Single point mutation in the Rieske iron–sulfur subunit of cytochrome b_6/f leads to an altered pH dependence of plastoquinol oxidation in *Arabidopsis*

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Abstract The *pgr1* mutant of *Arabidopsis thaliana* carries a single point mutation (P194L) in the Rieske subunit of the cytochrome b_6/f (cyt b_6/f) complex and is characterised by a reduced electron transport activity at saturating light intensities in vivo. We have investigated the electron transport in this mutant under in vitro conditions. Measurements of P700 reduction kinetics and of photosynthetic electron transport rates indicated that electron transfer from cyt b_6/f to photosystem I is not generally reduced in the mutant, but that the pH dependence of this reaction is altered. The data imply that the pH-dependent inactivation of electron transport through cyt b_6/f is shifted by about 1 pH unit to more alkaline pH values in *pgr1* thylakoids in comparison with wild-type thylakoids. This interpretation was confirmed by determination of the transmembrane ΔpH at different stromal pH values showing that the lumen pH in *pgr1* mutant plants cannot drop below pH 6 reflecting most likely a shift of the p*K* and/or the redox potential of the oxidised Rieske protein.

Key words: Cytochrome b_6/f complex; pH regulation; Photosynthetic electron transport; Plastoquinol oxidation; Rieske iron–sulfur protein; *Arabidopsis thaliana*

1. Introduction

Photosynthesis of higher plants is driven by light-induced electron transfer from the water oxidising photosystem II (PSII) to the ferredoxin reducing photosystem I (PSI). Electron transport between the two photosystems is mediated by the cytochrome b_6/f complex (cyt b_6/f) which transfers electrons from plastoquinol to plastocyanin. This reaction is accompanied by proton release into the thylakoid lumen. Plastoquinol oxidation by cyt b_6/f is the rate limiting step of photosynthetic electron transport in higher plants and is controlled by the lumen pH (see [1] for an overview).

The Rieske iron–sulfur protein (ISP) plays a central role in electron and proton transfer in all *bc* and *bf* complexes. Crystal structures of the mitochondrial cyt bc_1 complexes from beef and chicken heart [2–5] indicated a pronounced conformational flexibility of the ISP within the complex which is assumed to be essential for both electron and proton transfer [3,4]. From the high similarity of the [2Fe–2S] cluster-binding domain of the ISP of the bovine bc_1 and spinach b_6/f complexes a similar flexibility and role have been concluded for the ISP in chloroplasts [6,7].

In *bc* complexes of *Rhodobacter sphaeroides* and bovine mitochondria, a histidine ligand to the [2Fe–2S] cluster, H152 in *R. sphaeroides* and H161 in bovine mitochondria, has been identified as the group responsible for the p*K* of about 7.5 of the oxidised ISP [8,9] giving rise to the pH dependence of electron transfer through the *bc* complex. It has been shown for different organisms that site-directed mutagenesis of amino acids close to this region can induce modifications of the ISP redox potential [10–12]. In *R. sphaeroides*, the Y156W exchange was accompanied by a shift of the pH dependence of quinol oxidation by about 1 pH unit to more alkaline pH [12].

In contrast, the p*K* of the ISP redox potential in the two acidophilic prokaryotes *Sulfolobus acidocaldarius* and *Thiobacillus ferrooxidans* is generally shifted by almost 2 pH units to more acidic pH [13,14], reflecting an adaptive response to the acidic environment of these organisms [14]. Similarly, the acidic p*K* of about 6.5 for the oxidised ISP in chloroplasts of higher plants has been related to the pH dependence of the chloroplast ISP redox potential [15], in agreement with the pH dependence of plastoquinol oxidation [16] and cyt *f* reduction [17].

Recently, a point mutation in the ISP (P194L) has been shown to affect thermal dissipation of absorbed light energy in the *pgr1* mutant of *Arabidopsis thaliana* [18]. Physiological characterisation of this mutant under in vivo conditions indicated that photosynthetic electron transport was unchanged at low light intensities, but significantly reduced under saturating light conditions. In addition, the reduced capacity of thermal energy dissipation in high light was accompanied by the retardation of violaxanthin de-epoxidation kinetics, both indicating a limited acidification of the thylakoid lumen under these conditions in the mutant [18]. In the present study, we have analysed the electron transport in isolated thylakoids from wild-type (WT) and *pgr1* mutant plants and focused on the pH dependence of plastoquinol oxidation.

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Abbreviations: cyt b_6/f, cytochrome b_6/f complex; ISP, iron–sulfur protein; PSI, photosystem I; PSII, photosystem II; WT, wild-type; 9-AA, 9-aminoacridine

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2. Materials and methods

2.1. Plant materials and growth conditions

A. thaliana WT (ecotype Columbia *gl1*) and *pgr1* were cultured in soil under greenhouse conditions (80–100 μmol photons m^{-2} s^{-1}, 16 h light/8 h dark cycles) for 4–6 weeks.

2.2. Chloroplast preparation

Leaves from 4–6 weeks old plants were homogenised in a medium containing 330 mM sorbitol, 20 mM Tricine/NaOH pH 7.6, 5 mM EGTA, 5 mM EDTA, 10 mM $NaCO_3$, 0.1% (w/v) BSA, and 330 mg l^{-1} ascorbate. After centrifugation for 5 min at $2000\times g$, the pellet was resuspended in 300 mM sorbitol, 20 mM HEPES/KOH pH 7.6, 5 mM $MgCl_2$, and 2.5 mM EDTA. With this procedure, high yields of broken chloroplasts were obtained, which retained high photosynthetic activity for several hours.

2.3. Measurement of 9-aminoacridine (9-AA) fluorescence

Fluorescence of 9-AA was measured with the instrumental setup as described [19]. Measurements were performed in a medium containing 50 mM Tricine/NaOH pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM phenazine methosulphate and thylakoids equivalent to 25 μg chlorophyll ml^{-1}. After registration of the basal fluorescence, 9-AA was added to yield a final concentration of 25 μM.

The maximum ΔpH was calculated from the quenching of 9-AA fluorescence under illumination with saturating actinic red light ($\lambda > 630$ nm) following the approach by Schuldiner et al. [20] and applying the calculations from Laasch et al. [21].

2.4. Photosynthetic electron transport rates

Electron transport rates under saturating illumination with red light ($\lambda > 630$ nm) were derived from measurements of photosynthetic oxygen evolution using a Clark type oxygen electrode. Thylakoids equivalent to 10 μg chlorophyll ml^{-1} were used for all measurements. Linear electron transport ($H_2O \rightarrow K_3[Fe(CN)_6]$) was measured in a medium containing 330 mM sorbitol, 40 mM HEPES/NaOH pH 7.6, 10 mM NaCl, 5 mM $MgCl_2$, 2 μM gramicidin D, and 5 mM NH_4Cl using 0.5 mM $K_3[Fe(CN)_6]$ as terminal electron acceptor.

2.5. Measurements of P700 reduction kinetics

The transient oxidoreduction of P700 [22] was measured flash-spectrophotometrically (for methodological details see [23]). Thylakoids equivalent to 10 μg chlorophyll ml^{-1} were suspended in a medium containing 5 mM $MgCl_2$, 10 mM NaCl, 0.3 M sorbitol. 5 mM Tricine/NaOH was used as buffer in the pH range from 7.0 to 8.0, 5 mM MOPS/NaOH in the pH range from 6.0 to 7.0 and 5 mM MES/NaOH in the pH range from 5.0 to 6.0. 200 μM $K_3Fe(CN)_6$ was added as electron acceptor.

3. Results

3.1. Kinetics of P700 reduction

Electron transfer from cyt b_6/f to PSI has been investigated in isolated thylakoids from WT and *pgr1* mutant plants of *Arabidopsis* by flash-spectrophotometric measurements of P700 reduction. In comparison with WT thylakoids, the reduction of P700 in the mutant was differentially altered in respect of its dependence on the pH of the suspension medium (Fig. 1). At pH 7.5 and 8, the kinetics of P700 reduction were nearly identical in both types of plants. Obviously, electron transfer through the Rieske protein was not disturbed in general by the P194L exchange in this subunit. At pH values between 7.0 and 6.0, however, the kinetics of P700 reduction were strongly retarded in the mutant, but only slightly reduced in WT thylakoids. After further acidification of the reaction medium to pH 5, the electron transfer kinetics were also slowed down in WT thylakoids (see Fig. 1). These data imply that the general function of the Rieske protein in electron transfer is not impaired in the *pgr1* mutant but only the pH dependence of this reaction.

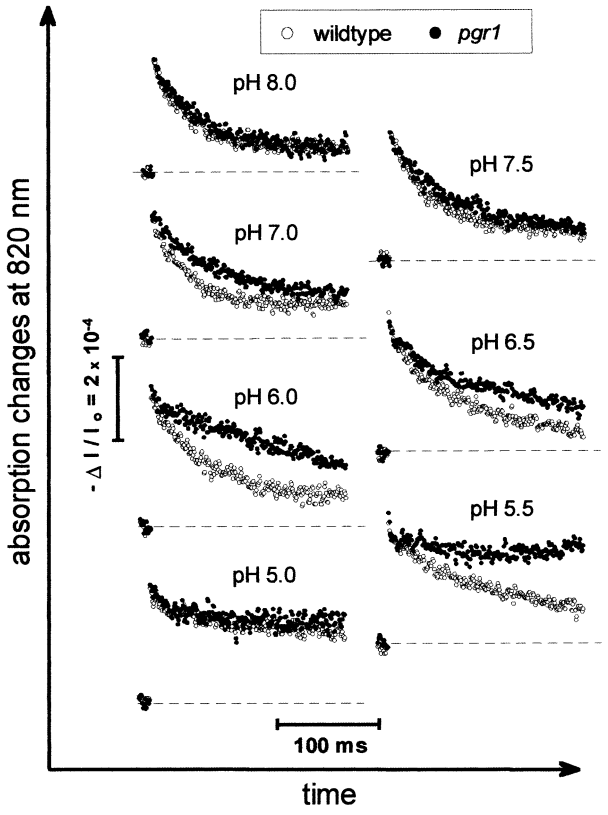

Fig. 1. Flash-induced transient oxidation and rereduction of P700. Time resolution 200 μs per point. Every fifth point is shown. Samples were excited with saturating single turnover flashes ($\lambda > 610$ nm). 40 signals were averaged under repetitive excitation (0.1 Hz). The dotted lines indicate the level of fully reduced P700 prior to excitation. 10 measuring points before excitation are shown for each signal. Upward-directed signals indicate oxidation of P700.

3.2. Transmembrane proton gradient

The pH of the thylakoid lumen generated by illumination of isolated thylakoids has been estimated from changes in the fluorescence of 9-AA. The pH of the suspension medium (and thus at the stromal side of the thylakoid membrane) was varied between pH 8 and 7 (Fig. 2). In both types of plants the apparent transmembrane ΔpH was reduced with decreasing medium pH showing that the lumen pH and no other factors like electron transport capacity or proton conductance of the thylakoid membrane were limiting further acidification. Otherwise, one would have expected that, particularly in the mutant, the apparent transmembrane ΔpH would have remained constant, independent of the medium pH. The data further show that the apparent transmembrane pH in the mutant is about 1 pH unit smaller than in WT thylakoids so that the minimal lumen pH in WT and mutant thylakoids under saturating illumination can be estimated with about pH 5 and 6, respectively.

3.3. Rates of linear electron transport

Rates of linear photosynthetic electron transport in thylakoids from both types of plants have been determined from polarographic measurements of oxygen evolution. As shown in Fig. 3, the pH dependence of electron transport from PSII (electron donor: H_2O) to PSI (electron acceptor: $K_3[Fe(CN)_6]$) was clearly different in both genotypes. Under uncoupled conditions, no transmembrane proton gradient can

be generated under illumination so that the lumen pH is expected to be identical with the pH adjusted in the reaction medium. In WT thylakoids, maximum activities of uncoupled electron transport were yielded in a broad range between pH 6.25 and 8 and dropped to lowest values at pH 5 (Fig. 3, filled circles). In contrast, inhibition of electron transport in the mutant started at pH 7.25 and reached lowest values already at pH 6 (Fig. 3, filled squares). Obviously, the pH-dependent inactivation of linear electron was shifted by about 1 pH unit to alkaline pH values in the mutant in comparison with WT thylakoids.

Under coupled conditions, a transmembrane proton gradient is built up under illumination so that the actual lumen pH is influenced by two parameters, the pH adjusted in the medium and the light-induced acidification. Light-induced lumen acidification is supposed to generate a lumen pH of about 5 in WT thylakoids and of about 6 in mutant thylakoids (see Fig. 2). Thus no strong dependence of the electron transport rates on the medium pH can be expected under coupled conditions. This is indeed reflected by the results given in Fig. 3 showing more or less similar rates of electron transport over the whole pH range for thylakoids of both types of plants, although the rates obtained with *pgr1* thylakoids were significantly lower than those determined for WT thylakoids at most pH values.

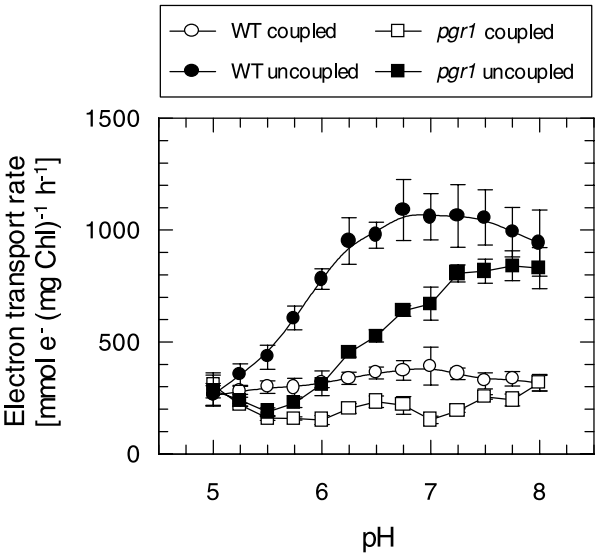

Fig. 3. pH dependence of linear electron transport. Electron transport rates from PSII (electron donor H_2O) to PSI (electron acceptor $K_3Fe(CN)_6$) were calculated from measurements of oxygen evolution under saturating red light ($\lambda > 610$ nm). Coupled (open symbols) and uncoupled (filled symbols) rates were determined with the same sample by adding the uncouplers gramicidin D (2 μM) and NH_4Cl (5 mM) after measuring coupled electron transport for 2 min. Mean values ± S.D. of four independent experiments are shown.

4. Discussion

The presented data show that the reduction of photosynthetic electron transport in the *pgr1* mutant found in vivo under saturating light conditions [18] originates from an altered pH dependence of plastoquinol oxidation. The similar maximum rates of linear electron transport at pH 8 and the similar ratio of uncoupled/coupled electron transport rates at this pH in both genotypes (Fig. 3) support the view that electron transfer in general and the membrane properties are not altered in the mutant. Since plastoquinol oxidation is the rate limiting step of linear photosynthetic electron transport in isolated thylakoids [22], the differences between WT and mutant plants in the pH dependence of electron transport can thus be directly related to the P194L mutation in the ISP of the cyt b_6/f complex. This conclusion is corroborated by the measurements of P700 reduction in single turnover experiments (Fig. 1). The identical half-times of 20–30 ms for the rereduction of P700 at pH 8 in both genotypes are in agreement with the kinetics of earlier determinations in spinach and pea [22,24] and with the rates of cyt *f* reduction in leaves from different plants [1] underlining the fully functional electron transport capacity in mutant thylakoids at neutral and alkaline lumen pH.

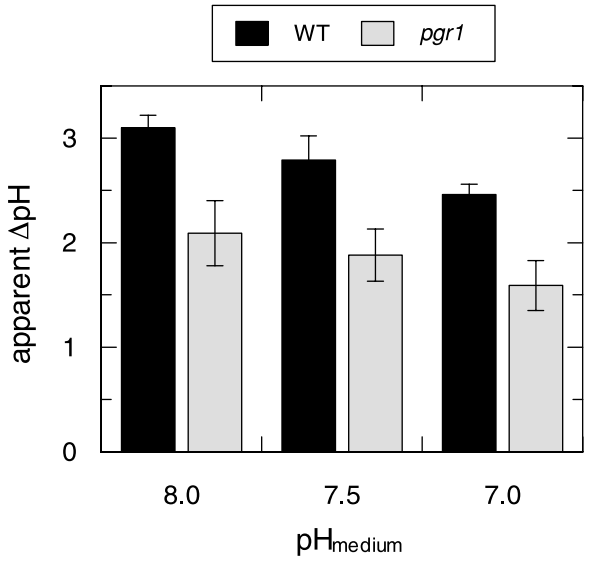

Fig. 2. Measurement of the transmembrane ΔpH. The maximum light-induced (1500 μmol photons m^{-2} s^{-1}) pH difference across isolated thylakoid membranes from WT and *pgr1* plants was estimated from the quenching of 9-AA fluorescence. Mean values ± S.D. of three to five independent experiments are shown.

The determination of the transmembrane ΔpH at varying pH values on the outer, stromal side of the thylakoid membrane (Fig. 2) has shown that the lumen pH remains nearly constant independent of the outer pH. Although no calibration of the 9-AA signal in *Arabidopsis* thylakoid membranes has been established so far, the similarity of the obtained results with those reported for spinach thylakoids [25] supports the reliability of the calculations. Therefore, we can conclude from these data that the lumen pH in *pgr1* plants is adjusted to about pH 6 at saturating illumination under both in vivo and in vitro conditions. This implies that at a lumen pH of about 6 proton release from plastoquinol oxidation at the cyt b_6/f complex is in equilibrium with proton reflux to the stromal side of the membrane. As estimated from Fig. 1 the half-time of P700 reduction and thus of plastoquinol oxidation is about 150 ms at this pH in *pgr1* thylakoids, so that a reduction of electron transport by a factor of 5–10 in comparison with the maximum kinetics at pH 8 is sufficient to prevent lumen acidification below pH 6. A similar reduction of the kinetics was found for WT thylakoids when

the pH was dropped below 5.5 (Fig. 1), in agreement with the data from the literature (see [1] and references therein).

The shift in the pH dependence of cyt b_6/f activity in the mutant in comparison with WT plants implies that the P194L mutation induced a p*K* shift of about 1 pH unit in an amino acid of the ISP, which is essential for electron transfer and thus plastoquinol oxidation. As the p*K* of a histidine ligand to the [2Fe–2S] cluster (H161 in the ISP of bovine mitochondria) is supposed to be responsible for the p*K* of the oxidised ISP [8,9], it is likely that the P194L mutation altered the p*K* of this ligand (corresponding to H178 in *A. thaliana*). It is tempting to speculate that the shift in the pH dependence of cyt b_6/f activity in the mutant is accompanied by the reduction of the redox midpoint potential of the ISP in *pgr1* plants. However, it is known from specific mutagenesis of the ISP in *R. sphaeroides* that changes in the redox potential of the ISP are not necessarily paralleled by changes of the p*K*, while on the other hand a shift of the p*K* by about 1 pH unit to more alkaline pH may be accompanied by a reduction of the E_m by about 120 mV [15]. Thus it is clear that direct determination of the E_m is necessary to evaluate the influence of the P194L mutation on the redox potential of the ISP.

Alignment of the ISP sequences from different organisms [6,18,26] revealed that the mutation in *pgr1* plants is located in the 'proline loop' of the ISP, 16 amino acids separated from one of the histidine ligands to the [2Fe–2S] cluster (H178 in *A. thaliana* or H161 in bovine mitochondria), which is located in the 'ligand loop II'. It can be speculated from our data that amino acids in the proline loop may influence the p*K* of this histidine and thus the pH dependence of electron transfer through the ISP. As estimated from the published structure of the ISP in bovine mitochondria [9], however, the spatial distance of about 10 Å between H178 and P194 seems to be too large to allow a direct interaction of these two residues. Thus it looks more likely that an indirect interaction – possibly through H141, another ligand to the [2Fe–2S] cluster, which is located between P194 and H178 – is responsible for the observed shift in the pH dependence of plastoquinol oxidation. It is interesting to note that in *S. acidocaldarius*, an acidophilic archaeon, an altered p*K* of the oxidised ISP is also paralleled by the exchange of the corresponding proline to valine in the Rieske ISP encoded by the soxL gene [26]. In conclusion, the *pgr1* mutant is an interesting tool to study the pH regulation of electron and proton transfer through the cyt b_6/f complex and the role of amino acids in the 'proline loop' in determining the p*K* of the oxidised ISP.

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