers (2006) found that NO reaction with $O_2^{-\cdot}$ yields $ONOO^-$, which leads to oxidative damage even under normal conditions with low amounts of $ONOO^-$. High NO concentrations may lead to increased $ONOO^-$ production, which results in enhanced lipid peroxidation, oxidation of stromal and thylakoid proteins, and damaged electron transport in the chloroplast (Jasid *et al*., 2006). NO also inhibits cytochrome *c* oxidase in mitochondria (Millar and Day, 1996), leading to an increase in $O_2^{-\cdot}$ levels, and thus $ONOO^-$, which can then enter the chloroplast. $O_2^{-\cdot}$ produced in the chloroplast may also be increased by NO-mediated inhibition of the Calvin–Benson cycle (Abat *et al*., 2008; Abat and Deswal, 2009). Therefore, NO released by photodegradation of GSNO used in the present experiments may have yielded $ONOO^-$.

However, $\Phi_{PSII}$ and $q$P returned to their original values after wash out of NO (Fig. 5A, C, black symbols), which suggests that $ONOO^-$ was not produced in amounts high enough to damage photosynthetic linear electron transport irreversibly. Therefore the reduction of $F_v/F_m$ seems to be caused by the non-relaxing $q$E component rather than the presence of $q$I.

## Conclusion

In summary, the present results clearly show that NO, beyond its signalling role in stomatal closure, reversibly inhibits photosynthetic electron transport of guard cells. This decreases the amount of ATP and NADPH for osmoregulation, which in turn decreases starch formation and the synthesis of malate$^{2-}$ and sucrose for turgor maintenance. NO also leads to the slow and continuous increase of NPQ, mainly through its $q$E component.

Sufficiently large concentrations of bicarbonate abolish NO-induced inhibition of linear electron transport, completely restoring $q$P and $\Phi_{PSII}$, which suggests that the primary mechanism of inhibition is the displacement of bicarbonate from the non-haem iron (II) in the $Q_AFe^{2+}Q_B$ complex.

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