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Estimation of threshold of lumenal pH inducing thermal dissipation of absorbed light energy using an *Arabidopsis* cytochrome b_6f mutant

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Introduction

Lumenal pH controls the efficiency of photosystem (PS) II photochemistry by dissipating excitation energy as heat, which prevents production of toxic active oxygen (Niyogi, 1999). Low lumenal pH induces thermal dissipation in two ways, conversion of violaxanthin (Vx) to zeaxanthin (Zx) via antheraxanthin (Ax) in the so-called xanthophyll cycle (Demmig *et al.*, 1987) and conformational changes of light-harvesting complex II (Horton *et al*., 1996). The amount of thermal dissipation is monitored as energy dependent nonphotochemical quenching of chlorophyll (Chl) fluorescence (NPQ) (Krause and Weis, 1991). Since thermal dissipation competes with photochemistry, precise energy dissipation is required for plant to maintain maximum photosynthesis under fluctuating light condition.

In this report, we characterized an *Arabidopsis* mutant, *pgr1 (proton gradient regulation)*. The *pgr1* phenotype was rather specific to thermal dissipation, although the mutation was in the Rieske subunit of cytochrome (Cyt) b_6f functioning in electron transport chain. We estimated lumenal acidification required for inducing thermal dissipation, with using *pgr1* completely lacked energy dependent NPQ. The results suggest physiological significance of photosynthesis regulation by lumenal pH.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana wild type (ecotype Columbia *gl1*) and *pgr1* previously referred to as CE10-10-1 (Shikanai *et al*., 1999) were cultured in soil under the culture room conditions (50 μE m^{-2} s^{-1}, 16 h-light / 8 h-dark cycles at 23ºC) for 25 days.

Physiological analysis

Chl fluorescence was measured with a pulse-amplitude modulation (PAM) Chl fluorometer (Walz, Effeltrich, Germany) with an emitter-detector unit ED101 as described (Schreiber *et al*., 1986).

For Vx de-epoxidation experiment, leaf discs were floated on water in a temperature-controlled cuvette (20°C) and illuminated at 1,500 μE m^{-2} s^{-1}. At different times, leaf discs were frozen in liquid N_2 then extracted into 1 mL 80% (w/v) acetone. After short centrifugation, pigments were separated by reverse-phase HPLC as described (Färber *et al*., 1997).

Measurement of 9-aminoacridine (9-AA) fluorescence were performed in a medium containing 50 mM Tricine/NaOH pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM phenazine methosulfate and thylakoids equivalent to 25 μg Chl 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 (λ > 630 nm) (Laasch *et al*., 1993; Lohse *et al*., 1989; Schuldiner *et al*., 1972).

Results

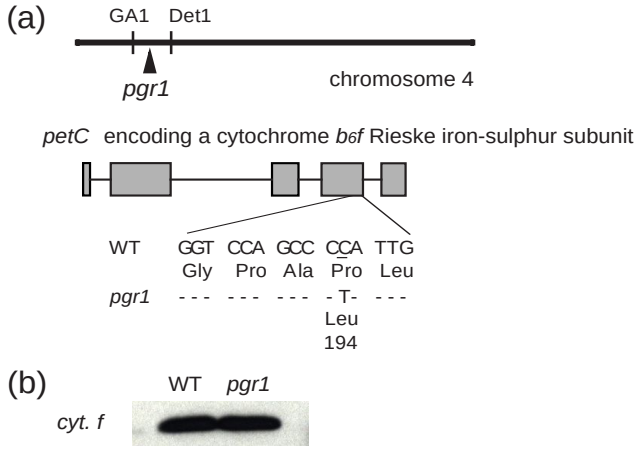

Figure 1. Map-based cloning of *pgr1*. (a) *pgr1* mutation was single nucleotide substitution in the forth exon of *petC* (C to T) resulting in a change from Pro 194 to Lue. Boxes stand for exons. (b) Western analysis of Cyt *f* in the wild type (WT) and *pgr1*.

pgr1 was isolated as a mutant lacking NPQ with using a screening system of Chl fluorescence imaging (Shikanai *et al*., 1999). The *pgr1* locus was mapped on a bacterial artificial chromosome (BAC) clone, F4C21, between molecular markers GA1 and Det1 on chromosome 4 (Figure 1a). From the genome sequencing, one nucleotide substitution was found in the forth exon of *petC* encoding Cyt b_6f Rieske iron-sulfur protein (C to T) (Figure 1a). The mutation led an amino acide alteration in a highly conserved domain which is likely to be important to fix a loop forming a 2Fe-2S center (Pro 194 to Lue) (Carrell *et al*., 1997). To conform that impaired NPQ phenotype in *pgr1* due to the mutation in *petC*, *pgr1* was transformed with wild-type *petC* gene. The extent of NPQ was partially restored in the transformants (data not shown). Partial complementation is explained by the semi-dominant nature of the *pgr1* mutation. Western analysis showed normal accumulation of Cyt *f* in *pgr1,* suggesting that the *pgr1* mutation affects the activity of Cyt b_6f rather than the complex stability (Figure 1b) *pgr1* can glow as well as the wild type on the soil and its maximum activity of PS II estimated from Fv/Fm was not affected. Although quantum yield of PS II ($\Phi_{PS\ II}$) was the same as in the wild type at low light intensity, it was affected at high light intensity. *pgr1* was significantly impaired in NPQ induction at high light intensity (Table I). NPQ formed during 5-min actinic light irradiation did not relax in the dark period of 3 min (data not shown) indicating that the remaining NPQ was not related with energy dependent NPQ. These results suggest that in *pgr1,* electron transport is limited by mutated Cyt b_6f at high light intensity so that ΔpH is insufficient to induce NPQ.

Table I. Steady-state Chl fluorescence parameters after 5-min irradiation. (n=4)

		low light (80 μE m^{-2} s^{-1})	high light (1000 μE m^{-2} s^{-1})	
	Fv/Fm	$\Phi_{PS\ II}$	$\Phi_{PS\ II}$	NPQ
Wild type	0.790±0.005	0.707±0.005	0.166±0.019	1.46±0.11
pgr1	0.793±0.009	0.716±0.016	0.066±0.018	0.36±0.03

To estimate the lumenal pH at high light intensity in *pgr1*, we analyze the kinetics of Vx de-epoxidation *in vivo*, which is known to be strongly retarded at a lumenal pH ≥ 6.2 (Pfündel and Dilley, 1993; Jahns and Heyde, 1999). The conversion of Vx to Zx was significantly slower in mutant plants than in the wild type (Figures 2). About 50% of the total Vx was converted into Zx and Ax within 10 min in the wild type, while the same degree of conversion was achieved in *pgr1* after 120 min. These results corroborate our assumption that the pH generation is impaired in *pgr1*. In comparison with earlier data (Pfündel and Dilley, 1993; Jahns and Heyde, 1999), the observed reduction of the de-epoxidation kinetics indicates that the lumenal pH in *pgr1* should be in the range between 6.0 and 6.4 even under saturating light conditions.

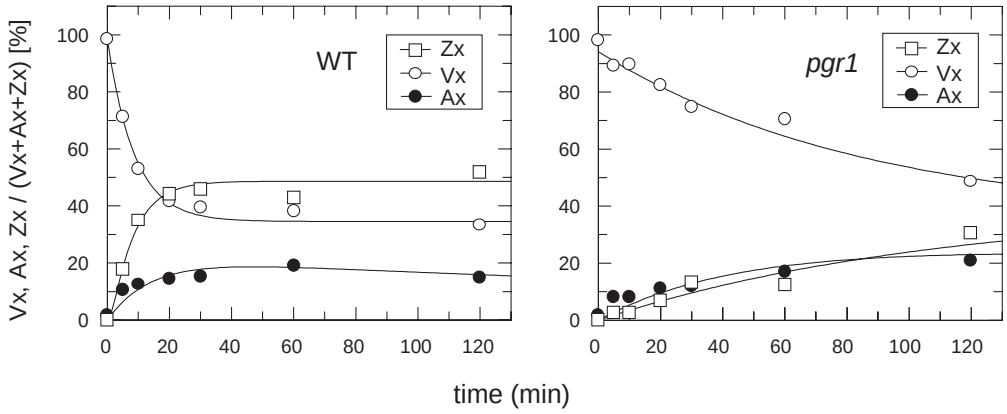

Figure 2. Time course of Vx de-epoxidation in the wild type (WT) and *pgr1*.

To evaluate the activity of ΔpH generation more quantitatively, we estimated the ΔpH from the quenching of 9-AA fluorescence in isolated thylakoids (Table II). The maximum transmembrane ΔpH was 1 unit lower in *pgr1* than that in the wild type. Based on these results, the lumenal pH at saturating light intensities can be estimated roughly at about 5.0 in the wild type and 6.0 in *pgr1*, which is fully consistent with the former *in vivo* analysis of the Vx de-epoxidation.

Table II. Measurement of ΔpH in isolated thylakoids. Transmembrane ΔpH bult up in saturating light at pH 8.0 was estimated from the quenching of 9-AA fluorescence. (n=3-6)

	Wild type	*pgr1*
maximum ΔpH	3.10 ± 0.12	2.09 ± 0.32

Discussion

pgr1 completely lacked energy-dependent NPQ induction because of insufficient ΔpH generation. The mutation in the Rieske subunit restricts the electron transport through the Cyt b_6f complex so that the lumenal pH cannot drop below pH 6.0. Surprisingly photosynthetic growth was not affected; although ΔpH generation was reduced in *pgr1*. It is considered that the ΔpH built up in *pgr1* is too small to induce thermal dissipation but is large enough to drive ATP synthesis. In other words, there is a critical threshold of lumenal pH around 6.0 for the induction of thermal dissipation, which make it possible to maintain the maximum photosynthesis under fluctuating light conditions. The results also indicate that the maximum activity of the Cyt b_6f complex is necessary for rapid acclimation to changes in light conditions.

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