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Photochemistry of PSII in CYP38 *Arabidopsis thaliana* Deletion Mutant

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Summary

Chloroplast protein CYP38 is a cyclophilin-like peptidyl-prolyl *cis-trans* isomerase involved in photosystem II (PSII) assembly. It also serves as a regulator of thylakoid protein phosphatase. In this work the efficiency of PSII in CYP38 deficient *Arabidopsis thaliana* M13 plants has been analyzed by measuring *in vivo* chlorophyll *a* (Chl *a*) fluorescence transient (OJIP test). Significant differences in overall photosynthetic performance (PI_{ABS}), absorption (ABS/RC), trapping (TR_o/RC), electron transport (ET_o/RC), and dissipation (DI_o/RC) were observed between *A. thaliana* M13 and the wild type (WT) plants. Increased Chl *a* and Chl *b* levels, as well as decreased Chl *a*/Chl *b* ratio were measured in M13 plants, indicating the adjustment of PSII antenna for increasing light absorption capability. Based on the obtained results, it can be concluded that the deficiency in CYP38 protein leads to impaired function of PSII due to the conversion of a certain fraction of active reaction centres to dissipative ones. This leads to a decrease in overall photosynthetic performance (PI_{ABS}) in M13 plants. Such effect was due to lowering of TR_o/DI_o parameter, which was influenced mostly by significant increases in energy dissipation (DI_o/RC) and in trapping of electrons (TR_o/RC) per active reaction centre.

Key words: chlorophyll fluorescence, photosystem II, photosynthesis regulation, OJIP test, TLP40

Introduction

Biogenesis of photosynthetic membranes of chloroplasts is still a poorly understood series of molecular events. The dual genetic origin and the enormous physiological versatility, in particular its ability to manage short- and long-term changes in the light environment, are outstanding characteristics of this specialized biomembrane. Various mechanisms can regulate the distribution of excitation energy between the two photosystems, and others can convert excess excitation energy into thermal energy. A significant number of auxiliary enzymes are believed to be involved in these physiological processes. These include, for instance, protein kinases and phosphatases, chaperones, a substantial number of proteases that catalyze regulated protein degradation, and various other protein components that are required during biogenesis of the photosynthetic multisubunit complexes.

One such auxiliary component is chloroplast protein TLP40 (thylakoid lumen PPIase of 40 kDa). TLP40 is a cyclophilin-like peptidyl-prolyl *cis-trans* isomerase which

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shows protein folding activity characteristic of immunophilins (*1*). This protein was co-isolated with thylakoid membrane phosphatase from spinach (*2*). TLP40 is implicated in the regulation of protein dephosphorylation during the process of photosynthetic state transitions (*3*). The activation of phosphatase coincides with the temperature-induced release of TLP40 from the membrane into thylakoid lumen. Apparently, dissociation of TLP40 caused by an abrupt elevation in temperature and activation of the membrane protein phosphatase trigger the accelerated repair of photodamaged PSII and operate as possible early signals initiating other heat shock responses in chloroplasts (*3*). Gene encoding CYP38 protein, an ortholog of TLP40 from spinach, is located on the 3rd chromosome of *Arabidopsis thaliana*. It has been demonstrated that CYP38 is involved in the assembly and maintenance of PSII in *Arabidopsis thaliana* (*4*).

The aim of this work is to characterize the changes in PSII photochemistry caused by deficiency of CYP38 protein, with a special emphasis on particular processes in photosystem II such as absorption of light energy, trapping, efficiency of the conversion of excitation energy to electron transport and dissipation of excess excitation energy. For this purpose, *in vivo* measurement of chlorophyll *a* fluorescence transient, a kind of fast fluorescence measurement named OJIP test that enabled following the fate of excitation energy inside the PSII and reaching conclusions on its functioning, has been used. The OJIP test was established by Strasser and Strasser (*5*) and it uses a pulse of saturating light to induce transient increase in chlorophyll *a* fluorescence (the so-called Kautsky transient) in the dark-adapted plant material. When fluorescence values are plotted on the logarithmic scale of time, this transient reveals two steps (J and I) between the initial (O) and maximum (P) fluorescence values. The raw fluorescence data are then used to calculate some parameters that describe the functionality of PSII (Table 1). The most significant parameter in OJIP test that gives the information about overall photosynthetic performance is the performance index (PI_{ABS}). PI_{ABS} comprises three main processes in PSII: light absorption (ABS), trapping of excitation energy (TR) and conversion of excitation energy to photosynthetic electron transport (ET) (*6*). Furthermore, the OJIP test facilitates the calculation of dissipation (DI) of excess light energy absorbed by PSII. Our hypothesis is that deficiency of CYP38 protein might cause certain discrepancy between light harvesting and electron transport processes in PSII that would lead to photoinhibition or at least to its down-regulation due to increased processes of light energy dissipation.

Table 1. OJIP test parameters and expressions

Parameters	Description
F_o	fluorescence intensity at 50 μs (O step)
F_{300}	fluorescence intensity at 300 μs
F_J	fluorescence intensity at 2 ms (J step)
F_I	fluorescence intensity at 30 ms (I step)
F_m	maximal fluorescence intensity (P step)
F_V	maximal variable fluorescence; $F_V=F_m-F_o$
t_{max}	time needed to reach F_m
V_J	variable fluorescence at J step; $V_J=(F_J-F_o)/(F_m-F_o)$
V_I	variable fluorescence at I step; $V_I=(F_I-F_o)/(F_m-F_o)$
S_m	normalized total complementary area above OJIP transient; $S_m=\text{area}/(F_m-F_o)$
M_o	net rate of reaction centres closure; $M_o=(TR_o/RC)-(ET_o/RC)=4(F_{300\mu s}-F_o)/(F_m-F_o)$
N	turnover number; $N=S_m\cdot[(dV/dt)_0]/V_J$
RC/CS_o	density of reaction centres; $RC/CS_o=F_v/F_m\cdot(V_J/M_o)\cdot ABS/CS_o$
F_v/F_m	maximum quantum yield of PSII; $F_v/F_m=[1-(F_o/F_m)]=TR_o/ABS$
ABS/RC	absorption per active reaction centre; $ABS/RC=M_o\cdot(1/V_J)\cdot[1/(F_v/F_m)]$
TR_o/RC	trapping per active reaction centre; $TR_o/RC=M_o\cdot(1/V_J)$
ET_o/RC	electron transport per active reaction centre: $ET_o/RC=M_o\cdot(1/V_J)\cdot(1-V_J)$
DI_o/RC	dissipation per active reaction centre; $DI_o/RC=(ABS/RC)-(TR_o/RC)$
PI_{ABS}	performance index; $PI=(RC/ABS)\cdot(TR_o/DI_o)\cdot[ET_o/(TR_o/ET_o)]$
RC/ABS	density of reaction centres on chlorophyll basis; $RC/ABS=(RC/TR_o)\cdot(TR_o/ABS)=[(F_J-F_o)/4(F_{300\mu s}-F_o)]\cdot(F_V/F_m)$
TR_o/DI_o	flux ratio trapping per dissipation; $TR_o/DI_o=F_V/F_o$
$ET_o/(TR_o/ET_o)$	electron transport beyond Q_A^-; $ET_o/(TR_o/ET_o)=(F_m-F_J)/(F_J-F_o)$

Materials and Methods

To characterize the function of CYP38 protein *in vivo*, a knock-out *Arabidopsis thaliana* line M13, which was screened for T-DNA insertion, has been analysed. Plants were grown for 4 weeks under photons of 80 μmol/(m^2·s) with a long day cycle (16-hour day, 60 % of humidity, 20 °C and 8-hour night, 70 % of humidity, 18 °C) in a growth chamber. Genomic DNA was extracted from leaves of 2- to 3-week-old plants using QIAGEN DNeasy Plant Mini Kit (Hilden, Germany). Genotype was determined by PCR amplification with T-DNA primer (LBb1_Salk 5′-GCG TGG ACC GCT TGC TGC-3′) and gene-specific forward (TLP40_B_F 5′-CGG TTC CTC TAG AGA TTA TGG-3′) and reverse (TLP40_B_R 5′-CCT GAG TCA TTC TCA AAC TCC-3′) primers. Primers were designed to amplify the wild type (WT) and T-DNA insertion region of the At3g01480 locus. Gene was interrupted by T-DNA insertion at position 1962 bp (6th exon) in M13 line (Fig. 1), as confirmed by PCR and sequencing. Absence of CYP38 protein was tested by Western blotting (Fig. 2). The T-DNA insertion line in the Columbia background for At3g01480 (SALK_029448) was obtained from NASC (*7*).

Chlorophyll *a* fluorescence transient from F_o to F_m (Table 1) was measured using Plant Efficiency Analyser (PEA, Hansatech, UK). WT and M13 mutant line of *Arabidopsis* plants (five plants of each) were adapted to

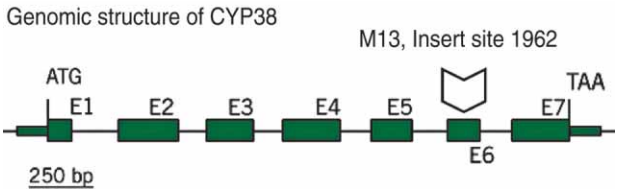

Fig. 1. Plant genomic structure of the CYP38 allele is shown with the T-DNA insertion. The gene is composed of 7 exons and encodes 1314 bp of coding sequence. Exons are shown as green bars and introns are represented as lines. Gene coding CYP38 protein, ortholog of TLP40 from *Spinacia oleracea*, is located on the 3rd chromosome of *Arabidopsis thaliana*

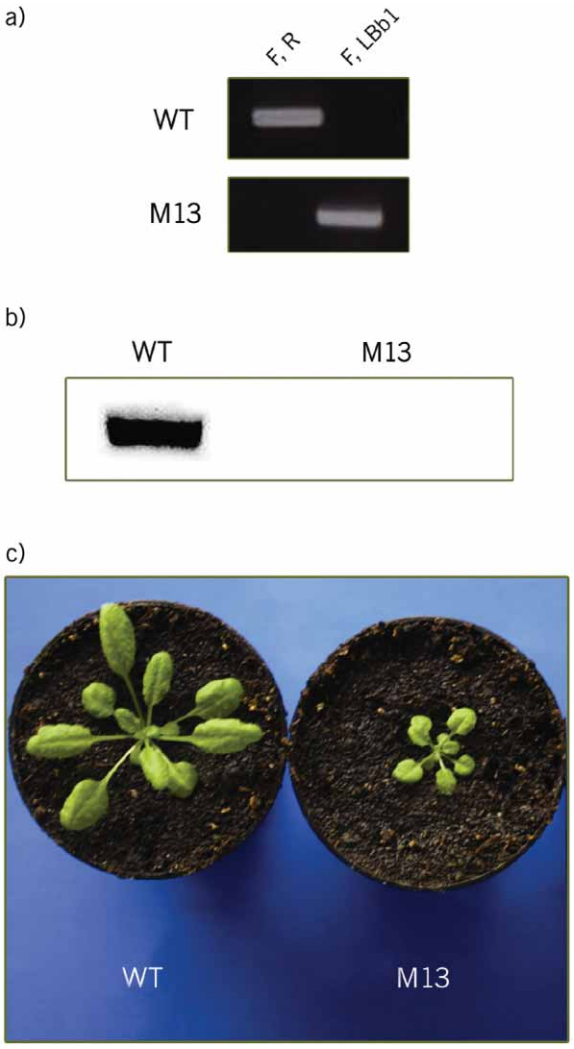

Fig. 2. The knock-out *Arabidopsis thaliana* line Salk_029448 (M13) screening for T-DNA insertion and corresponding phenotype: (a) DNA gel analysis of PCR amplified fragments from wild type (WT) and knock-out mutant M13 (homozygous) plants, (b) Western blotting analysis of CYP38 presence in WT and M13 total leaf protein extracts, (c) phenotypes of WT and M13 transgenic plants

the dark for about 30 min before measurements. Chlorophyll fluorescence transients (OJIP) were induced by applying the pulse of saturating red light (peak at 650 nm, photons of 3000 μmol/(m^2·s)). Changes in fluorescence were measured for 1 s, starting from 50 μs after the onset of illumination. During the first 2 ms, changes were recorded every 10 μs and afterwards every 1 ms. The obtained data were used in OJIP test (*6*) in order to calculate several biophysical parameters of PSII functioning (Table 1).

For the determination of chlorophylls *a* and *b*, the leaf tissue was extracted with cold anhydrous acetone using pestle and mortar. The absorbance was measured at different wavelengths (661.6 and 644.8 nm). The concentrations of chlorophyll *a* and chlorophyll *b* were calculated according to Lichtenthaler (*8*).

Quantitative data concerning the OJIP test and chlorophyll concentrations were statistically evaluated using *t*-test.

Results and Discussion

Genetic, protein, and phenotypic characterization of *A. thaliana* wild type (WT) and mutant (M13) lines is shown in Fig. 2. PCR amplification revealed that T-DNA insertion in At3g01480 locus is homozygous (Fig. 2a). Western analysis using TLP40 specific antibodies confirmed the complete absence of CYP38 protein in M13 plants (Fig. 2b). *Arabidopsis* M13 mutant phenotype showed dwarf, stunted growth and low survival of seedlings (Fig. 2c). Although the insertion in M13 line was at a different position in the At3g01480 locus (Fig. 1) as compared to the mutant line described by Fu *et al.* (*4*), the observed phenotype (Fig. 2c) corresponded.

Polyphasic chlorophyll *a* fluorescence transient of the investigated plants is shown in Fig. 3a. Both WT and M13 plants revealed characteristic OJIP curve shape with differences in fluorescence transient for J-I and I-P steps (Fig. 3b). This resulted in a substantial increase in variable fluorescence at 30 ms (V_I), while variable fluorescence at 2 ms (V_J) revealed small, but significant increase (Table 2). According to Strasser *et al.* (*9*) I and P steps indicate the presence of fast and slow reducing plastoquinone (PQ) reaction centers, therefore reflecting PQ pool heterogeneity. Also, M13 plants had the normalized total complementary area above OJIP transient (S_m) and the time needed to reach F_m (t_{max}) for 2 and 2.5 times higher, respectively, in comparison with WT (Table 2). The S_m value gives measure for the energy needed to close all reaction centres (*6*). Number of Q_A reduction events between F_o and F_m, given by turnover number (N), was twice higher in M13 plants than in WT (Table 2). This corroborated the observed differences in OJIP curve shape (Fig. 3), indicating differences in the functioning of PSII reaction centres between M13 and WT plants. M13 plants were characterized by considerable decrease in F_m as well as by small but significant increase in F_o (Fig. 3a and Table 2). This was the reason for reduced values of the maximum quantum yield of PSII (F_v/F_m) in M13 plants, which appeared to be significantly lower than in WT plants (Table 2). Also, it was lower than 0.75, the value considered as boundary value for fully functional PSII (*10*). Increase in F_o could be due to the disconnection of antennae from their reaction centres (*11*), but also due to the accumulation of reduced form of Q_A (*12*).

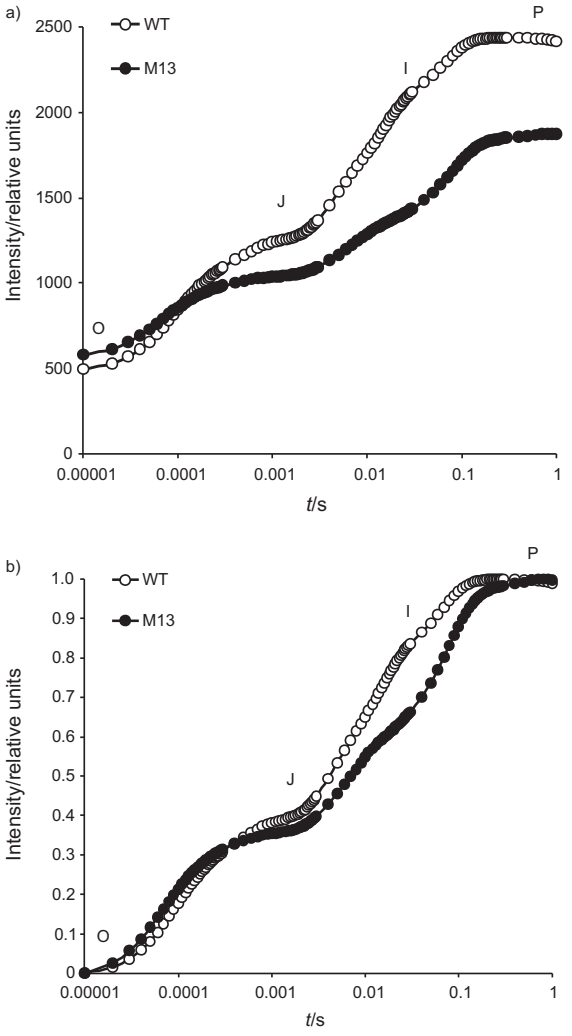

Fig. 3. OJIP chlorophyll *a* fluorescence transients: (a) without normalization and (b) O-P normalized

Table 2. Chlorophyll *a* fluorescence parameters in wild type (WT) and mutant (M13) plants of *Arabidopsis thaliana*

Parameters	WT	M13	t	p(t)
F_o	507±53	634±85	2.831	**
F_m	2401±280	1886±258	3.027	**
F_v/F_m	0.788±0.013	0.662±0.012	15.966	***
V_J	0.402±0.025	0.356±0.020	3.262	**
V_I	0.853±0.037	0.670±0.018	9.906	***
t_{max}	288±64	720±164	5.476	***
S_m	15.340±1.005	29.817±4.279	7.365	***
N	45.516±3.299	97.795±14.297	7.967	***
RC/CSo	135.05±17.105	130.047±15.898	0.480	NS

NS – not significant; **p(t)<2 %, ***p(t)<1 %
For parameter descriptions see Table 1

Although there was no difference in the density of active reaction centres (RC/CS_o) (Table 2), the analysis of specific energy fluxes per Q_A-reducing reaction centre revealed significant differences in absorption (ABS/RC), trapping (TR_o/RC), electron transport (ET_o/RC) and dissipation (DI_o/RC) between M13 and WT plants (Fig. 4a). The expression ABS/RC gives the total absorption of chlorophylls in PSII antennae divided by the number of Q_A--reducing reaction centres. This is a good measure for average functional antenna size (*13*). Also, increased chlorophyll *a* and chlorophyll *b* levels were measured in M13 plants (Table 3). Enhanced biosynthesis of chlorophylls

Table 3. Concentration of chlorophylls (mg/g FM) in wild type (WT) and mutant (M13) plants of *Arabidopsis thaliana*

	Chl *a*	Chl *b*	Chl *a*+*b*	Chl *a*/Chl *b*
WT	0.744±0.043	0.345±0.022	1.089±0.065	2.155±0.022
M13	0.815±0.033	0.420±0.018	1.235±0.050	1.942±0.022
t	4.239	6.635	5.084	13.017
p(t)	***	***	***	***

Chl *a* – chlorophyll *a*, Chl *b* – chlorophyll *b*, Chl *a*+*b* – total chlorophylls, Chl *a*/Chl *b* – chlorophyll *a* to chlorophyll *b* ratio; ***p(t)<1 %

would be a metabolic adjustment of M13 mutants for increasing light absorption capability. Interestingly, M13 decreased chlorophyll *a*/chlorophyll *b* ratio, leading to a conclusion that PSII antennae were increased in size (*14*). This was in accordance with increased value of ABS/RC parameter in M13 plants (Fig. 4a). The observed increase in antenna size in M13 would in turn cause the overloading of the PSII with electrons, leading to a modification of a certain number of RC from active to dissipative ones. Such dissipative reaction centres are formed under the influence of high proton gradient across the thylakoid membrane (ΔpH) which converts the violaxanthin to zeaxanthin, enabling them to dissipate most of the energy as heat (*15*). Higher ABS/RC values in M13 than in WT corroborated the increase in dissipation of excitation energy (DI_o/RC) compared to WT. M13 plants had twofold increased DI_o/RC value (Fig. 4a). Functioning of photosynthetic apparatus in the investigated plants was monitored by performance index (PI_{ABS}). The PI_{ABS} values in WT were twofold higher than in M13 plants, indicating their considerably better overall photosynthetic performance (Fig. 4b). Performance index (PI_{ABS}), established by Strasser *et al.* (*16*), combines several parameters that describe three main functional characteristics of PSII reaction centre, namely the absorption of light energy (RC/ABS), trapping (TR_o/DI_o) and efficiency of the conversion of excitation energy to electron transport ($ET_o/(TR_o-ET_o)$). This parameter was shown to be very sensitive to different kinds of environmental stresses (*17,18*). The main contribution to the decrease of PI_{ABS} in M13 plants was due to lowering of TR_o/DI_o parameter (Fig. 4b), which was influenced mostly by higher increase in energy dissipation (DI_o/RC) rather than by trapping of electrons (TR_o/RC) per active reaction centre. Similar response of PSII reaction centres was reported for several plant species as the consequence of plant exposure to elevated ozone levels (*19*). However, M13 plants were shown to have increased electron transport beyond Q_A^- ($ET_o/(TR_o-ET_o)$) (Fig. 4b). Since the clear relationship between photosynthetic electron trans-

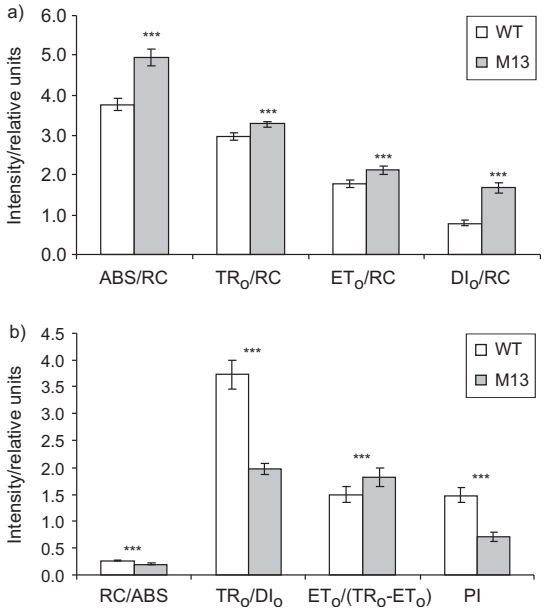

Fig. 4. Specific fluxes or specific activities per active reaction centre (a) and performance index (PI) and its components (b); **p(t)<2 %, ***p(t)<1 %

port and CO_2 fixation was established (*20*), it might be possible that mutant line has comparable or even higher CO_2 assimilation rate based on a leaf area basis. On the other hand, vastly different phenotype of the two investigated lines would be in accordance with decreased overall photosynthetic performance in M13 line, revealed to be regulated at the PSII point. This phenotypic difference might also be due to other pleiotropic effects involved, such as metabolic deficiency, or retrograde signal transduction.

Conclusion

Our results indicate that reduced photosynthetic performance of M13 plants was the consequence of conformational changes in PSII that led to the modification of a certain fraction of active reaction centres, which made them functioning as dissipative ones. It has also been revealed that overall photosynthetic performance (PI_{ABS}) in M13 plants was decreased due to lowering of TR_o/DI_o parameter. Precise regulatory mechanisms of described downregulation of photosystem II performance in M13 plants remain to be elucidated. Thus, further investigation will be focused on the effects of different irradiance intensities on the processes of light harvesting and non-photochemical quenching of chlorophyll fluorescence. Furthermore, since CYP38 is likely involved in redox-regulation of thylakoid protein phosphates, we will investigate the influence of CYP38 deletion on state 1 to state 2 transitions.

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