Journal of Integrative Plant Biology 2006, **48** (12): 1409–1414

Decrease of Photosystem II Photochemistry in *Arabidopsis ppt1* Mutant Is Dependent on Leaf Age

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Abstract

In the present study, we compared the effect of leaf age at one development stage on the photosynthetic behavior of a T-DNA-tagged allele of the *ppt1* mutant. Chlorophyll fluorescence (F_v/F_m, where F_m is the maximum fluorescence yield and F_v is variable fluorescence) and fluorescence decay kinetics of the *ppt1* mutant were different in leaves of different ages. The steady state levels of thylakoid membrane proteins in 40-day-old leaves were decreased compared with those in 20-day-old leaves and changes in photosystem (PS) II proteins were correlated with those of the F_v/F_m ratio in the *ppt1* mutant. Increased accumulation of leaf sugars was accompanied by decreased photosynthetic gene transcripts and protein content in 40-day-old leaves of the mutant. Thus, the results of the present study provide evidence for the phosphate translocator in maintaining normal photosynthesis at a late leaf age.

Key words: *Arabidopsis thaliana*; leaf age; photosynthesis; photosystem II; *ppt1* mutant.

Ma JF, Guo JK, Peng LW, Chen CY, Zhang LX (2006). Decrease of photosystem II photochemistry in *Arabidopsis ppt1* mutant is dependent on leaf age. *J Integr Plant Biol* **48**(12), 1409–1414.

doi: 10.1111/j.1672-9072.2006.00386.x; available online at www.blackwell-synergy.com, www.jipb.net

Photosynthesis is not only affected by plastid developmental stage, but also by several other metabolic processes. Mutation of a gene encoding a protein with a structural or regulatory function can interfere with chloroplast biogenesis or function, with photosynthesis, and finally, influence plant development. The *Arabidopsis ppt1* (or *cue1*) mutant is defective in AtPPT1 (at5g33320), which has been isolated in a screen for chlorophyll *a*/*b* binding protein (CAB) underexpressors (Li et al. 1995; Knappe et al. 2003). The affected gene in *cue1* is a phosphoenolpyruvate (PEP)/phosphate translocator (PPT) of the plastid inner envelope membrane (Streatfield et al. 1999). It appears that PEP is essential for plastid development and most non-green plastids have been reported to be incapable of producing PEP from Calvin cycle intermediates (Flügge 1995; Voll et al. 2002). As a key metabolite, PEP acts as a precursor and intermediate for primary and secondary plant products via the shikimate pathway, which is profoundly important to plant life (Herrmann 1995; Fischer et al. 1997; Lara et al. 2002).

Previous studies have demonstrated that the *ppt1* mutant is severely compromised in establishing photoautotrophic growth (Li et al. 1995). The morphological and gene underexpression phenotypes of *ppt1* are light intensity dependent (Streatfield et al. 1999). The chlorophyll and carotenoid components of the light-harvesting and photoprotective machinery are also diminished in *ppt1* (Knappe et al. 2003). Overexpression of heterologous PPT2 or orthophosphate dikinase (PPDK) can partially complement the phenotype of *ppt1*. Thus, the *cue1* mutant phenotype cannot simply be explained by a general restriction of the supply of plastids with PEP via the PPT (López-Juez 1998; Streatfield et al. 1999; Voll et al. 2003).

In the present study, we characterized a T-DNA-tagged allele of the *ppt1* mutant that was selected on the basis of its high chlorophyll fluorescence phenotype. We compared the photosynthetic behavior of different aged leaves of the *ppt1* mutant at one developmental stage. The results provide

Received 20 Mar. 2006 Accepted 16 May 2006

Supported by Trans-Century Training Programme Foundation for the Talents, The Project-sponsored by SRF for ROCS and the Key Project of Chinese Ministry of Education.

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evidence for PPT in maintaining normal photosynthesis at a late leaf age.

Results

The *ppt1* allele used in the present study was selected on the basis of its high chlorophyll fluorescence phenotype (Peng et al. 2006) and it possesses a representative reticulate phenotype of the *ppt1* mutant. Reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that, in the mutant, the expression of the corresponding *PPT1* gene was barely detectable compared with that in the wild-type plant (data not shown). Shoot development was similar between the mutant and wild type, although the growth mass of the mutant was less than that of the wild type. Under the growth conditions in the present study, the wild-type and mutant plants developed flowers within 8 weeks. At week 6, all leaves of the wild type were healthy and the leaf phenotype of the *ppt1* mutant differed between young and mature leaves (Figure 1A). Fluorescence imaging analysis of *Arabidopsis* leaves of different developmental stages also confirmed this phenotype (data not shown). It is possible that the photosynthetic performance was altered in the mutant. Thus, we used rosette leaves from 6-week-old plants for further experiments. The positions of the leaves within the rosette were color coded using different colored threads (Figure 1B). Experiments were performed with leaves of different ages derived from one rosette; leaf age of approximately 20 and 40 d was represented by orange and blue threads, respectively.

Photosynthetic characterization

Chlorophyll fluorescence imaging is useful because it allows us to determine the maximal efficiency of photosystem (PS) II photochemistry in intact whole plants (Gray et al. 2003). Six-week-old wild-type plants retained a uniform F_v/F_m ratio (where F_m is the maximum fluorescence yield and F_v is variable fluorescence) with a red-orange color in the image (Figure 1B). However, in the mutant, chlorophyll fluorescence F_v/F_m ratio decreased from the central to the marginal. Distinct color differences were observed in images between young leaves (orange) and mature leaves (dark blue) of 6-week-old *ppt1* mutant plants.

Based on the above observations, we further analyzed chlorophyll fluorescence kinetics. The F_v/F_m ratio, reflecting maximal PSII photochemical efficiency, was not changed in different aged leaves of the wild type (0.80). However, the F_v/F_m ratio decreased from 0.75 in 20-day-old leaves of 6-week-old *ppt1* mutant plants to 0.62 in 40-day-old leaves.

Fluorescence decay kinetics of the *ppt1* mutant

To determine the rate of electron transfer from the first (Q_A) and second (Q_B) plastoquinone electron acceptors more precisely, the kinetics of reoxidation of Q_A^- by Q_B was measured. Figure 2 shows the chlorophyll fluorescence decay kinetics in 20- and 40-day-old leaves of the wild type and *ppt1* mutant. Under normal conditions, after a single flash, the largest contribution to the chlorophyll decay kinetics is typically electron transfer from Q_A^- to the secondary electron acceptor Q_B (Trìlek et al. 1997; Diner 1998; Meetam et al. 1999) and the

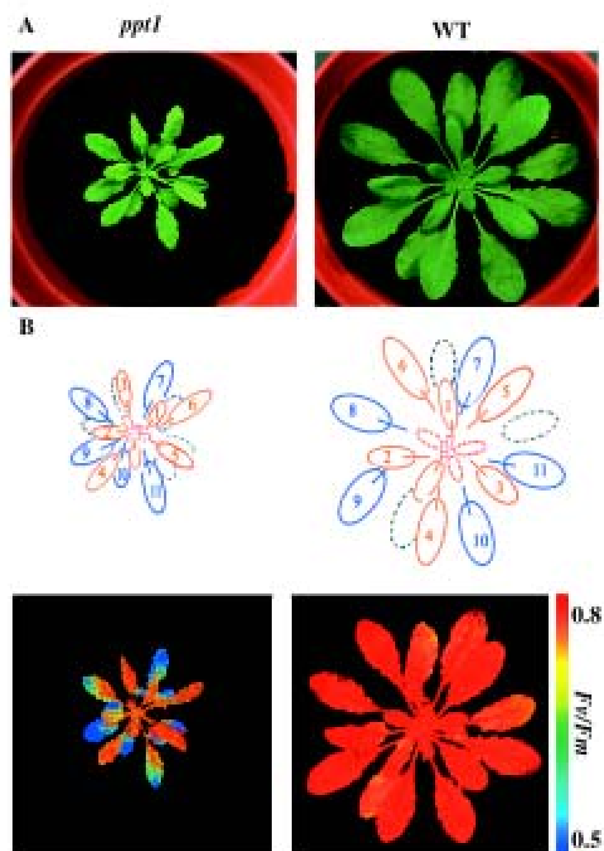

Figure 1. Phenotype of *ppt1* mutant and wild-type (WT) plants and chlorophyll fluorescence imaging.

(A) Six-week-old plants grown in the growth chamber.

(B) Leaf material that was used for the analyses was pooled according to the schematic drawing of each leaf marked with a colored thread at the time of formation.

Youngest and old leaves, illustrated by the red and black dotted lines, were not included in the analyses. Leaves of 6-week-old plants were pooled into young (leaves 1–6; orange thread) and mature (leaves 7–12; blue thread) groups. The lower part shows the chlorophyll fluorescence imaging for whole rosette of WT and *ppt1* plants.

fluorescence decay kinetics could be fitted well by four exponential components according to the two-electron gate model (Crofts and Wraight 1983; Skotnica et al. 2000). The 20- and 40-day-old leaves in wild-type plants exhibited nearly identical kinetics for the four components and amplitudes of the relative contributions (Table 1). In 20-day-old leaves of the *ppt1* mutant, the electron transfer in the acceptor side was slightly affected compared with that in the wild type. More pronounced inhibitory effects on the electron transfer in the acceptor side were observed in 40-day-old leaves (Figure 2; Table 1).

Levels of thylakoid protein components and chloroplast gene transcripts in the *ppt1* mutants

We next examined the steady state levels of photosynthetic thylakoid protein accumulation. Immunoblot analysis with antibodies against CP47, PsaA/B, ATPase, light-harvesting complex (LHC) II and the 33 kDa protein showed that the steady state levels of these photosynthetic proteins were largely unchanged in 20-day-old leaves of the *ppt1* mutant compared with wild-type leaves. However, the amount of proteins in 40-day-old leaves was reduced to approximately 50% of that in wild-type leaves (Figure 3).

Levels of the plastid-encoded and nuclear-encoded chloroplast transcripts were also investigated using RNA gel blot hybridization. We showed that the transcripts of *psb*A, *psb*C and *psbEFJL* operon were not altered, whereas those of the *psaA* and *petA* were slightly reduced in 20-day-old leaves of the *ppt1* mutant. All the transcripts investigated herein were decreased by approximately 20%–50% in 40-day-old leaves of the *ppt1* mutant (Figure 4).

Pigment, protein and carbohydrate content in the *ppt1* mutant

Measurement of protein contents of 20- and 40-day-old leaves of the rosette (6-week-old plants) showed that the protein content in mutant plants decreased in 40-day-old leaves compared with 20-day-old leaves in the same rosette (Table 2). Chlorophyll content per unit fresh weight (FW) was slightly increased in 40-day-old leaves but was unchanged in wild-type plants. The level of sugars in 20-day-old leaves of the *ppt1* mutant was less than that in the wild type and then increased in 40-day-old leaves (Table 2). The decrease in starch and sucrose accumulation in the *ppt1* mutant was accompanied by a significant increase in the glucose content, but not in the fructose content.

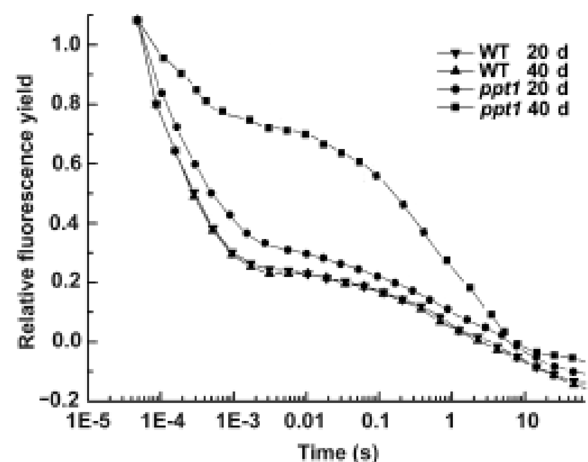

Figure 2. Fluorescence decay kinetics from wild-type (WT) and *ppt1* mutant *Arabidopsis* leaves.

The decay of chlorophyll *a* fluorescence was measured using a Dual-Modulation Kinetics Fluorometer. Before measurement, *Arabidopsis* leaves were dark adapted for 20 min in water.

Table 1. Kinetic parameters of chlorophyll fluorescence decay kinetics after a single turnover flash from 6-week-old rosette leaves of the wild type and *ppt1* mutant

Leaves	Fast component		Middle component				Slow component
	Amp (%)	τ_1 (μs)	Amp (%)	τ_2 (ms)	Amp (%)	τ_3 (ms)	Amp (%) τ_4 (> 8 s)
Wild type							
20 days old	57 ± 2	230 ± 3	15.6 ± 1.4	1.65 ± 0.06	14.7 ± 1.7	420 ± 7	12.7 ± 1.7
40 days old	57 ± 1	240 ± 3	15.5 ± 1.5	1.74 ± 0.08	15.0 ± 1.6	420 ± 9	12.5 ± 1.7
ppt1 mutant							
20 days old	50 ± 2	287 ± 4	14.0 ± 2.0	7.19 ± 0.14	17.9 ± 2.7	480 ± 10	18.1 ± 2.4
40 days old	24 ± 2	364 ± 11	12.4 ± 2.5	15.07 ± 0.21	38.2 ± 3.9	620 ± 15	25.4 ± 4.3

Data are the mean ± *SD* from six decay curves.

Experiments were performed at room temperature.

Amp and τ_i are the amplitude and lifetime of the fluorescence decay, respectively.

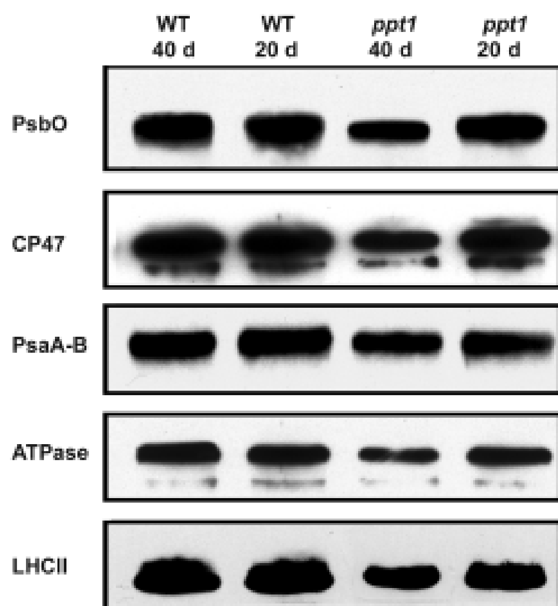

Figure 3. Immunodetection of photosynthetic proteins.

Thylakoid membrane proteins of wild-type (WT) and *ppt1* mutant plants were separated by sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis and blots were probed with specific antibodies against PsbO, Cp47, PsaA/B, ATPase and light-harvesting complex (LHC) II.

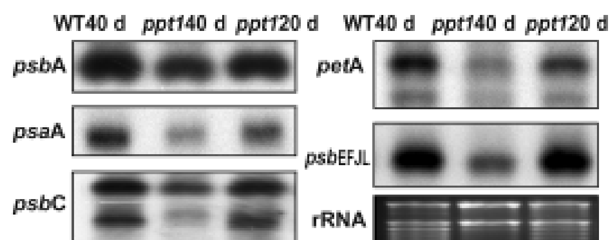

Figure 4. Northern blotting analysis of transcripts in wild-type (WT) and *ppt1* mutant plants.

Transcripts of the *psbA*, *psbC*, *psaA, psbEFJL* and *cytb6* genes were detected by probing the filter with the appropriate gene-specific probes. To control for RNA loading, 2 μg samples of total rRNA from WT and *ppt1* mutant plants were denatured and run on a native 1 × 3-[*N*-morpholino]propanesulphonic acid (MOPS) gel.

Discussion

In present study, we found that the chlorophyll fluorescence F_v/F_m ratio and fluorescence decay kinetics of the *ppt1* mutant were altered at different developmental stages of the leaves. This indicates that the photosynthetic capacity decreased in the *ppt1* mutant with increased leaf age, whereas in the wild-type plant function was optimal in both 20- and 40-day-old leaves. Examination of the steady state levels of thylakoid membrane proteins showed that protein levels in 40-day-old leaves were decreased compared with those in 20-day-old leaves and that the changes in PSII proteins were correlated with those in the F_v/F_m ratio in the *ppt1* mutant. These results indicate that the amount of photosynthetic protein complexes was degraded and the function of PSII was perturbed during leaf development in the leaves of the *ppt1* mutant.

Alteration of carbohydrate metabolism was observed in 40-day-old leaves in comparison with 20-day-old leaves of the *ppt1* mutant. Carbon metabolite control of photosynthesis also operates at the whole-leaf level by influencing leaf development and senescence (Matthew and Till 2003). It is well known that the concentration of leaf sugars can increase during leaf

Table 2. Chlorophyll and protein content and soluble sugar levels of *Arabdiopsis thaliana* wild type and the *ppt1* mutant 20 and 40 d after germination

Parameters	20 d		40 d	
	Col-0	*ppt1*	Col-0	*ppt1*
Chl content (mg/g FW)	1.65	0.87	1.59	0.92
Chl *a/b* ratio	2.97	2.86	2.95	2.78
Carotenoids (mg/g FW)	0.36	0.18	0.38	0.20
Protein (mg/g FW)	11.20	8.30	11.00	5.50
Glucose (μmol/g FW)	2.42	2.15	2.52	3.02
Fructose (μmol/g FW)	0.67	0.57	0.54	0.98
Sucrose (μmol/g FW)	0.81	0.61	0.76	0.88
Starch (μmol/g FW)	73.62	48.90	87.42	43.22
DW/FW ratio	0.081	0.094	0.085	0.100

Values are mean of three independent samples and the standard error of the measure in all cases was <10% of the mean.

Col-0, wild-type *Arabidopsis thaliana* (ecotype Columbia); Chl, chlorophyll; DW, dry weight; FW, fresh weight.

senescence (Crafts-Brandner et al. 1984), which leads to a decline in chlorophyll and photosynthetic proteins (Krapp and Stitt 1994). In addition, increased accumulation of glucose and fructose represses the transcription of photosynthetic genes (Sheen 1990). Indeed, in our experiments, the increased accumulation of leaf sugars was accompanied by a decrease in photosynthetic gene transcripts and protein content in 40-day-old leaves of the mutant (Figure 4; Table 2). The reduced photosynthetic gene expression in 40-day-old leaves of the *ppt1* mutants may account for the photosynthetic performance (Figures 1, 4).

The changes in photosynthetic performance with increasing leaf age in the mutant may point to the more active function of ATPPT1 in maintaining PSII function at a developmental stage. At the early stage of leaf development, only a finite function of PPT1 is required to establish photoautotrophic growth. In fact, the expression of *AtPPT1* is barely detectable in mesophyll cells of young *Arabidopsis* leaves, but spreads into the bundle sheath with leaf development (Knappe et al. 2003). It is speculated that *AtPPT1* is involved in the synthesis of signal molecules and that the expression of *AtPPT1* in leaves of different ages depends on the different development status (Voll et al. 2003).

Taking these observations together, we suggest that PPT takes part in the whole network of plant development, including mediating signals controlling gene expression and maintaining photosynthetic performance. In addition, defective PPT can lead to decreased capacity and efficiency of photosynthetic energy conversion for catabolism of chloroplasts and other components of the photosynthetic apparatus in the leaf.

Materials and Methods

Plant materials

The *ppt1* mutant was isolated in a collection of pSKI015 T-DNA-mutagenized *Arabidopsis thaliana* (ecotype Columbia) lines from *Arabidopsis* Biological Resource Center based on the high chlorophyll fluorescence phenotype in the dark under long wavelength UV radiation (Peng et al. 2006). *Arabidopsis thaliana* was grown in soil under a 10 h light/14 h dark cycle with a photon flux density of 120 μmol/m^2 per s at a constant temperature of 22 °C. Experiments were performed in leaves of different ages derived from one rosette; leaf age of approximately 20 and 40 d was color coded using different colored threads (orange and blue, respectively; Figure 1B). Leaves that were too young or too old (marked with red and black threads) were not used. To avoid changes in expression that may be due to circadian regulation, leaves were always harvested 3 h after the beginning of illumination.

Chlorophyll fluorescence images analysis

Chlorophyll *a* fluorescence images and numeric data were captured *in planta* at room temperature using a commercially available modulated imaging fluorometer (FluorCam; Photon System Instruments, Brno, Czech Republic), as described by Gray et al. (2003). Values of minimum fluorescence yield (F_o) and F_m were averaged to improve the signal-to-noise ratio. Image data for each experiment were normalized to a false color scale, whose extremes were arbitrarily assigned values of 0.5 (lowest) and 1 (highest). This resulted in the highest and lowest F_v/F_m values being represented by the red and blue extremes of the color scale, respectively.

Chlorophyll fluorescence analysis

Chlorophyll fluorescence was measured using a PAM 2000 portable chlorophyll fluorometer (Walz, Effeltrich, Germany) connected to a leaf-clip holder (2030-B; Walz) with a trifurcated fiberoptic (2010-F; Walz; Peng et al. 2006). After determination of potential PSII efficiency, the re-oxidation kinetics of Q_A^- were measured as the decay of chlorophyll *a* fluorescence using a Dual-Modulation Kinetics Fluorometer (Model FL_200; Photon System Instruments; Lazar 1999; Strasser et al. 2000). The instrument contained red light-emitting diodes for both actinic (40 μs) and measuring (2.5 s) flashes and was used in the 100 μs–100 s time range. Before measurements were made, *Arabidopsis* leaves were dark adapted for 20 min in the presence of water.

Quantification of protein, chlorophyll and sugars

Plant tissues were frozen and ground in liquid nitrogen. Protein was extracted in 50 mmol/L HEPES-KOH (pH 7.4), 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L EGTA, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5 mmol/L dithiothreitol, and 1% (w/v) sodium dodecyl sulfate (SDS) and determined with the BCA Protein Assay Kit (Beyotime Biotechnology, Nanjing, China). Chlorophyll was extracted in 80% acetone (3 mg tissue/1 mL acetone) and quantified according to Lichtenthaler and Wellburn (1983).

Arabidopsis leaves for sugar determination were harvested in the morning, between 3 and 4 h into the photoperiod. Sugars were extracted and determined as described by Scholes et al. (1994).

Other experimental methods

For SDS-polyacrylamide gel electrophoresis (PAGE), thylakoid proteins were solubilized and separated by SDS-PAGE using 15% (w/v) acrylamide gels containing 6 mol/L urea. For

immunoblotting, proteins were transferred to nitrocellulose membranes, probed with specific antibodies and visualized by the enhanced chemiluminescence method. Nucleic acid preparation and analysis were performed essentially as described previously (Peng et al. 2006).

Acknowledgements

The authors are grateful to Nottingham Stock Center for the *Arabidopsis* seeds.

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(Managing editor: Ping He)