



# ROS-mediated enhanced transcription of CYP38 promotes the plant tolerance to high light stress by suppressing GTPase activation of PsbO2

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As a member of the Immunophilin family, cyclophilin38 (CYP38) is discovered to be localized in the thylakoid lumen, and is reported to be a participant in the function regulation of thylakoid membrane protein. However, the molecule mechanisms remain unclear. We found that, CYP38 plays an important role in the process of regulating and protecting the plant to resist high light (HL) stress. Under HL condition, the gene expression of CYP38 is enhanced, and if CYP38 gene is deficient, photochemistry efficiency, and chlorophyll content falls distinctly, and excessive reactive oxygen species synthesis occurs in the chloroplast. Western blot results showed that the D1 degradation rate of *cyp38* mutant plants is faster than that of wide type plants. Interestingly, both gene expression and activity of PsbO2 were drastically enhanced in *cyp38* mutant plants and less changed when the deleted gene of CYP38 was restored under HL treatment. This indicates that CYP38 may impose a negative regulation effect on PsbO2, which exerts a positive regulation effect in facilitating the dephosphorylation and subsequent degradation of D1. It is also found that, under HL condition, the cytoplasmic calcium ( $[Ca^{2+}]_{cyt}$ ) concentration and the gene expression level of *calmodulin 3* (*CaM3*) arose markedly, which occurs upstream of CYP38 gene expression. In conclusion, our results indicate that CYP38 plays an important role in plant strengthening HL resistibility, which provides a new insight in the research of mechanisms of CYP38 protein in plants.

**Keywords:** *Arabidopsis*, high light, CYP38, PsbO2, reactive oxygen species, D1 protein

## Introduction

The light-dependent reactions and subsequent oxygen evolution in photosynthesis are performed by four multi-subunit protein complexes within the thylakoid membranes of chloroplast. They are Photosystem I (PSI) and Photosystem II (PSII), cytochrome *b<sub>6</sub>f* complex, and CFo-CF<sub>1</sub> complex (Shi and Schröder, 2004; Barber, 2006; Nelson and Yocum, 2006). Among these multi-subunit protein complexes, PSII serves as a light-driven water-plastoquinone oxidoreductase in the thylakoid membranes of oxygenic photosynthetic organisms, included in the process of oxygen-evolution and a membrane-embedded light-harvesting chlorophyll a/b complex (LHCII) that directs the light energy to the PSII core. As the essence of photosynthesis, light also can lead

to inactivation of PSII and causes damage to the D1 protein in the reaction center, especially triggered by high light (HL) conditions. Due to such light-induced damage, D1 turns over at a higher rate than all other thylakoid polypeptides (Mattoo et al., 1981). Photodamage to PSII and attendant turnover of D1 protein for PSII repairing occur at all degrees of light intensity. It requires a number of processes to repair photodamaged PSII, which include (i) phosphorylation of the damaged D1 protein and migration from the grana to the stroma membrane regions of thylakoid, (ii) dephosphorylation of p-D1 (p-D1 instead of phosphorylated D1), and then its degradation, (iii) reassembly of new D1 protein into functional PSII complexes (Adir et al., 1990; Barbato et al., 1992; Prasil et al., 1992; Aro et al., 1993; Rintamäki et al., 1996). However, these processes need much more precise molecular regulations which have not yet been completely unveiled.

Initially, immunophilins are found to be cellular receptor proteins for immunosuppressive drugs: FK506 and cyclosporin A (Schreiber, 1991). The receptor proteins for FK506 and cyclosporin A, respectively, called FK506-binding proteins (FKBPs) and cyclophilins (CYPs), are indicated as immunophilins collectively. Now it is known that these proteins widely exist in organisms, from bacteria and fungi to animals and plants. Genes encoding 52 putative immunophilins have been identified in *Arabidopsis*, including 29 CYPs, and 23 FKBPs (He et al., 2004). Thus far, the group is the largest family of immunophilin identified in any organism. One feature of *Arabidopsis* immunophilin family that arrests attention is that a large fraction is confined to a particular locality of the chloroplast and that up to 11 FKBPs and 5 CYPs were predicted to exist in the thylakoid lumen. The sequences divergence implies that they may have highly specialized functions in thylakoid lumen (Romano et al., 2005; Edvardsson et al., 2007).

The CYP38 protein is a complex immunophilin residing in the *Arabidopsis* thylakoids lumen. Its ortholog from *Spinacia*, TLP40, is also discovered to be located in the thylakoids lumen (Fulgosi et al., 1998), which is intended to function in the phosphorylation of thylakoid proteins (Vener et al., 1999; Rokka et al., 2000). Recently, it is shown that CYP38 takes up a vital role in the assembly and maintenance of PSII supercomplexes in *Arabidopsis* (Fu et al., 2007), and its crystal structure is also found (Vasudevan et al., 2012). CYP38 deficiency is found to be able to severely decrease the *in vivo* phosphorylation of PSII core proteins (Sirpiö et al., 2008). Sirpiö et al. (2008) also suggests that during assembly of PSII, CYP38 protein directs the appropriate folding of D1 (and CP43) into PSII, thereby making the accurate assembly of the water-splitting Mn<sub>4</sub>-Ca cluster able, even with high PSII turnover. It is still a void in many studies, however, when it comes to a mechanistic analysis of plant response to HL stress, especially to how the signaling pathway of CYP38 protects the PSII.

PsbO, referred to as the 'manganese-stabilizing' protein, has been proved to bind with high affinity to GTP (Spetea et al., 2004) and function as a GTPase (Lundin et al., 2007a). From cyanobacteria to higher plants this extrinsic subunit is conserved. There are two different protein isoforms, PsbO1 and PsbO2, have been discovered in *Arabidopsis*, and they differ only by 10 amino acids in the mature form. It is found in further study that the PsbO2 component demonstrated considerably higher

GTPase activity than PsbO1 protein (Lundin et al., 2008). The different functions of PsbO1 or PsbO2 are understood with the help of two T-DNA insertion mutant lines of *Arabidopsis* deficient in either isoform: it seems that PsbO1 mainly supports PSII activity, whereas the PsbO2 regulates the dephosphorylation and turnover of D1 protein (Lundin et al., 2007b). However, studies on the molecular mechanisms that underlie the roles of PsbO2 in response to abiotic stresses are poor.

It is believed that reactive oxygen species (ROS) and calcium (Ca<sup>2+</sup>) are quite essential signaling messengers in plant cells (Bowler and Fluhr, 2000; Romeis et al., 2001). calmodulin (CaM), a ubiquitous second messenger, serves as a crucial sensor for Ca<sup>2+</sup> in signal transduction in plants. CaM in *Arabidopsis* varies in different isoforms and they can interact with their particular targets upon different exogenous stimuli (Bowler and Fluhr, 2000; Romeis et al., 2001; Li et al., 2012). However, analysis of the ROS and Ca<sup>2+</sup>-CaM cascade response to HL, especially the signaling pathway, is still lacking.

This paper probes into the possible molecular mechanisms of the protective roles of CYP38 on PSII under the HL stress process. The result of the study showed that CYP38 gene transcription was up regulated in HL condition. It is also discovered that PsbO2 may a target inhibited by CYP38, and the role of CYP38 in HL-induced defense response is important.

## Materials and Methods

### Plant Materials, Growth Conditions, and Chemicals

Wild-type *Arabidopsis* ecotype Columbia (Col-0) plants, *cyp38* (SALK\_029448), *psbo1* (SALK\_093396), *psbo2* (SALK\_024720) knockout mutants and the CYP38 complementation line (*cyp38-C*), were used in this study. The seeds of *cyp38* and the *cyp38-C* line were obtained from Professor Eva-Mari Aro. and mutant seeds *psbo1*, *psbo2* were obtained from the European *Arabidopsis* Stock Center. Following 2 d of cold stratification, seeds were grown in an environmentally controlled growth room at 22°C with a 16/8 h day/night cycle and 82% relative humidity. Fluo-3-AM and Singlet Oxygen Sensor Green Reagent (SOSG) were obtained from Molecular Probes (Eugene, OR, USA). BAPTA-AM, cyclopiazonic acid (CPA), ascorbic acid (AsA), 2',7'-dichlorofluorescein diacetate (H<sub>2</sub>DCFDA) and cyclosporin A (CsA), were purchased from Sigma-Aldrich (Shanghai, China).

### High Light Treatment

*Arabidopsis* plants were fully exposed to HL (2000 μmol photons m<sup>-2</sup> s<sup>-1</sup>) supplied by light-emitting diode panels (Photon System Inst.) (Zhao et al., 2014). Protoplasts at a chlorophyll concentration of 0.2 mg ml<sup>-1</sup> were illuminated with white light at 2000 μmol photons m<sup>-2</sup> s<sup>-1</sup> at 20°C (Herbstová et al., 2012). Control samples of Protoplasts were kept in the dark or normal light, and otherwise identical conditions. Isolated thylakoid membranes (0.1 mg Chl ml<sup>-1</sup>) were illuminated for up to 1 h with 2000 μmol photons m<sup>-2</sup> s<sup>-1</sup> at 20°C. The *Arabidopsis* plants used for qRT-PCR were illuminated with 2000 μmol photons m<sup>-2</sup> s<sup>-1</sup> at 20°C for up to 15 h.

## DAB Staining

For ROS detection, the leaves from wide type (WT) plants and *cyp38* mutant plants were incubated in DAB solution (1 mg ml<sup>-1</sup>) in the dark for 8 h (Liu et al., 2007). The leaves were then cleared using 95% ethanol for 10 min at 80°C to remove the chlorophyll completely.

## Measurement of Chlorophyll Content and Photochemical Efficiency

Chlorophyll from leaves, Protoplasts or thylakoid membranes was extracted by 95% ethanol at 80°C. The chlorophyll content in each unit mass or unit volume of sample was determined as described by Lichtenthaler (1987), and then counting or concentration adjusting was performed, so that it would be more convenient to release the data or perform the next stage of the experiment. Integrity of PSII affected the ratio of maximum variable fluorescence to maximum yield of fluorescence (Fv/Fm), which was measured by a PAM fluorometer (Walz GmbH, Effeltrich, Germany) (Ralph et al., 2005).

The content was determined spectrophotometrically using the formula  $\text{Chl (a + b)} = 5.24 A_{664.2} + 22.24 A_{648.6}$ , the content of Chlorophyll (%) =  $\text{Chl (a + b)} \times V \times 10^{-3}/m$ , where Chl (a + b) equaled the chlorophyll concentration in  $\mu\text{g ml}^{-1}$ ,  $A$  represented the absorption,  $V$  was the volume of the 95% ethanol and  $m$  symbolized the mass of individual leaves.

## Total RNA Extraction and Quantitative Reverse Transcript-PCR (qRT-PCR)

Following the protocol of the manufacturer of TRIzol reagent (Sigma), total RNA was extracted, at times as indicated subject to different treatments, from detached leaves utilizing the said reagent. The RNA concentration and purity were determined, with OD at 260 nm. Total RNAs were transcribed reversely into cDNAs using an M-MLV kit (Takara), and the cDNAs were adopted as polymerase chain reactions templates. For qRT-PCR assay, SYBR green RT-PCR amplifications were conducted using a total volume of 20  $\mu\text{l}$  that contains 10  $\mu\text{l}$  of SYBR Green real-time PCR Master mix (Toyobo), 5  $\mu\text{l}$  of cDNA, 0.5  $\mu\text{l}$  each of forward and reverse primers (10  $\mu\text{M}$ ), and 4  $\mu\text{l}$  of distilled water. The *Actin* (Zhou et al., 2013) was amplified as an endogenous control. PCR amplification was performed with the light cycler 2.0 instrument (Roche Applied Science). The gene-specific primers for RT-PCR analysis were employed as depicted in the Supplementary Table S1. Each measurement of gene expression profiles was carried out at least three times with independent experimental replicates.

## Treatment with CsA

Before HL treatment, the *Arabidopsis* leaves, protoplasts or thylakoid membranes from different samples were pre-sprayed with solutions containing CsA. CsA was used at a final concentration of 10  $\mu\text{M}$ .

## Isolation of Thylakoid Membranes and GTP Hydrolysis Assay

Thylakoid membranes from the WT, *cyp38*, *psbo1*, and *psbo2* were isolated as previously described (Robinson et al., 1981).

To assess the GTP hydrolysis activity of PsbO protein, PSII membranes were isolated from different type plants finally at a same chlorophyll content for each sample as previously described (Berthold et al., 1981; Lundin et al., 2007a). GTP hydrolysis assay using a QuantiChrom™ GTPase Assay Kit (DATG-200, Hongyue, China).

## Western Blot

For Western blotting, the protein samples of thylakoid membranes, at a consistent chlorophyll content, treated with or without HL (2000  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) were transferred to poly (vinylidene difluoride) membranes (PVDF-Plus; Micron Separations, Westborough, MA, USA), and reacted with specific anti-D1 antibody (Cell Signaling Technology) followed by an enhanced chemiluminescence (ECL-Plus) detection system (Amersham Biosciences, Chalfont St Giles, UK), then analyzed by chemiluminescence imaging (LAS-1000; Fuji, Japan).

## Protoplast Isolation

Protoplast isolation as described previously (He et al., 2007; Li et al., 2012). The purified protoplasts were suspended in the W5 solution (154 mM NaCl, 5 mM KCl, 125 mM CaCl<sub>2</sub>, 5 mM Glc, and 1.5 mM MES-KOH, pH 5.6).

## Laser Confocal Scanning Microscopy (LCSM)

Reactive oxygen species production was determined by detecting the fluorescence of DCF, the product of oxidation of H<sub>2</sub>DCFDA, as described previously (Gao et al., 2008). Ca<sup>2+</sup> content of the protoplasts was determined by detecting the fluorescence of Fluo-3, the product of Fluo-3-AM (Zhang et al., 1998). With or without HL treatment, the *Arabidopsis* protoplasts were incubated with H<sub>2</sub>DCFDA or Fluo-3-AM at a final concentration of 5  $\mu\text{M}$ . The intracellular ROS production and distribution, as well as the chloroplast autofluorescence and Fluo-3 fluorescence, were visualized under the Zeiss LSM 510. The fluorescence intensity at 525 nm was used to determine the relative DCF and Fluo-3, and chloroplast autofluorescence was visualized at 650 nm with a long pass filter, all this with an excitation wavelength of 488 nm.

## Flow Cytometry Analysis

Singlet oxygen (<sup>1</sup>O<sub>2</sub>) react with the SOSG to produce SOSG endoperoxides (SOSG-EP), as described previously (Shen et al., 2011). To the changes in DCF, Fluo-3, and SOSG-EP, flow cytometry FACScanto II (Becton Dickinson, Mountain View, CA, USA) equipped with BD FACSDiva acquisition soft-ware was used. Protoplasts were incubated with 10  $\mu\text{M}$  of SOSG, 5  $\mu\text{M}$  H<sub>2</sub>DCFDA, or 5  $\mu\text{M}$  Fluo-3-AM for 30 min at room temperature and then analyzed by flow cytometry. The fluorescence of SOSG-EP was excited with a 504 nm laser. Autofluorescence of the unstained protoplasts under the same physiological conditions were used as controls. Histograms were processed with FCS Express software. Fluorescent efflux was measured by counting cells in the H region of the plot (Chen et al., 2006).

## Results

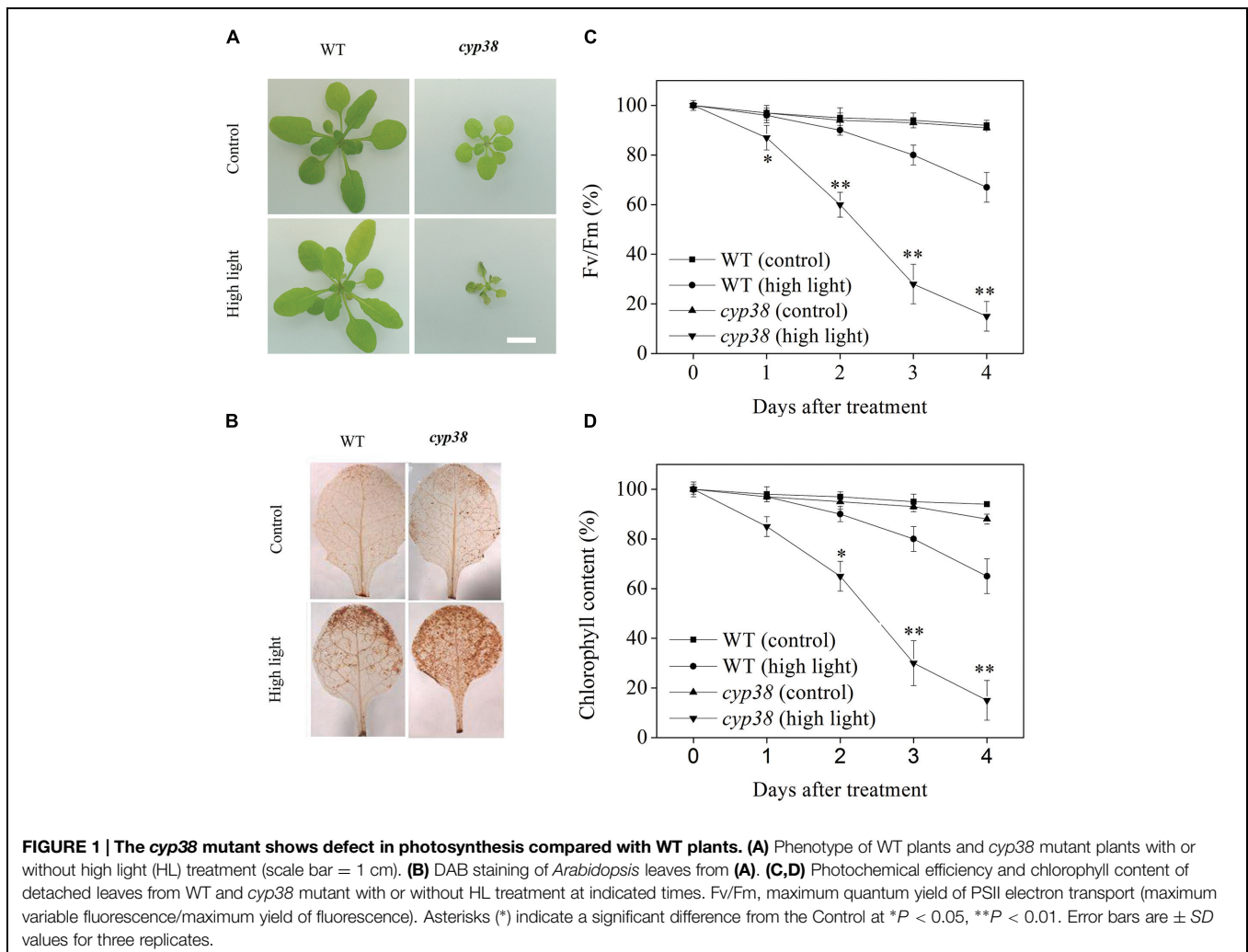
### HL Causes Inhibition of Photosynthesis in *Arabidopsis*

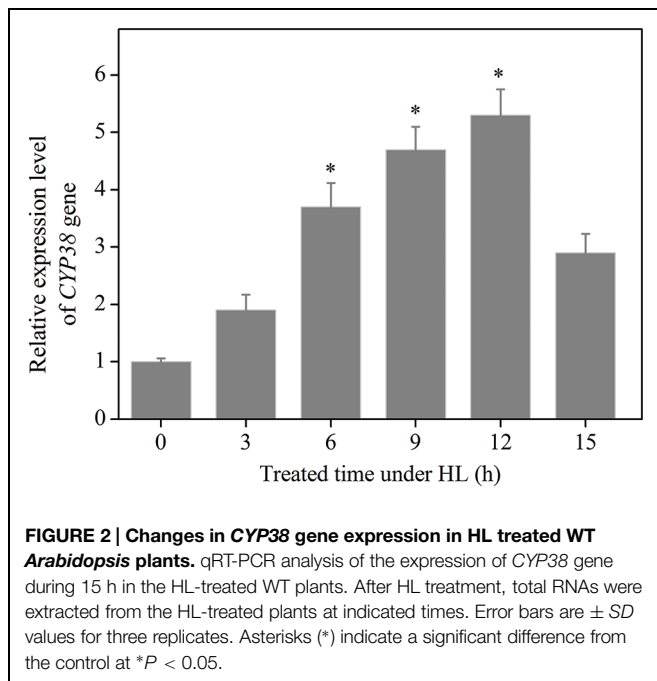
It is reported in literatures that deletion of CYP38 causes stunted growth of the plants and the *cyp38* mutant plants is highly sensitive to HL (Fu et al., 2007; Sirpiö et al., 2008). When *Arabidopsis* plants were treated under HL condition, as shown in the **Figure 1A**, *cyp38* mutant plants soon wilt and finally die. However, when the *CYP38* gene was restored, the wilting and death of the plants (*cyp38-C* plants) were eased off (data not shown). Further DAB staining showed that, under HL condition, as the *cyp38* mutant was dying, its leaves accumulated more ROS in comparison with WT (**Figure 1B**). Stunted growth, as well as high sensitivity to HL of the *cyp38* mutant, illustrate that, because of CYP38 deletion, the photosynthetic activity of plants is more likely to be damaged by oxidation induced by HL. For further data analysis, we have simultaneously detected the chlorophyll content of leaves and photochemistry efficiency of PSII. As shown in the **Figures 1C,D**, under HL treatment, there was dramatic decline in chlorophyll content and photochemistry efficiency of

the *cyp38* mutant. After HL treatment for 3 days, the chlorophyll contents in WT plants and *cyp38* mutant plants, respectively, fell down to 80 and 28% of the original levels, and the photochemistry efficiencies, respectively, fell down to 83 and 30% of the original levels.

### CYP38 is Involved in Response to HL Stress Condition in *Arabidopsis*

The data given above illustrates that, in terms of protecting photochemistry efficiency and HL resistance of plant, CYP38 plays an important role. In order to further confirm with experiment that CYP38 essentially participates in the protection of photosynthetic activity under HL, we detected the change of *CYP38* gene expression level in WT plant after HL treatment. qRT-PCR results showed that, after HL treatment, *CYP38* gene expression arose, and rose to a level four times higher than that in the control group at 6 h (**Figure 2**). To verify the authenticity of Result of RT-PCR, we added *Rbcl* gene (Kress and Erickson, 2007) and *UBQ* gene (Miki et al., 2005), respectively, as control groups (Supplementary Figure S4), and found that *CYP38* gene expression levels are consistent with the *Actin* group.





On the contrary, the gene expression under low light (LL) is not significantly different from that of control genes (Supplementary Figure S1). In sum, CYP38 may, as an important regulation molecule, participate in the protection of *Arabidopsis* plant under HL condition.

### Lack of CYP38 Gene Increases HL-Induced Chloroplast ROS Production

Under HL condition, the process of damage to photosynthetic activity of *cyp38* mutant plants, as well as death of the plants later on, was characterized by outbreak of excessive ROS in leaves (Figure 1B). These illustrate that CYP38 plays a positive role in maintaining the redox state of the leaves. Cells are the most fundamental unit for plants' function. In order to carry out deeper research on relevant mechanisms, it is necessary to do ROS distribution analysis at cellular or even sub-cellular level. The imaging results showed that, after 30 min of HL treatment, there was generation of ROS in protoplasts derived from WT plants; after 60 min, significance in quantity was observed (Figure 3A). The inhibition of photosynthetic chain can induce  $^1\text{O}_2$  to synthesize. To clarify the type of ROS, it is found after flow cytometry analysis that, lots of  $^1\text{O}_2$  was synthesized in the cell during the early stage of HL processing (Figures 3B,C). As  $^1\text{O}_2$  is of highly oxidation activity, high cell synthesis is the cause of overdue impairment of cells. Under HL condition, the cell generated ROS, indicating that HL causes change of the redox state of cells. In comparison to this, a higher outbreak of protoplast ROS derived from *cyp38* mutant plants occurred as early as 30 min after HL treatment, while DCF fluorescence imaging and chloroplasts were subject to co-localization (Figure 3A). Furthermore, CsA, as the non-selective combinator and inhibitor of CYPs (Handschumacher et al., 1984; Steinmann et al., 1991), can be used for pre-treatment of

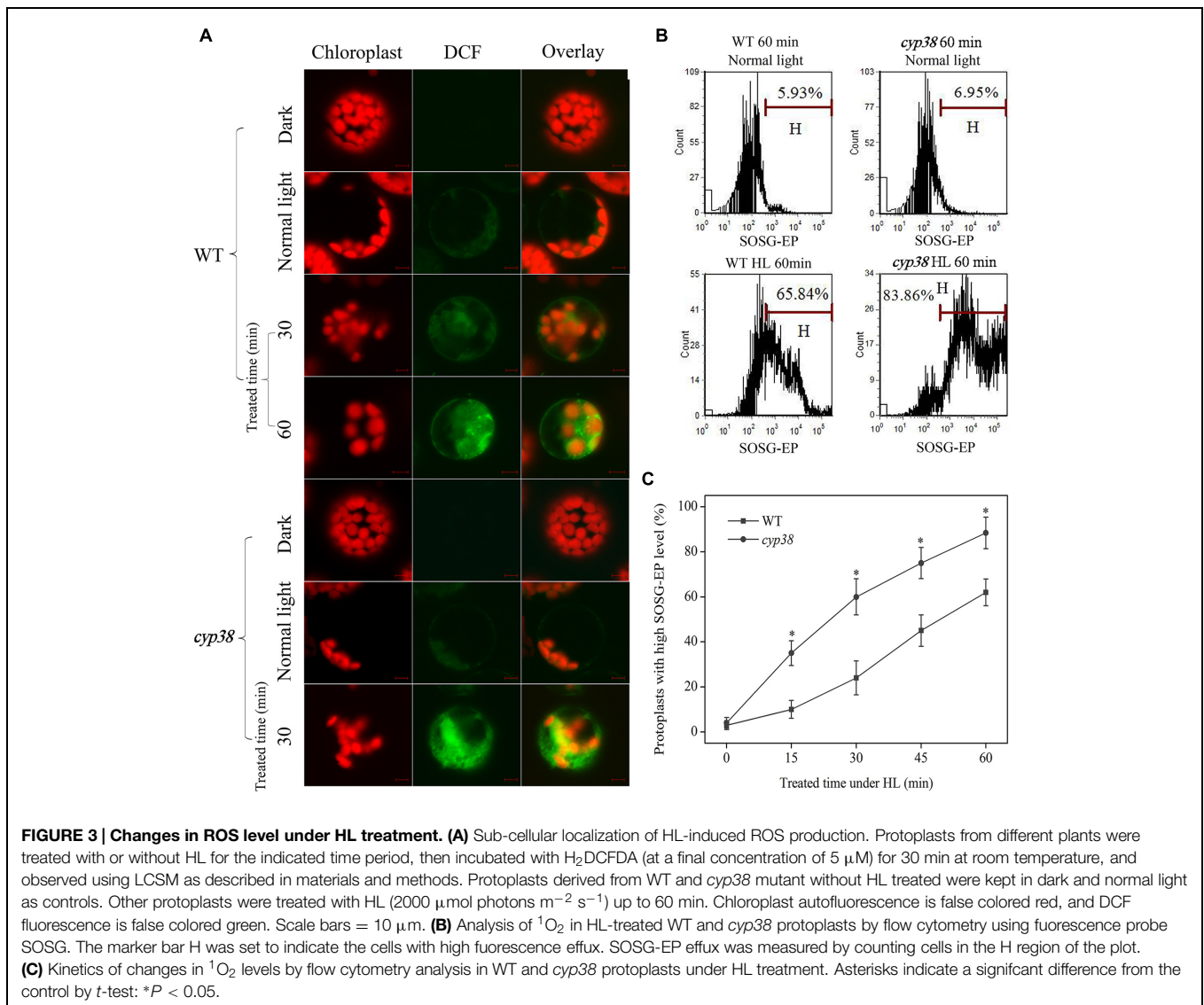
protoplasts derived from WT plants (Supplementary Figure S2). It was discovered that, after 30 min of illumination (HL) in the pre-treatment, the ROS which was larger than that non-pretreated group. The outbreak of ROS which is subject to co-localization with chloroplasts in CYP38-deletant under HL may be an important reason for lack of photosynthetic efficiency and poor HL resistibility in *cyp38* mutant plant.

### D1 Protein is Drastically Decreased in CYP38-Deletion Plants Under HL Stress Condition

Oxygen-evolving complex (OEC), as an important constituent of PSII, can become a major damaged point (Hakala et al., 2005), whether under biotic or abiotic stress, leading to the decrease in activity of oxygen evolution. Studies also proved that, D1, as one of the core proteins of PSII, boasts the fastest turnover rate and is the part which is most likely to be damaged by oxidation (Greenberg et al., 1989; Jegerschoeld et al., 1990). Our research proved that CYP38 may play a certain role in maintaining the redox state of chloroplast, and deletion of this protein induce excessive generation of chloroplast ROS (Figure 3). In order to offer more in-depth information, the thylakoid membranes of WT and *cyp38* mutant were extracted in this study, and treated under HL up to 60 min, in order to detect the oxygen evolution activity and remaining D1 protein content. As shown in the Figure 4A, under HL treatment, the activity of oxygen evolution of *cyp38* mutant fell distinctly. Instead, the WT showed a lag phase of 15 min, and reached similar level as in *cyp38* mutant after 45 min of HL treatment. Both the D1 contents of WT and *cyp38* mutant declined, yet there was distinctive difference between them. In 60 min, the inventories of D1 protein in the extracted membranes of WT and *cyp38* mutant, respectively, fell down to 70 and 15% of the original levels (Figures 4B,C). The detection of the level of remaining protein after HL treatment showed that D1 protein degraded rapidly in *cyp38* mutant. This may be caused by over-oxidation of chloroplasts caused by CYP38 deletion. The mechanism behind the more significant reduction in D1 proteins in *cyp38* mutant as compared with WT was further investigated.

### HL-Induced PsbO2 Transcription and Activation Can be Enhanced by CsA

D1 dephosphorylation is a step of the degradation damaged D1, and degradation is impossible if dephosphorylation is abnormal (Rintamäki et al., 1996). PsbO2, as a membrane protein of PSII, is proved to can positively regulate D1 dephosphorylation in recent research report (Lundin et al., 2007b). Our data given above indicates that CYP38 participates in the turnover regulation of D1 (Figure 4). In addition, the results of Sirpiö et al. (2008) indicate that CYP38 imposes regulation effect on D1 phosphorylation. In order to elucidate if there is an interrelation between CYP38 and PsbO2, CsA, the common combinator and inhibitor of CYPs (Handschumacher et al., 1984; Steinmann et al., 1991), was used. First, the change in transcript level of PsbO2 was detected using qRT-PCR. Results showed a significant increase in PsbO2 expression in detached *Arabidopsis* leaves pretreated with CsA at the 6 h and 9 h under HL treatment, compared to that of the non-pretreated samples (Figure 5A). While the transcription of *PsbO1*



has no much change under HL or CsA pretreated conditions. The change of PsbO activity under HL was analyzed using two T-DNA insertion mutant lines, *psbo1* and *psbo2*. In CsA-pretreated PSII membranes, the distinctive increase of PsbO activity was found at the 6 h after HL treatment in *psbo1* mutant compared to that WT and *psbo2* mutant (Figure 5B). These data indicates that CYPs cascades are involved in HL-induced PsbO activation of and transcription, and PsbO2 may be the main target.

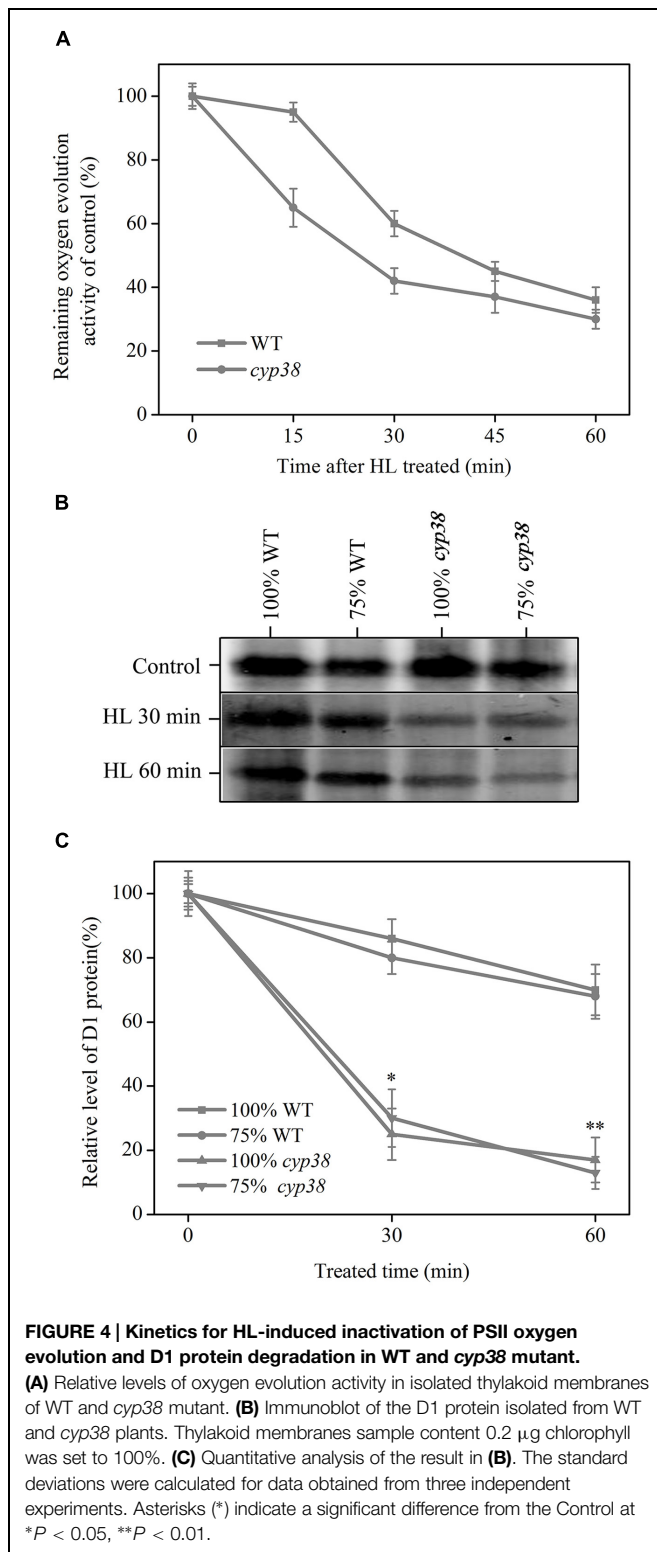
### CYP38 is Required for Inhibiting HL-Induced PsbO2 Activity

Data given above illustrates that CYPs may participate in regulation of HL-induced gene expression and activity of PsbO2 (Figure 5). However, further clarification is needed in order to confirm whether CYP38 participates in the process. In order to explain whether CYP38 involves regulation activity of PsbO2, *cyp38* mutant and *cyp38-C* were utilized simultaneously in the experiment. First of all, under HL condition, we detected *PsbO2*

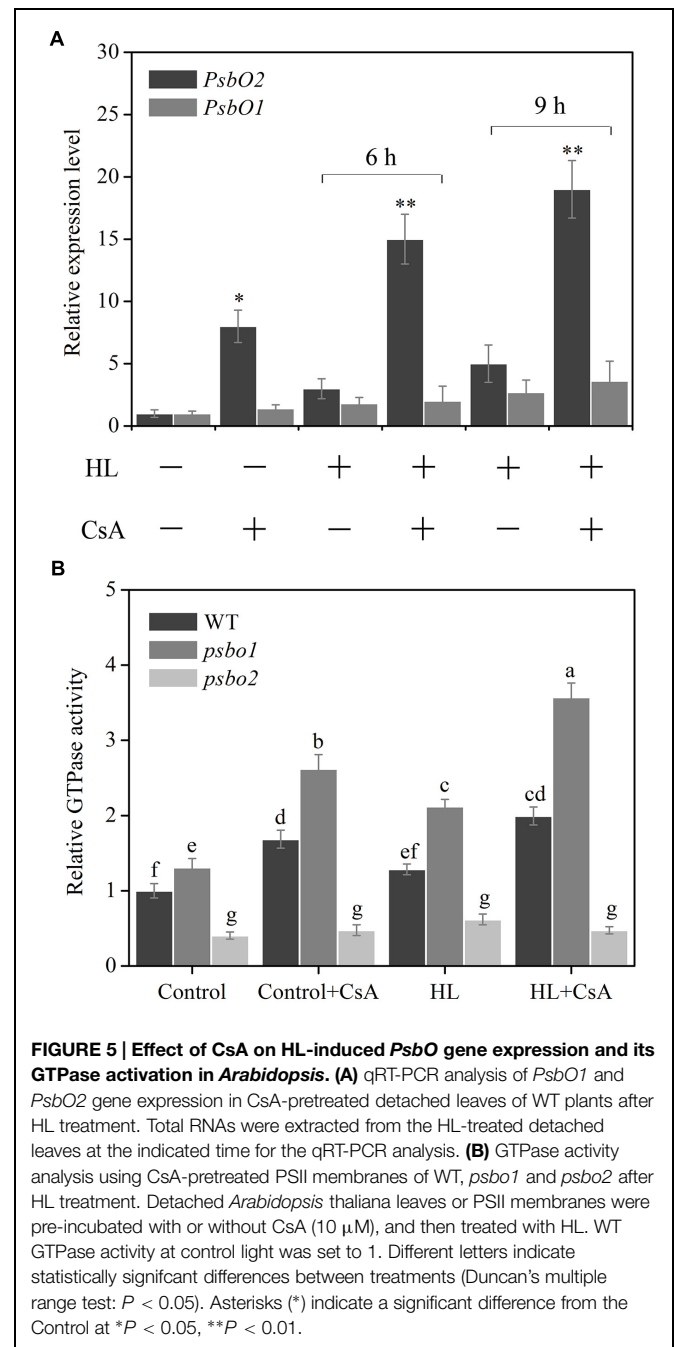
gene expressions in *cyp38* mutant and *cyp38-C* (Figure 6A). The results showed that *PsbO2* gene expression in *cyp38* mutant was higher than that of WT and *cyp38-C*, and such distinction became even larger after HL treatment. However, contrary to this, there was no distinctive change of *PsbO1* gene expression was observed either in *cyp38* mutant or *cyp38-C* (Supplementary Figure S3). The detection and analysis of PsbO activity was carried out at the same time. In *cyp38* mutant, PsbO activity rose distinctly after HL treatment (Figure 6B). In *cyp38-C*, PsbO activity was similar to that of WT. In sum, CYP38 has an inhibiting effect on PsbO activity and influence of its transcription, the main target is PsbO2.

### Gene Expression of CYP38 is Related to Ca<sup>2+</sup>-CaM3

Reactive oxygen species, as a widely studied oxidative stress molecular, can also function as cellular second messenger. Recently, it is proved that ROS plays an important role in cell

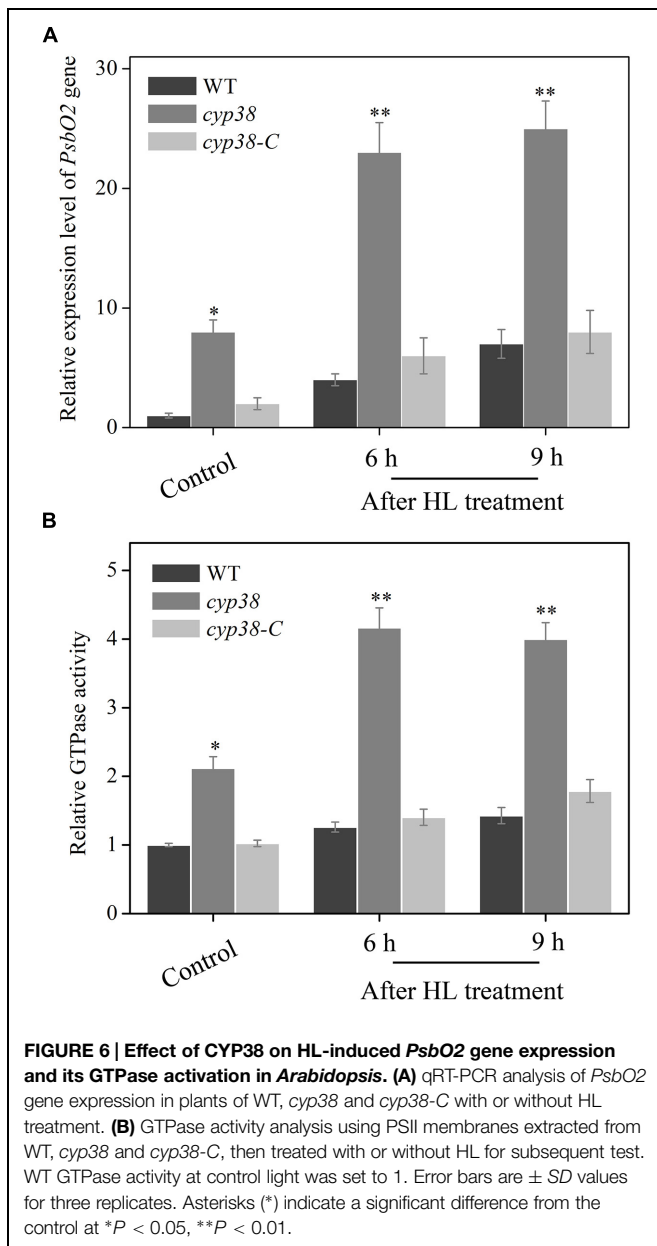


signal transduction, one target being  $\text{Ca}^{2+}$  (Mori and Schroeder, 2004; Bose et al., 2014). The results showed that, there was a significant increase in the level of ROS in the plant cells under HL conditions, subsequent to the cytoplasmic calcium  $[\text{Ca}^{2+}]_{\text{cyt}}$



content increased gradually; after the removal of ROS by AsA, the level of  $\text{Ca}^{2+}$  significantly dropped (Figures 7A,B). These results indicated that ROS may be an important signal molecular to increase the concentration of the  $[\text{Ca}^{2+}]_{\text{cyt}}$ .

$\text{Ca}^{2+}$ , as an important second messenger in plant cells, can function under abiotic stresses (Bowler and Fluhr, 2000; Li et al., 2012). We detected an increase in  $[\text{Ca}^{2+}]_{\text{cyt}}$  and up-regulation of the *CAM3* gene transcript level under HL condition (Figures 7A,C). In order to verify whether this change associated with the function of CYP38, the AsA, BAPTA-AM (Yue et al., 2012), CAP and *cam3* mutant was used. Under HL condition,



either scavenging  $[Ca^{2+}]_{cyt}$  with BAPTA-AM or abolishing ROS by treatment with AsA dramatically declined *CYP38* gene expression (Figures 7D,E). The *CYP38* gene expression was also dropped in *cam3* mutant plants. On the contrary, rapid  $[Ca^{2+}]_{cyt}$  elevation was induced by CPA can increase its transcription. The results above indicate that gene expression of *CYP38* is related to cell redox signal induced  $[Ca^{2+}]_{cyt}$ -CaM3 cascade.

## Discussion

This study is an attempt to understand the possible molecular mechanisms in order to explain how *CYP38* plays an important role in protection of PSII in *Arabidopsis* under HL treatment.

The results shown in this paper provide evidence for the cellular signaling cascade of the function of *CYP38* in *Arabidopsis* in response to HL stress.

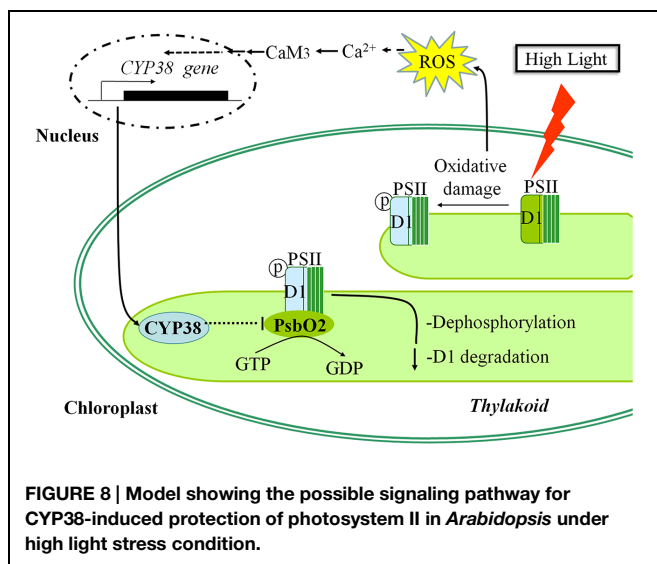
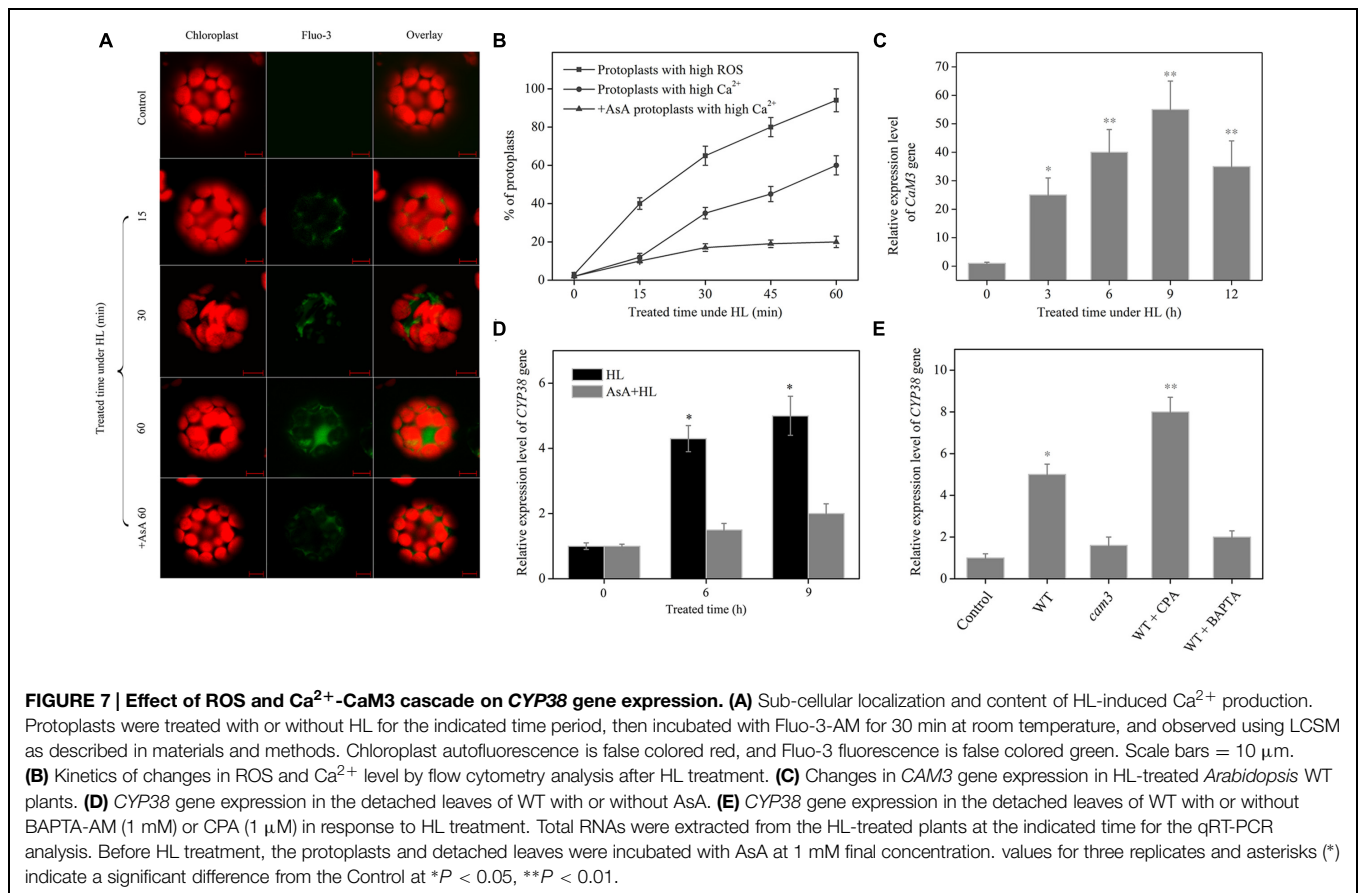
Recent studies have reported that deletion of *CYP38* in *Arabidopsis* results in stunted growth and *CYP38*-deletant is highly sensitive to HL environment (Fu et al., 2007; Sirpiö et al., 2008; Figure 1A). The present study observed that, under HL condition, *cyp38* leaves were more likely to be damaged by oxidation than WT leaves (Figure 1B). At the same time, experiment at the sub-cellular level showed that, in the protoplasts derived from *cyp38* mutant, there was excessive outbreak of ROS which mainly subject to co-localization with chloroplasts (Figure 3). Further detected the change of *CYP38* gene expression under HL, and discovered that its gene expression was increasing under HL condition compared with controlled and low light condition (Figure 2; Supplementary Figure S1), indicating that *CYP38* plays an important role in the protection of the chloroplasts in HL response.

Sirpiö et al. (2008) have demonstrated that *CYP38* deletion drastically decreases the *in vivo* phosphorylation of D1 protein in *Arabidopsis*. This research discovered that, under HL treatment, the degradation rate of D1 protein in *cyp38* mutant was distinctly higher than that of WT (Figure 4). Literature reports in the past pointed out that, first of all, the damaged D1 undergoes phosphorylation (Koivuniemi et al., 1995; Rintamäki et al., 1995a). As a signal for location change, the D1 undergoing phosphorylation is guided and migrated from grana membranes to stroma lamellae, where D1 undergoes dephosphorylation and later on be degraded (Tikkanen et al., 2008). To sum up, *CYP38* deletion causes the inhibiting effect on thylakoid membrane phosphatase to disappear, and correspondingly facilitate dephosphorylation of p-D1, and therefore strengthen degradation of the damaged D1. *PsbO2* also has been showed to have positive regulation effect on dephosphorylation and turnover of the PSII reaction center D1 protein (Lundin et al., 2007b). *CYP38* and *PsbO2* play important roles in D1 turnover process, which indicates that there may be some relevance between them (see the discussion below).

*CYP38* protein is identified to be present in the thylakoid lumen. However, the research of Sirpiö et al. (2008) show that considerable amount of *CYP38* protein is also associated with PSII monomer. As far as we know, these associated proteins of *CYP38* with PSII monomer may participate in relevant regulation of membrane protein, rather than merely being a structural composition. Our data presented that *PsbO2* gene expression and its GTPase activation were increased by CsA pro-treatment in WT plants compared with control (Figure 5). Further detection in *cyp38* mutant discovered similar results. However, in *CYP38* complementation line *PsbO2* gene expression and its GTPase activation fell down (Figure 6). We speculate that, the change of *PsbO2* transcription mainly because of the redox diversification of the plant cell related to *CYP38*. All these indicated that *CYP38* may, through inhibition of *PsbO2* activity and then influence the dephosphorylation rate of the damaged D1.

Previous studies discovered that, under HL condition, the degradation rate of p-D1 is lower than that of D1 (Rintamäki et al., 1995b). Therefore, as a mode of existence, p-D1 may





be more stable than D1. We consider that the high gene expression of CYP38 under HL and inhibition of PsbO2 prevent dephosphorylation-based degradation of p-D1 protein, and as a result they transform D1 to p-D1 which is relatively more stable. In this way, the excessive degradation loss caused by HL is avoided. Other reports note that PSII existing in the form

of p-D1 may no longer have the activity for transformation of light energy, however, the energy dissipation of such PSII can be enhanced (Critchley and Russell, 1994), which is beneficial for the adaptation to HL stress. From another perspective, CYP38 expression drops down to certain levels under LL conditions compared with HL (Figure 2; Supplementary Figure S1), which may release inhibition of PsbO2 and therefore enhance turnover of the damaged D1, facilitate PSII repair and increase efficiency of photosynthetic activity.

When plants are under abiotic threatening circumstances (such as excessive illumination), concentration of chloroplasts ROS grows and Ca<sup>2+</sup> levels in the cytosol increases as well (Baxter et al., 2014). If the balance between light absorption and the use of light energy is disturbed, photosynthesis will be further inhibited (Asada, 2006). In addition, the lack of light energy consumption leads to photoinhibition of PSII and enhances generation of <sup>1</sup>O<sub>2</sub>, which results in sequential reduction to superoxide anion radical (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxyl radicals ( $\bullet$ OH; Foyer and Noctor, 2000). This study shows that a large amount of <sup>1</sup>O<sub>2</sub> is generated in the cells under excessive light. DAB-stained leaves also tell that a great amount of H<sub>2</sub>O<sub>2</sub> is produced under the same condition (Figures 1 and 3). Recently, some articles claim that H<sub>2</sub>O<sub>2</sub> 'signatures', including antioxidant defense signal and well-known cytosolic calcium 'signatures' (Bose et al., 2014; Ozgur et al., 2014; Pottosin et al., 2014), may operate in plant signaling networks.

$\text{Ca}^{2+}$ , as an important messenger in plant cells (Hashimoto and Kudla, 2011), can function under various stimuli (Bowler and Fluhr, 2000; Romeis et al., 2001; Li et al., 2012). Under HL stress, we found that concentration of  $[\text{Ca}^{2+}]_{\text{cyt}}$  and *CAM3* gene expression increased, and inhibited  $[\text{Ca}^{2+}]_{\text{cyt}}$  by BAPTA-AM or deficient *CaM3*, which drastically decreased *CYP38* gene expression in *Arabidopsis* under HL condition, however, pre-incubated with CPA, which induced rapid elevation of  $[\text{Ca}^{2+}]_{\text{cyt}}$ , and drastically increased *CYP38* gene expression. Additionally, the level of  $[\text{Ca}^{2+}]_{\text{cyt}}$  and *CYP38* gene expression were significantly inhibited after removal of ROS by AsA, indicating that ROS generation may be a upstream signaling in these process (Figure 7). Our data present a cellular signaling cascade, composed of ROS production,  $[\text{Ca}^{2+}]_{\text{cyt}}$  increase and the upward regulation of *CaM3* transcript level, which function in the upstream of *CYP38* gene expression in response to HL.

## Conclusion

Our data showed that *CYP38*-mediated inhibition of PsbO2 activity modulated the tolerance of *Arabidopsis* to HL stress. According to our experimental results, a potential cascade of cellular events under HL stress was given (Figure 8): Firstly, HL stress induced ROS production in chloroplast, causing change to chloroplastic redox state. This signal might cause the

increase of  $[\text{Ca}^{2+}]_{\text{cyt}}$  and the activation of  $\text{Ca}^{2+}$ -CaM3 cascade, and subsequently facilitated *CYP38* expression. The increase in *CYP38* greatly inhibited PsbO2 activity, which weakened the dephosphorylation of D1 protein and kept it in a relatively stable phosphorylation state to avoid degradation. These results present a possible mechanism for plants to respond to external HL stress, and shed a new insight into the cellular signaling cascade of *CYP38*.

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## Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00777>

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