

# Putrescine stimulates chemiosmotic ATP synthesis

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## Abstract

Putrescine is a main polyamine found in animals, plants and microbes, but the molecular mechanism underlying its mode of action is still obscure. *In vivo* chlorophyll a fluorescence in tobacco leaf discs indicated that putrescine treatment affects the energization of the thylakoid membrane. Molecular dissection of the electron transport chain by biophysical and biochemical means provided new evidence that putrescine can play an important bioenergetic role acting as a cation and as a permeant natural buffer. We demonstrate that putrescine increases chemiosmotic ATP synthesis more than 70%. Also a regulation of the energy outcome by small changes in putrescine pool under the same photonic environment (i.e., photosynthetically active radiation) is shown. The proposed molecular mechanism has at least four conserved features: (i) presence of a membrane barrier, (ii) a proton-driven ATPase, (iii) a  $\Delta pH$  and (iv) a pool of putrescine.

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## 1. Introduction

Putrescine (Put) is an ubiquitous diamine [ $NH_2(CH_2)_4NH_2$ ] which was first identified in microbes in the late 1800s [1]. Many contributions focused on the effects of Put in prokaryotic and eukaryotic cells (about 9750 citations for the keyword ‘Putrescine’ in Pubmed). Nevertheless, the mechanism underlying its mode of action still remain obscure hindering research in different disciplines from plant physiology to human oncology. Put belongs to the main polyamines along with spermidine and spermine and also it is their precursor. Polyamines are linked with many cellular processes such as cell division, growth and senescence [2–4] and their major biosynthetic enzyme, ornithine decarboxylase is regulated by

one of the most fast and efficient ways known to date [5]. In plants, Put is formed directly by the decarboxylation of ornithine, via ornithine decarboxylase (ODC; EC 4.1.1.17), or indirectly from arginine by arginine decarboxylase (ADC; EC 4.1.1.19) via agmatine (Agm) [6,7].

Due to their cationic character, polyamines also bind to negatively charged molecules like nucleic acids, affecting both RNA translation and DNA conformation [8]. Mutants of polyamine biosynthetic enzymes usually have growth problems like a double mutant in *E. coli* which cannot synthesize Put and spermidine and show a 70% inhibition in growth which is reversible upon exogenous polyamine supply [8]. In a cell wall-deficient *Chlamydomonas* strain, the increased endocellular spermine arrests the cell cycle, which could be re-established by subsequent addition of spermidine or, to a lesser extent, also by addition of Put [9]. In unicellular green algae, Put reported values varies between 170 nmol up to 3  $\mu$ mol per billion cells of *Chlamydomonas* [9] and *Scenedesmus* [10], respectively, and is higher in dividing than in non dividing cells [9,10]. In higher plants Put concentration distribution is difficult to be determined, because a significant quantity can be stored in the vacuole, but probably the content of Put is in the micromolar range or fractional millimolar range [11]. In

**Abbreviations:** PS, photosystem; Put, Putrescine; PAR, photosynthetically active radiation; NPQ, non-photochemical quenching;  $F_v/F_m$ , maximum photosynthetic efficiency;  $F_v = F_m - F_0$ , maximum variable fluorescence;  $F_m$ , maximum fluorescence (dark);  $F'_m$  maximum fluorescence (light);  $F_0$ , initial fluorescence; LEF, linear electron flow; Fecy, Ferricyanide; MV, methylviologen;  $\Delta\psi$ , electrical component of the proton motive force; Chl, chlorophyll; ATP, adenosine triphosphate, HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid), Tricine (N-tris-(hydroxymethyl)methylglycine)

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various types of stress such as  $K^+$  deprivation, [12], low pH [13], heavy metals [14], ozone [15], Put increases many times. In a double *Arabidopsis* mutant of spermidine synthase, Put is accumulated and the seeds are abnormally shrunken and they have embryos that are arrested morphologically at the hearttorpedo transition stage [16]. Again in *Arabidopsis* plants, overexpression of spermidine synthase enhances tolerance to multiple environmental stresses [17]. There is also evidence that Put play a photoprotective role during exposure of the photosynthetic apparatus to high light intensity or UVB radiation [18,19]. In spite of the above findings, the mechanism of Put action in the plant cell, especially in chloroplasts is not yet understood. A widely known but rather overlooked chemical property of the Put pool is the dynamic equilibrium between the neutral base and its protonated forms,  $pK_2=9.04$  [20]. This simple property increases the complexity of a system when a membrane barrier (for instance the thylakoid membrane) is present. In this contribution, our scope was to investigate the role of Put in the photosynthetic apparatus of higher plants. *In vivo* studies emphasized Put effect on thylakoid membrane energization and *in vitro* studies allowed a better understanding of the underlying causes. An attempt to highlight the sequence of molecular events has led to the suggestion of a novel model concerning the mode of Put action which is a reasonable but simplified hypothesis that can serve as a working basis for further experimentation.

## 2. Materials and methods

### 2.1. Plant material

Tobacco plants (*Nicotiana tabacum* cv. Xanthi) grown in a greenhouse for 6–8 weeks were transferred for 2 days in a thermostated chamber (25 °C) under continuous light (50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). For leaf discs experiments healthy leaves were cut with a special knife. Discs of 10 mm diameter were freely floating in distilled water in petri dishes at 25 °C with or without Put and illuminated for 24 h with 50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Put stock solution (Sigma) was freshly made and the working concentration was 1 mM. Tobacco was substituted with market swiss-chard (*Beta vulgaris*) in photophosphorylation experiments.

### 2.2. Chloroplast isolation

Chloroplasts were isolated by grinding leaves of tobacco (*Nicotiana tabacum* cv. Xanthi) in 50 mM Tricine buffer, pH 7.6 (adjusted with KOH), containing 0.33 M sucrose. The slurry was filtered through cheesecloth and chloroplasts were precipitated by centrifugation at 3000 g for 7 min. The pellet was resuspended in a small aliquot of the isolation medium (Chl up to 2 mg  $\text{mL}^{-1}$ ). A low cation, low osmoticum buffer (100 mM sorbitol, 10 mM Tricine pH 7.6 adjusted with KOH) suitable for evaluation of the cationic effect of Put on thylakoid membranes was the reaction mixture. Chlorophyll concentration was estimated according to Holden [21].

### 2.3. Fluorescence measurements

In order to determine the maximum photosynthetic efficiency ( $F_v/F_m$ ) samples were dark adapted for 15 min and fluorescence was measured using a Handy-PEA Fluorophotometer (Hansatech, King's Lynn, UK). For determination of non-photochemical quenching ( $\text{NPQ} = F_m/F_m' - 1$ ) [22] leaf discs were illuminated with red light of different intensities (10, 30, 60, 100, 350, 500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) for 5 min using Handy-PEA (multi-hit mode) and every 30 s a saturating pulse of 3000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  was applied for determination of  $F_m'$ . After the 5 min induction period of NPQ continuous light was switched off and only saturating pulses were applied every 20 s for 5 more min. The effect of Put treatment was repeatedly evaluated in leaf discs and results were showing the same motif. In order to estimate, in arbitrary units, the heat overall produced during the NPQ-induction period of illumination (300 s duration), we suggest the introduction of an index  $I_\Theta$  ( $\Theta$  stands for heat in greek),  $I_\Theta = (\Delta t) \times (\text{NPQ})_t$  that corresponds to the total area between NPQ induction curve and the time axis for this period of 300 s.

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### 2.4. Polarographical measurements

Polarographical measurements were carried out using a Clark type electrode, as previously described [18]. The light source was a M100 projector (Mille Luce) providing 1000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  of white light. The infrared part of the applied irradiation was filtered off by inserting a 2%  $\text{CuSO}_4$ -containing cuvette (4 cm path length) into the light beam. Coupled linear electron flow (LEF) and PSII activity of tobacco chloroplasts supported by methylviologen (MV) and ferricyanide (Fecy) [23], respectively, were measured in a reaction medium containing 100 mM sorbitol, 10 mM Tricine, 2 mM ADP, 2 mM  $\text{Na}_2\text{HPO}_4$ , (pH 7.6 adjusted with KOH). Experiments were performed three times in different tobacco preparations with similar results.

### 2.5. Photophosphorylation measurements

Chloroplasts were extracted from healthy leaves of swiss-chard as in ref. [24]. Chloroplasts were prepared in a grinding buffer containing 0.4 M sucrose, 40 mM tricine, 10 mM NaCl, 10 mM sodium ascorbate, (pH 7.8 adjusted with KOH). After grinding, the suspension was filtered and chloroplasts were pelleted by centrifugation. The competent for photophosphorylation preparations obtained by this treatment were stable for at least 8 h. Photophosphorylation was measured in a medium containing 200 mM sorbitol, 12 mM HEPES, 10 mM tricine, 10 mM KCl, 5 mM  $\text{MgCl}_2$ , 2 mM  $\text{K}_2\text{HPO}_4$ , 1 mM sodium ascorbate and 0.6 mM ADP (pH 7.8 adjusted with KOH). 300 nM valinomycin (in ethanol, final concentration 0.25%) was added in order to dissipate  $K^+$  gradients, while phenazine methosulphate (40  $\mu\text{M}$ ) was the electron acceptor. Put stock solutions were freshly prepared and the incubation time in the dark was 1 min. Illumination was provided for 3 min in waterjacketed chamber (thermostated at 23 °C) under continuous stirring and the reactions were stopped after switching off the light by mixing with 10%  $\text{HClO}_4$  (final pH <1). After neutralization with KOH and centrifugation in order to pellet the remainings of the thylakoids and  $\text{KClO}_4$ , the ATP content of the supernatant was determined by a sensitive kit using the luciferin–luciferase system (FL-ASC Kit, Sigma) in a LS50-B Luminometer Spectrometer (Perkin Elmer) according to the manufacturer's instructions. The quantification was done by adding 3 times small amount of ATP stock solutions of known concentration (freshly prepared). The preparations were essentially free of adenylate kinase activity as previously described [24]. The stimulation was validated for three different swiss-chard preparations.

## 3. Results

### 3.1. *In vivo* studies: Put treatment affects NPQ

We followed changes of tobacco leaf discs Chl a fluorescence during the first 24 h of Put treatment. Our main objective was the detection – by minimally invasive means – of a possible impact of Put treatment on photosynthetic processes. Six different light intensities (10, 30, 60, 100, 350 and 500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) used for NPQ induction were from one fifth up to 10-fold of incubation intensity (50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and kinetics were measured at the 8th and 24th hour. Results concerning the induction and relaxation of non photochemical

quenching are presented in Fig. 1. For the first 8 h, the Put treatment had marginal effects in NPQ (Fig. 1B). However, after 24 h, the kinetics were different in shape for Put-treated leaf discs (Fig. 1D). In untreated discs, during the first minute of measurements, photoprotective mechanisms ( $\text{NPQ}_{30\text{s}}$  up to 0.6) were transiently activated and at light intensities lower than  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  gradually inactivated ( $\text{NPQ}_{300\text{s}} < 0.2$ ). But this is not the case for Put-treated discs, which exhibited a mild, progressive, intensity-dependent activation of NPQ with no sign of inactivation during the light phase. Control  $F_V/F_M$  values of leaf discs during the 24 h were not declining ( $F_V/F_{M8\text{h}} = 0.801 \pm 0.007$ ,  $n=6$  and  $F_V/F_{M24\text{h}} = 0.811 \pm 0.010$ ,  $n=6$ ) indicating that senescence was negligible during this period. Remarkably, NPQ curves in Put-treated discs lost the characteristic peak evident at the first 30 s of light adaptation ( $\text{NPQ}_{30\text{s}} < 0.1$ ), which normally lasts only few seconds when the actinic light is of low intensity (see Fig. 1C, D). At 24 h in Put-treated discs the maximal NPQ values were lower (0.6 maximum value) than control (maximum  $\text{NPQ} > 1$ ). Also, the

thermal losses ( $I_\Theta$ ) for Put-treated discs during the 5 min light period at  $500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  were about 66% less than  $I_\Theta$  of the corresponding control (see inset Fig. 1D). So, the NPQ analysis indicated that Put treatment somehow affected the thylakoid membrane energization. In order to clarify the role of Put directly on the membrane we performed *in vitro* experiments.

### 3.2. In vitro studies

A major aim of the *in vitro* experiments was to test the effect of Put on chemiosmotic ATP production which is a process strictly related to the membrane energization [25]. Due to the cationic character of Put, one should also evaluate possible coulombic effects. Those coulombic effects were evaluated before photophosphorylation measurements. Data from these studies are illustrated to the following three sections. Given that chloroplast isolation procedures deplete small solutes like Put, one has to exogenously add this natural amine in order to simulate *in vivo* conditions. Low salt media were used for

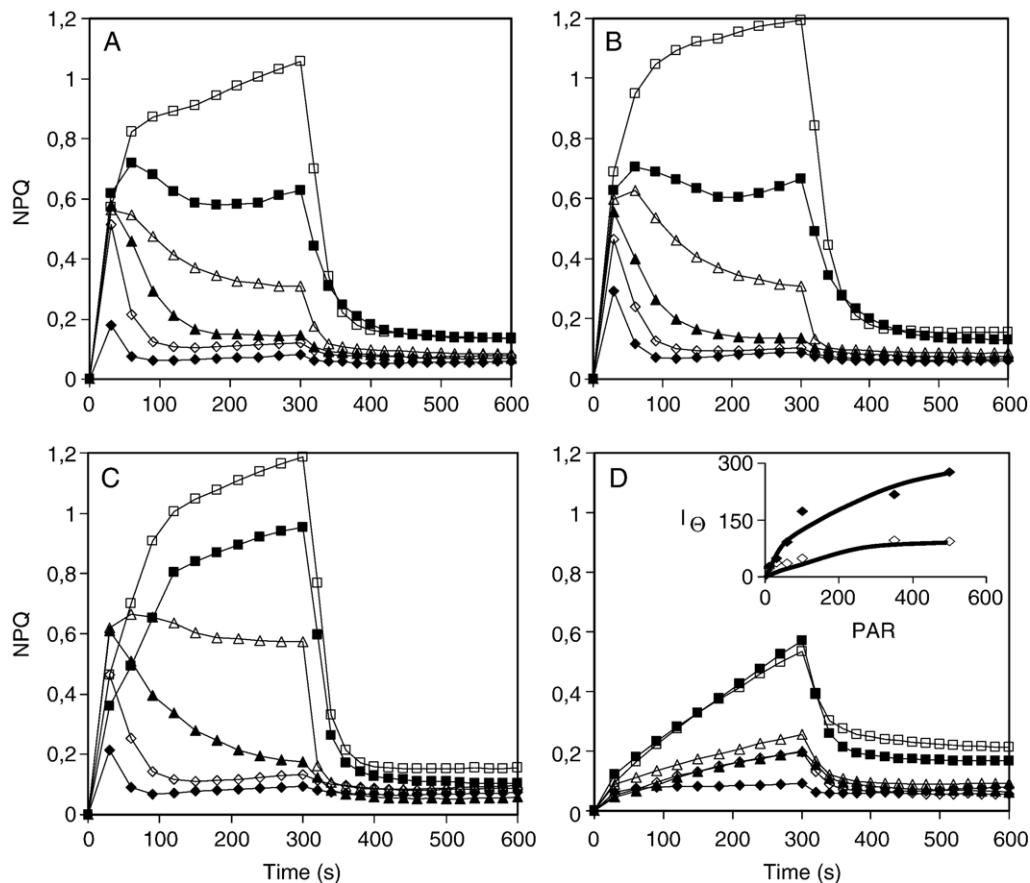


Fig. 1. Time course for induction and relaxation of non photochemical quenching of Chl fluorescence (NPQ) in tobacco leaf discs. Leaf discs were incubated for 24 h at  $25^\circ\text{C}$  at  $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . (A, B) Induction (for 300 s) at different light intensities and relaxation of NPQ (for additional 300 s) of control (A) leaf discs and putrescine-treated 1 mM (B) after 8 h of incubation. (C, D) Induction and relaxation of NPQ of control (C) leaf discs and putrescine-treated (D) after 24 h. Symbols (numbers correspond to PAR values in  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ): closed diamonds: 10; open diamonds: 30; closed triangles: 60; open triangles: 100; closed squares: 350; open squares: 500. Panel D inset: light response curves for  $I_\Theta$  estimating thermal losses during NPQ induction measurement (closed diamonds: control, open diamonds: putrescine-treatment).  $I_\Theta = (\Delta t) \times (\text{NPQ}_t)$  corresponds to the total area between NPQ induction curve and the time axis for this period of 300 s. The  $I_\Theta$  index allows comparison between different samples. Here we apply  $I_\Theta$  approach for data illustrated in panels C and D. Putrescine treatment reduces thermal losses even at light intensities 10-fold higher than the intensity of incubation PAR  $I_{\Theta\text{con}500} > I_{\Theta\text{put}500}$ .

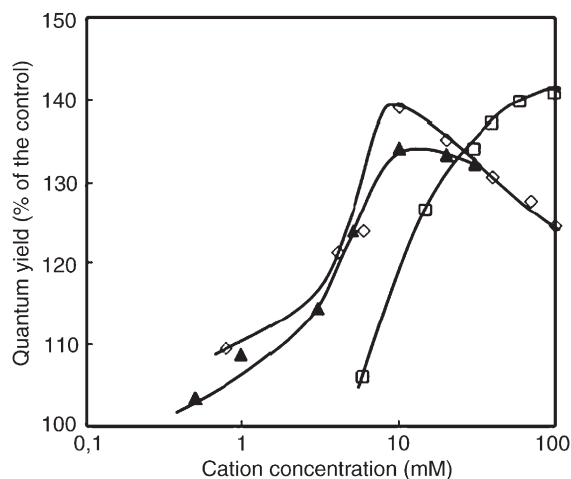


Fig. 2. Stimulation of maximum quantum yield of PSII by different concentrations of putrescine (triangles) in low-salt tobacco chloroplasts (working concentration  $10 \mu\text{g Chl ml}^{-1}$ ). For comparison  $\text{Mg}^{2+}$  (diamonds) and  $\text{K}^+$  (squares) were also used (chloride salts) and semilogarithmic plot was preferred for clarity reasons. Fluorescence was measured in a reaction medium containing 100 mM sorbitol, 10 mM Tricine (pH 7.6 KOH). The values are expressed as percentage from the initial values measured prior to cation (Put,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ) addition, which were used as control. Control values (100%) of maximum quantum yield ( $F_v/F_m$ ) in low salt were  $0.393 \pm 0.016$  ( $n=3$ ).

isolation and resuspension (see Material and methods) in order to evaluate the cationic effect of Put. On the contrary, classical media with carefully selected total cation content were used in order to avoid cationic effects during experiments which focused on the equilibrium of the base.

### 3.2.1. Put cationic effect on quantum yield

In isolated chloroplasts Chl a fluorescence decreased upon suspension in low cation media [26]. Recovery of fluorescence can

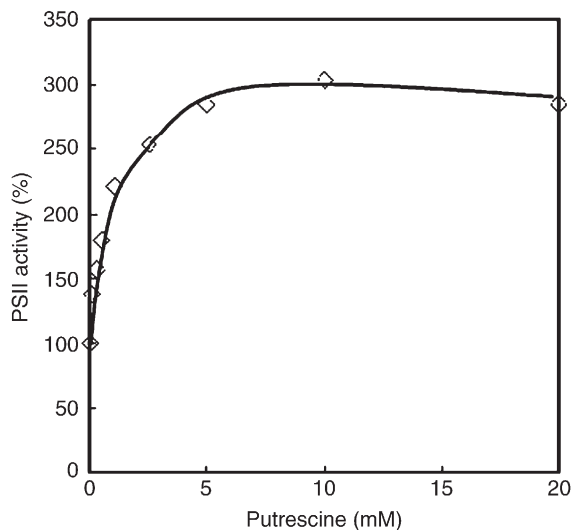


Fig. 3. Stimulation of PSII activity by different concentrations of putrescine in low-salt tobacco chloroplasts. Polarographical measurements performed in a reaction medium containing 100 mM sorbitol, 10 mM Tricine, 2 mM ADP, 2 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.6/KOH. The values are expressed as percentage from the initial values measured prior to Put addition, which were used as control. Control value (100%) was  $82 \mu\text{mol O}_2 \text{ mg Chl}^{-1} \text{ h}^{-1}$  and the electron acceptor was 3 mM Fecy. Standard deviation was less than point size.

occur upon cation addition. In Fig. 2 is shown that in low salt chloroplasts  $F_v/F_m$  ratio increased upon addition of Put. Put is clearly more effective than equimolar monovalent cations ( $\text{K}^+$ ) and slightly less effective as bivalent cations ( $\text{Mg}^{2+}$ ). 10 mM of Put had a maximal effect on  $F_v/F_m$ . When added in concentration smaller than 1 mM, Put had only a slight effect on  $F_v/F_m$ , whereas it increased about 35% the efficiency of photosystem II (PS II) at 10 mM concentration. The shape of Put kinetics curve is similar to that of  $\text{Mg}^{2+}$  but different when compared to  $\text{K}^+$ . Interestingly, a concentration of 10 mM Put has the same effect with 3 times more  $\text{K}^+$  (Fig. 2). The higher efficiency resulted in higher oxygen evolution rates for PSII as seen in Fig. 3.

### 3.2.2. Put effect on linear electron flow (LEF)

Chloroplasts were tested for their oxygen evolution ability in the presence of different concentrations of Put. Fig. 3 shows the effect of Put on PS II activity. In Fecy supported oxygen evolution the effect of Put was marginal at low concentration, whereas it presented a maximum oxygen evolution rate (300% increase) at concentration of 5 mM or higher. The effect of Put in MV supported linear electron flow is demonstrated in Fig. 4. 2 mM of Put is enough to stimulate maximum linear electron flow (200% increase).

### 3.2.3. Put effect on photophosphorylation

Given that photophosphorylation is easily biased *in vitro* by adenylate kinase activity, it would be ideal the preparations to have a negligible adenylate kinase activity. This was not possible with the tobacco chloroplasts so we searched for a better experimental system trying to avoid the use of inhibitors like di(adenosine-5')pentaphosphate. *Beta vulgaris* chloroplasts were not showing adenylate kinase activity in agreement with previous reports [24]. Another source of errors in an artificial

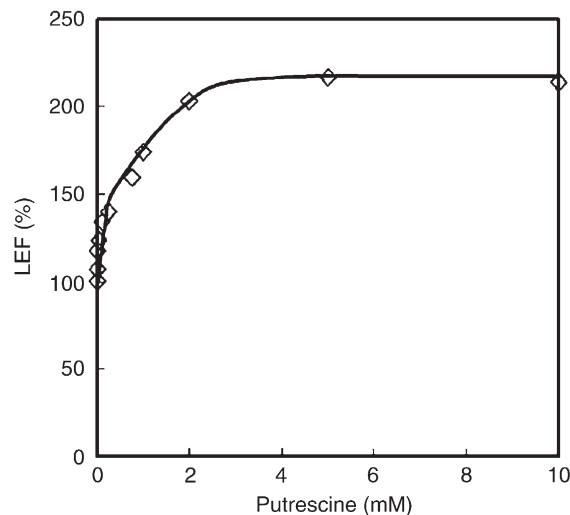


Fig. 4. Stimulation of linear electron flow (LEF) by different concentrations of putrescine in low-salt tobacco chloroplasts. Polarographical measurements performed in a reaction medium similar to that of Fig. 3. The values are expressed as percentage from the initial values measured prior to Put addition, which were used as control. Control value (100%) was  $76 \mu\text{mol O}_2 \text{ mg Chl}^{-1} \text{ h}^{-1}$  (uptake) and the electron acceptor was 100  $\mu\text{M}$  MV. Standard deviation was less than point size.



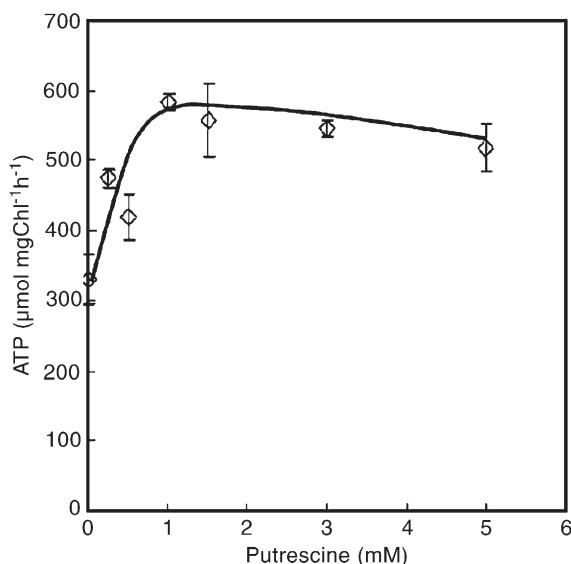


Fig. 5. Stimulation of ATP synthesis by different concentrations of putrescine in swiss-chard chloroplasts suspended in optimal-cation content media. Photophosphorylation was measured in a medium containing 200 mM sorbitol, 12 mM HEPES, 10 mM tricine, 10 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM K<sub>2</sub>HPO<sub>4</sub>, 1 mM sodium ascorbate and 0.6 mM ADP (pH 7.8/KOH). 300 nM valinomycin was present while phenazine methosulphate (40 μM) was the electron acceptor (working concentration 28 μg Chl ml<sup>-1</sup>). Standard deviation was depicted as vertical bars ( $n=3$ ).

phosphorylation system is an ion gradient due to K<sup>+</sup> of the medium. In order to secure that phosphorylation would not be influenced by K<sup>+</sup> gradients, valinomycin was included in the phosphorylation medium.

Put in cells occurs normally in the fractional millimolar range [11], subsequently this region of concentrations was also targeted. The selected pH was 7.8 that simulated the physiological pH of stroma. The results are illustrated in Fig. 5. Put effect on ATP synthesis exhibits a 3 phase kinetics ( $\alpha$ ,  $\beta$ ,  $\gamma$ ). At low concentrations (submillimolar) the ATP synthesis was slightly increased ( $\alpha$  phase). At 1 mM stimulation was optimal and reached a plateau ( $\beta$  phase). At higher concentration the ATP synthesis rate began to decline ( $\gamma$  phase). Maximum stimulation was more than 70% (see Fig. 5) and this corresponds to the steady state rate of phosphorylation.

#### 4. Discussion

A 24-h incubation of leaf discs with Put resulted in the disappearance of the early peak of NPQ (Fig. 1D) which is normally activated in order to protect via energy dissipation and is deactivated under low light intensity. The exact cause of this phenomenon is not clear. In general, the mechanism of dissipation is associated with a quenched state of PS II antenna proteins (mainly LHC II) [27,28] and this state is correlated to lumen acidification, the subsequent xanthophyll cycle and CP22 activation [29,30]. Given that CP22 is a lumen pH sensor [31] and its absence cause similar effects to Fig. 1D, the early peak perhaps can be seen as a sensitive indicator of lumen pH. Put treatment results in the disappearance of this peak, probably because the lumen was not acidified during the first minute of

illumination as much as in control plants. Thus, CP22 was not activated and an early NPQ peak did not appear. The two seemingly contrasting facts (light-driven proton release in lumen, but not extended acidification of the latter) can be interpreted if one accepts an increased internal buffering capacity. Buffering can sequester released protons and, subsequently, minimize lumen acidification. This first indication for an increased buffering capacity of lumen will be discussed further in conjunction with Put biochemical properties.

Besides the disappearance of the early NPQ peak a second indication of lower membrane energization is that Put treated discs after 24 h exhibited lower NPQ at the end of the 5 min light phase (up to 0.6 at 500 μmol photons m<sup>-2</sup> s<sup>-1</sup>). Relative thermal losses (inset Fig. 1D) of Put-treated discs for the whole period (5 min) of illumination remained low. Namely at 500 μmol photons m<sup>-2</sup> s<sup>-1</sup> losses were 66% less than the corresponding control albeit the measurement was performed in 10-fold more intense illumination than the incubation light regime. This conclusion agrees well with recent findings of our group that Put treatment, decreases the dissipation and protects photosynthesis under various stresses, such as UV irradiation or ozone [18,32]. Attention should be paid to the fact that lumen overacidification hinders PSII activity possibly due to donor side inactivation [33] and thus an increased buffering of lumen should protect this type of inactivation. Of course when buffering capacity is exceeded, lumen overacidification could occur and other mechanisms like non-radiative thermal dissipation of excess photon energy could be activated and protect the photosynthetic apparatus.

Clear conclusions concerning the mode of Put action cannot be drawn without quantification of the Put effect directly on photosynthetic subcomplexes (PSII, PSI, Cytb<sub>6</sub>f, ATPase). Due to the limited knowledge of Put effect directly on thylakoids, studies of fluorescence induction, classical Hill reactions as well as photophosphorylation rates in thylakoids were needed. In Fig. 2 it is shown that *in vitro* at pH 7.6 Put is a more efficient stimulator of quantum yield of PS II, than the monovalent K<sup>+</sup> and has a similar effect to the bivalent Mg<sup>2+</sup> (Fig. 2). The stimulation of PS II efficiency can be attributed to the cationic effect of Put (NH<sub>3</sub><sup>+</sup>(CH<sub>2</sub>)<sub>4</sub>NH<sub>3</sub><sup>+</sup>), since a similar effect can also be obtained by adding equivalent amounts of bivalent inorganic cations such as Mg<sup>2+</sup> although this could be coincidental. Hence, by adapting the low-salt dissociation cation reconstitution approach to a pH where about 99% of Put is protonated the cationic effect of Put could be quantified.

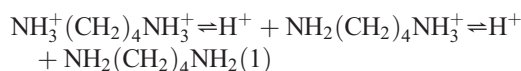
Higher maximum quantum yield (expressed as  $F_V/F_M$ ) due to addition of Put in isolated thylakoids is in good agreement with the increased PSII activity shown in Fig. 3. The Put treatment increases 3-fold PSII activity and more than 2-fold the linear electron flow (Fig. 4). This stimulation could be explained through a spatial segregation of PSII and PSI driven by electrostatic screening that is correlated to stacking of thylakoid membranes and spillover decreasing [34]. The thylakoid membranes are enriched in LHC II, which has a stroma exposed region that is negatively charged [35,36] and thus repulsion forces prevent stacking. Cations such as Mg<sup>2+</sup>

are known to provoke stacking [34] through a screening effect of those negative charges. It is possible the same to hold in this case, where Put increased  $F_V/F_M$  ratio and PSII activity to the same extent with equivalent concentration of  $Mg^{2+}$ . Given that plastidal transglutaminases could link Put in light harvesting proteins even in the dark – although at a lower rate than in light where activation of the enzyme is reported to occur – [37] our protocols reduced incubation time of thylakoids with Put in the dark in 2 min and up to 3–4 min in light during polarography. For these reasons Put covalent binding should be of minor importance to the reported effects in Figs. 2–4. Also Put-dependent  $F_V/F_M$  increase – as for previously reported  $Mg^{2+}$  dependent increase [38] – was due to stimulation of PSII centers mainly in grana, i.e., PSII $\alpha$  centers (unpublished data, Ioannidis and Kotzabasis). This might be an effect of Put with biological significance during *in vivo* stacking and unstacking both for short- or long-term changes. In all cases, one should be cautious when comparing whole plant data with thylakoid studies and be aware of the high degree of reductive thought in such connections. However, the importance of Put cationic role *in vivo* in physiological or pathological conditions is also strengthened by many previous studies. For instance, it is reported that plants respond to low  $K^+$  or low  $Mg^{2+}$  by accumulating many-fold more Put which indicates that plant cells can regulate through Put accumulation the cationic imbalance due to the reduced  $K^+$  or  $Mg^{2+}$  (for reviews see ref. [3] and ref. [39]). Recently, Serafini-Fracassini's group demonstrated the crucial effect of Put titer restoration on the growth, transglutaminase activity [40] and the thylakoid membrane aggregation under high salt stress [41] in a polyamine-deficient variant strain of *Dunaliella salina*.

Besides the cationic character, Put is a weak base and this has numerous implications that often are overlooked. Evaluation of such effects during steady state phosphorylation was possible by diminishing the cationic contribution of Put which was already quantified (Figs. 2–4). This was possible by working with optimal cation content media, with carefully selected pH and by selection of buffers that do not penetrate in lumen. As long as the lumen is buffered by weak bases like Put one might expect reduced rates of ATP synthesis. Remarkably, Put can stimulate ATP synthesis more than 70% when it is present in concentration of 1–2 mM (Fig. 5). The expected sequestration of Put by ATP molecules according to previous studies [42] could be about 4% when added Put is 0.25 mM and even less at higher concentrations and for this reason this effect should be of minor importance. However, the sequestration of Put by other molecules (i.e., Chls) might also decrease the active Put concentration to some extent. Small changes of available Put result in different energy output under the same light energy input (phase  $\alpha$  and  $\beta$ ). The normal Put concentration inside cells is in the fractional millimolar range, so we suggest that a small increase or decrease of available Put can fine tune ATP production. Putrescine rise during various stresses perhaps is related to ATP stimulation in order increased energy needs to be covered. Particularly, the shape of the curve exhibits at least three appealing traits. There is a drop ( $\alpha$  phase) in the ATP synthesis rates as the Put concentration lowers. *In vivo*, lower Put content characterizes the aged cells [43]. This is nicely correlated with the low metabolic

rates that are common during senescence. Optimal ATP rates were measured near 1.5 mM Put that might be near the value of endogenous Put of actively growing cells ( $\beta$  phase). In the  $\gamma$  phase the rates of ATP synthesis gradually decline. This curve might explain why the same amount of exogenously supplied Put can give opposite effects. This is a puzzling effect reported a number of times by polyamine researchers. If a sample is aged (low Put content) then n moles of supplementary Put result in a shift from  $\alpha$  phase to  $\beta$  phase (increased energy production). If a sample is robust/young the Put content is higher and when n moles of Put are exogenously supplied then the sample passes into the suboptimum region ( $\gamma$  phase) and the energy production lowers. However one should bear in mind that these results derive from an *in vitro* system and the values of Put concentration needed for a specific ATP synthesis rate may vary according to experimental conditions (i.e., Chl concentration, osmoticum, lumen volume, etc.). Therefore it cannot be directly correlated to the Put titer of cells which is also difficult to be determined.

The stimulation phase is most interesting due to the *in vivo* implications that rise. Stimulation of ATP synthesis – under similar to our conditions – by low molecular weight amines like imidazole, methylamine, ammonia is already reported and occur possibly through ion trapping [44,45]. The ion trapping mechanism was both theoretically described and experimentally demonstrated in thylakoid membranes for Put [46]. When chloroplasts are incubated in darkness with Put, the diamine is expected to be equally distributed at both sides of the thylakoid membrane ( $C_i = C_o$ ) [47]. The amine in each compartment (in lumen or in stroma) is in a dynamic equilibrium that is demonstrated below (see Eq. (1)). One should pay attention to a second type of equilibrium between the permeant uncharged amine in lumen and that in stroma (Fig. 6). By illuminating a sample, protons are released in lumen and shift the equilibrium to the left.



In lumen charged amines are produced, with a parallel depletion of uncharged forms. This disequilibrium forces a rapid

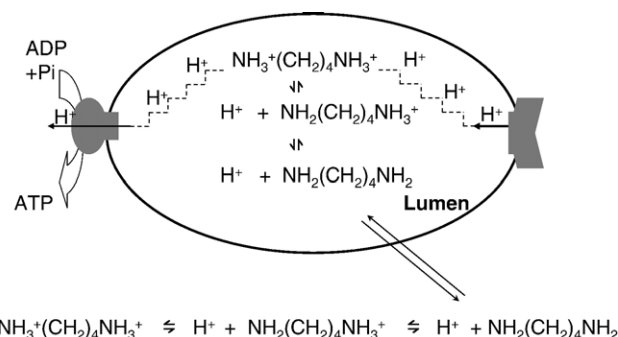


Fig. 6. Oversimplified hypothetical scheme showing the balance between fully- ( $NH_3^+(CH_2)_4NH_3^+$ ), semi- ( $NH_2(CH_2)_4NH_3^+$ ) and un-charged ( $NH_2(CH_2)_4NH_2$ ) putrescine. The equilibrium holds in lumen and in the external phase ("stroma"). During light activation, the protons ( $H^+$ ) released in lumen shift the equilibrium, pooling more neutral amine inside lumen. The concentration of Put in lumen is, probably, responsible for the stimulation of ATP synthesis.

influx of uncharged diamines from stroma (let us for a moment to assume that the membrane is impermeable to charged Put). The  $\Delta\text{pH}$  value defines the extent of trapping and the internal concentration of Put ( $C_i$ ) increases so much that the ratio  $C_i/C_o$  can be increased 500- to 3000-fold [46]. This gradient of Put buffers protons in lumen, but interestingly does not change the total cationic charge in lumen (see charges in equation 1). Finally, steady state ATP synthesis is stimulated not attenuated (Fig. 5). The assumption that thylakoids are impermeable to charged Put is an oversimplification. It is known that  $\text{Cl}^-$  channels of the thylakoid membrane open at 30 mV [48] and the influx of  $\text{Cl}^-$  (counter-ions) is expected to neutralize amine molecules and allow its efflux in stroma [49]. The voltage dependency of those channels may act as safety valve sensor that hinders Put overaccumulation in lumen and simultaneously fine tune membrane potential in values sufficient for ATP synthesis. In addition, other polymine-specific channels or translocators, as those reported for [50], yeast vacuole [51] and protozoan cell membrane [52] may contribute to Put movements across thylakoid membranes.

Also, the complexity of the *in vivo* system is severely increased by other factors. For example considering that the  $\text{pK}$  values of polyamines are spread over the range 7–11 and that the protonation sites are relevant to the chemical structures of polyamines, it seems likely that association and dissociation of polyamines with biological macromolecules can be controlled dynamically in correspondence with small changes in the local environment, such as ionic strength, pH, dielectric constant and temperature [53]. This association/dissociation of polyamines can decrease/increase active concentrations of free Put for instance and regulate chloroplast functionality. For a more precise discussion concerning polyamine binding constants to various macromolecules see review by Igarashi and Kashiwagi [4].

Taken together, these observations support the hypothesis that *in vivo* properly regulated Put levels result in an increased internal buffering capacity, increased PAR threshold for activation of heat dissipation and increased chemical energy production. Put might contribute to the regulation of chloroplast ionic strength that was reported to control parsing of pmf into  $\Delta\psi$  and  $\Delta\text{pH}$  [54]. Also our concept for an advanced role of Put in photoprotection is in accordance with the recent proposal that *in vivo* modulation of NPQ is regulated through the chloroplast ATP synthase and stromal metabolites [55]. If the demand for increased internal buffering capacity is high for high light grown plants and lowers as light intensity attenuates, then artificial elevation of the Put pool in low light adapted plants should increase their tolerance in high light. This Put mediated photoprotection is already reported to be true for green algae [18,19]. Finally, the concept of light-driven Put efflux on lumen brings again to the forth the crucial question and former review title “How acidic is the lumen?” [56]. Therefore, given that adaptation in different light environments adjusts the Put content of photosynthetic organisms [57], we enrich the question into: “How acidic is lumen for a given Put titer?”.

This study highlights that one of the various effects of polyamines as growth factors, besides those related to nucleic

acid synthesis and stabilization, protein synthesis and their post-translational modification is also to energize the cell through stimulation of ATP synthesis. Secondly, small changes in the putrescine pool fine tune the energy production in chloroplasts.

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