Evidence That Putrescine Modulates the Higher Plant Photosynthetic Proton Circuit

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Abstract

The light reactions of photosynthesis store energy in the form of an electrochemical gradient of protons, or proton motive force (pmf), comprised of electrical (Δψ) and osmotic (ΔφH) components. Both components can drive the synthesis of ATP at the chloroplast ATP synthase, but the ΔφH component also plays a key role in regulating photosynthesis, down-regulating the efficiency of light capture by photosynthetic antennae via the qE mechanism, and governing electron transfer at the cytochrome bc1 complex. Differential partitioning of pmf into ΔφH and Δψ has been observed under environmental stresses and proposed as a mechanism for fine-tuning photosynthetic regulation, but the mechanism of this tuning is unknown. We show here that putrescine can alter the partitioning of pmf both in vivo (in Arabidopsis mutant lines and in Nicotiana wild type) and in vitro, suggesting that the endogenous titer of weak bases such as putrescine represents an unrecognized mechanism for regulating photosynthetic responses to the environment.

Introduction

The light-driven transthylakoid proton motive force (pmf) plays several essential roles in photosynthesis [1]. Both the ΔφH (osmotic) and Δψ (electric) components of pmf contribute to ATP synthesis at the CF0-CF1 ATP synthase, in a thermodynamically equivalent fashion [2], but the ΔφH component of pmf is also a key signal for initiating photoprotection of the photosynthetic reaction centers through energy-dependent non-photochemical quenching (qE), a process that dissipates excess absorbed light energy as heat, thus protecting the photosynthetic apparatus from photodamage [3–5]. Acidification of the lumen also controls photosynthetic electron transfer by slowing the rate of plastoquinol oxidation at the cytochrome bc1 complex [6,7], preventing the accumulation of highly reducing species within photosystem I [8].

Differential partitioning of the thylakoid pmf into ΔφH and Δψ components has been observed in thylakoids [9] and in intact leaves [10] and was proposed to constitute an important fine-tuning mechanism for photosynthesis [11]. Under optimal conditions, when down-regulation is not needed, a large fraction of pmf can be stored as Δψ, leading to moderate lumen pH and low qE, even at high pmf (and thus high rates of ATP synthesis). In contrast, under environmental stresses—e.g., high light, low CO2/O2, phenolics), this mechanism may constitute a means to adjust the ΔφH/Δψ ratio in the short (seconds) and long term (hours to days).

Putrescine (Put) is a diamine [NH2(CH2)4NH2], which, along with spermidine and spermine, constitute the major polyamines in plants. Polyamines are important or even essential for many weak bases, such as polyamines, which occur normally in chloroplasts and may act as ‘permeant buffers’, specifically dissipating the ΔφH component and thus favoring Δψ. Because the titer of these weak bases can be regulated by the organism (by synthesis, degradation, transport, covalent binding to proteins, and phenolics), this mechanism may constitute a means to adjust the ΔφH/Δψ ratio in the short (seconds) and long term (hours to days).

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cellular processes, such as cell growth and stress tolerance [13–15]. Although the metabolism of polyamines is well understood, their mode of action is ill defined (for reviews see refs [16] and [17]). The proposed mechanism for putrescine action is similar to that by which amines dissipate the $\Delta \psi$ component of $pmf$ in isolated thylakoids [18]. Under physiological pH, the protonated forms of amines prevail but are in equilibrium with a small concentration of free base, which can permeate the membrane (see Figure S1). Because these forms are positively charged, they cannot readily cross the thylakoid membrane. Acidification of the lumen will displace the equilibrium toward the charged forms, in turn allowing diffusion of more free forms across the thylakoid membrane into the lumen. Net transfer of weak bases from stroma to lumen and conversion to the protonated forms dissipates $\Delta \psi$, but builds up a gradient of charged bases. Because the process is electroneutral with respect to the thylakoid membrane, weak bases do not dissipate (or augment) the $\Delta \psi$ component of $pmf$. However, weak bases in the presence of high concentrations of countercations, which are permeable through ion channels, can dissipate both the $\Delta \psi$ and $\Delta \rho$ components of $pmf$ [18]. It is important to note that the concentrations of permeable ions in chloroplasts in vivo is likely to be small [29], so that weak bases should primarily affect the $\Delta \rho$ component of $pmf$.

**Results and Discussion**

As a first test of the BWB hypothesis in vivo, we assessed the fractions of $pmf$ stored as $\Delta \psi$ and $\Delta \rho$ using in vivo spectroscopic techniques [7,10,19] in leaves depleted of or re-infiltrated with putrescine. We found that putrescine is highly mobile and readily diffuses into (Figure S2) and out of (Figure S3) leaves through cut petioles placed in water solutions. In figure S3 we report a decrease of the endogenous $\Delta \rho$ of putrescine. It is plausible to assume that at least in part this is due to loss of putrescine from the cut petiole. This leakage of endogenous putrescine from the leaf to the water is in line with the increase of putrescine $\Delta \rho$ of the solution from 0 $\mu$M to about 3.2 $\mu$M. Using cut tobacco leaves, which are stable in such solutions for long periods (e.g., Figure S3 which shows that photosystem II is stable in tobacco leaves for 48 h), we were able to deplete and replete putrescine levels, as confirmed by HPLC analysis [20]. Incubation for ~15 h after leaf detachment led to a 47% decrease in putrescine $\Delta \rho$, whereas feeding putrescine with 6 mM increased putrescine 4–5 times (Figures S2 and S3). After 16 h of incubation of the leaf petiole in a 3 mM putrescine solution, the putrescine $\Delta \rho$ was increased at the tip of the leaf about 2-fold. Figure 1a shows the change of the putrescine $\Delta \rho$ in vivo. The above experiments show a positive relationship between putrescine $\Delta \rho$ in leaves, altered by depletion, infiltration, or mutation, and the fraction of $pmf$ stored as $\Delta \rho$, consistent with the BWB hypothesis. At pH ~7.5 or lower, putrescine should predominantly be in its t+2 state ($pK_a = 10.5$, $pK_b = 9.04$), and its effects have been attributed to its ability to bind anions, nucleic acids, and other negatively charged molecules or domains [15,17,23]. It is possible that putrescine affects $pmf$ partitioning by scavenging anions that would otherwise permeate the thylakoid and dissipate $\Delta \psi$. To test these possibilities, we assayed the effects of putrescine on $\Delta \psi$/$pmf$ using ECS assays [9,24] in isolated spinach thylakoids in buffer with set ionic composition. The dependence of $\Delta \psi$/$pmf$ on putrescine concentration is shown in Figure 4 with selected ECS traces shown in the inset. Putrescine is a naturally occurring solute in chloroplasts but the endogenous pool is lost during isolation procedures, together with other stromal solutes. In the presence of 0 mM KCl and only a low dose of bivalent cations (i.e., 0.15 mM MgCl$_2$) (solution of low ionic strength), the fraction of $pmf$ stored as $\Delta \psi$ was about 0.2 for control, indicating that a significant decline occurred after isolation in comparison to the in vivo conditions ($\Delta \psi$/$pmf$=0.5) (Figure 4). This decline at least partly reflects altered ionic composition of the suspension buffer compared to chloroplasts in vivo [9], but could also reflect the loss of putrescine or other mobile buffers. Adding putrescine to the suspension buffer increased $\Delta \psi$/$pmf$ of thylakoids within a few seconds, indicating that putrescine can rapidly modulate $pmf$ partitioning. Addition of 220 $\mu$M putrescine led to an increase in $\Delta \psi$/$pmf$ to ~0.6, and putrescine concentrations above 2 mM led to $\Delta \psi$/$pmf$>0.9 (Figure 4). Our deconvolution suffers to some extent by drifts and noise due to in vitro conditions. Thus the absolute values of $\Delta \psi$/$pmf$ caused by putrescine could be slightly different to those of figure 4 (~15%). However, the effect of putrescine on thylakoidal energization is initially a rapid increase of $\Delta \psi$ and then at a second phase a plateau.
The fact that putrescine decreased $\Delta p\text{H}$ even in a solution with low concentration of scavangeable anions, supports the operation of the BWB mechanism, and is also consistent with the effects of exogenously added weak bases in photosynthetic prokaryotes [25] and thylakoids [26,27] in which amines have been shown to be concentrated by more than 100-fold on the low pH side of an energized membrane, with concomitant effects on $\Delta p\text{H}$. We would suggest that putrescine is a good compromise between balancing $\Delta p\text{H}$ and $\Delta\psi$ and avoiding secondary deleterious effects. In addition, putrescine levels in the cell is so finely tuned through multiple ways (synthesis through two highly regulated pathways, conversion from Spermidine, transport from other cell compartments or neighbour cells, release from conjugate with phenolics, binding to proteins etc [15,16]) that one can not find easily other cell metabolites that can adjust their level so rapidly and accurately to meet the ever changing demand. So although ammonia and methylamine could act in a similar way to putrescine their titer in cells is lower than that of putrescine and thus their importance in the BWB mechanism should be lower.

Assuming that putrescine is evenly distributed in leaves, we estimate its cellular concentration in tobacco leaves at about 275 mM (based on measured value of 250 nmoles Put/g fresh weight and assuming 90% of leaf mass is water; see also, ref. [28]). When thylakoids were suspended in buffer containing this concentration of putrescine in thylakoids, we observed about

**Figure 1. Regulation of the electric field component ($\Delta\psi$) of $pmf$.** Panel A shows typical deconvoluted traces obtained from intact tobacco leaves incubated with water (gray) or reinfiltrated with 3 mM putrescine (Put; black). Steady-state $pmf$ was probed at 8 different light intensities. Leaves with elevated putrescine (open symbols) show up to 40% higher $\Delta\psi/\Delta p\text{H}$ than the corresponding controls (closed symbols) (Panel B). Buffering of the thylakoid lumen by elevated putrescine levels allows more efficient electron transfer at higher light intensity in comparison to low putrescine levels (Panel C). D. Energy-dependent antenna down-regulation ($qE$) as a function of the $\Delta p\text{H}$ component of the light-induced $pmf$. The linear fit for control has a slope of 0.369 ($R^2 = 0.998$) and for putrescine treated a slope of 0.272 ($R^2 = 0.992$), showing that putrescine supply decreases sensitivity of $qE$ to ECSt by about 27%. All single points are means from 4 independent experiments performed with intact tobacco leaves and bars denote standard error.

doi:10.1371/journal.pone.0029864.g001

**Figure 2. The dependence of $\Delta\psi/\Delta p\text{H}$ in intact leaves on endogenous putrescine titer.** Partitioning of $pmf$ in excised tobacco leaves. Detachment of leaves and insertion of the petiole in distilled water leads to a gradual decrease of putrescine titer in the leaf, which in turn leads to a decrease in the $\Delta\psi$ fraction of $pmf$. Data correspond to the first two days after detachment ($Fv/Fm$ decrease during this period was no more than 10%) and bars denote standard error ($n = 2$). Linear regression of putrescine titer versus $\Delta\psi$ is shown as a solid line with a slope of 0.00173 ($R^2 = 0.995$).

doi:10.1371/journal.pone.0029864.g002
50% of pmf stored as Δψ and ΔpH (Figure 4), similar to what is observed in healthy, unstressed leaves [9,11]. However, it is important to note that the partitioning of pmf is also expected to be influenced by ionic composition, with increasing counterion concentration dissipating Δψ [9].

Putrescine concentrations in leaves are increased during environmental stress, in part due to up-regulation of chloroplast arginine decarboxylase (ADC, EC 4.1.1.19) [29–31]. One may thus expect to see a shift in the partitioning of pmf into Δψ under environmental stress. However, at least under drought stress in wild watermelon [32], short-term high light exposure in Arabidopsis [7], or low CO₂ and O₂ in tobacco [10], the opposite was observed, with a pronounced increase in ΔpH/Δψ. A reasonable explanation is that the initial increase in ΔpH/Δψ is due to stress-induced changes in ionic composition [9,11] and that putrescine may ameliorate these effects over the long term. At a mechanistic level, consistent with this view, Cruz et al. [9], showed that elevated lumen buffering capacity will increase the counterion chemical activity needed to dissipate the Δψ component of pmf, whereas Ioannidis et al. [12] showed that putrescine can overcome photosynthetic control and thus stimulate chemiosmotic ATP synthesis in thylakoids of higher plants [12].

In Arabidopsis grown under high salt stress, photosynthesis would likely need to operate under conditions where the ionic strength inside the plastid is high. In this case, pmf storage would be heavily regulated by the partitioning of pmf into Δψ and ΔpH.
biased toward ΔpH formation [9,33]. Consequently, energy dissipation would be more easily and strongly induced at low and moderate light intensities, severely limiting the productivity and growth of the plant, even if water and CO₂ were not limiting. Thus, the accumulation of putrescine observed in plants grown under high salt stress [34,35] and particularly in Arabidopsis through adc2 induction [36] could serve to increase the BWB effect, rebalancing pmf toward Δψ and optimizing the regulation of energy transduction. In this view, blocking this up-regulation of putrescine during salt stress, e.g., in the adc-2-1 mutant of Arabidopsis, leads to increased sensitivity to salt stress, which is restored upon addition of putrescine [36], whereas over-expressing adc increased tolerance to drought [13].

We present evidence that putrescine plays a role in modulating pmf partitioning in vivo via the BWB mechanism, possibly operating as a part of the regulatory network of photosynthesis. Putrescine levels in the leaf are known to be regulated at several levels, including rates of synthesis, catabolism, conversion to spermidine, conjugation, intracellular or extracellular transport, gene expression, and/or alloster [15]. Thus, putrescine homeostasis could provide the plant with an independent mechanism for adapting the qₑ response to pmf, optimizing the balance between energy transduction and dissipation under a variety of stress conditions.

Materials and Methods

Spectroscopy

We conducted time-resolved spectroscopic measurements for estimation of LEF, qₑ, ECS, and Δψ/pmf at room temperature using wild-type (Wt) Arabidopsis thaliana (Wassilewskija ecotype; WS) plants, the low putrescine adc-2-2 mutant [21], and wild-type Nicotiana tabacum cv Xanthi as previously described [10]. More particularly, Wt was defined for the following expression 0.84*PAR*(Fm’-Ft)/Fm’ where Fm’ is the maximal fluorescence value of a light adapted leaf after a saturating pulse (>7,000 μmol photons m⁻²s⁻¹) and Ft is the level of fluorescence immediately before the saturating pulse. For estimates of pmf partitioning, the ECS was measured over longer dark intervals (60 sec) and deconvoluted as previously described [10,11] using the following equation for tobacco: ECS₂₅₀ = A₂₅₀ − 0.5 × A₃₅₃ − 0.5 × A₁₅₀. For more precise deconvolution of ECS signals from Arabidopsis, we derived an equation from empirically determined, relative extinction coefficients at each wavelength, based on methods described previously [37,30]: ECS₂₅₀ = 0.17*A₁₅₀ + 0.61*A₂₅₀ − 1.17*A₃₅₃.

Determination of putrescine titer

Putrescine titer in leaves was estimated after benzoylation of the amines, separation in HPLC, and quantitation of the derivatives as previously described [20].

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In vitro experiments

Thylakoids were isolated from market spinach as previously described [9]. Freshly isolated thylakoids (10 μg/mL Chl) were treated with various doses of the amine salt in the dark and, after equilibration, were subjected to light for 16 s. The final volume was 3 mL of a working solution containing 0.125 mM tricine, pH 7.8, 0.15 mM MgCl₂, 0.3 M sucrose, 10% ficoll. Photosynthetic proton uptake was supported by 30 μM PMS. Spectroscopic measurements were performed, as above, with the sample contained in a cuvette (10-mm path length). For thylakoid samples, a simple deconvolution yielded consistent results: ECS₂₅₀ = A₂₅₀ − 1.2 × A₃₅₃.

Supporting Information

Figure S1 Simplified scheme for the regulation of pmf partitioning by Putrescine.
(DOC)
Figure S2 Assay for putrescine uptake into tobacco leaves by HPLC.
(DOC)
Figure S3 Effects on Δψ/pmf and PSII photochemical efficiency of infiltration of leaves with water.
(DOC)
Figure S4 Effect of putrescine titre on the dependence of energy-dependent antenna down-regulation (qₑ) on the Δψ component of light-induced pmf.
(DOC)
Figure S5 Energy-dependent antenna down-regulation (qₑ) as a function of total light-induced pmf.
(DOC)
Figure S6 Effects of putrescine titre on the apparent proton conductivity of the ATP synthase (gH⁻) in vivo.
(DOC)

Acknowledgments

We thank Prof. Dr. Polychronis Kostoulas (University of Thessaly) for the linear regression model and RIKEN and Profs. Dr. Urano and Dr. Shinozaki for the generous gift of adc-2-2 mutant.

Author Contributions

Conceived and designed the experiments: NI, DK. Performed the experiments: NI, JC. Analyzed the data: NI, JC, DK. Contributed reagents/materials/analysis tools: KK, DK. Wrote the paper: NI, JC, KK, DK.
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