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Involvement of DEG5 and DEG8 proteases in the turnover of the photosystem II reaction center D1 protein under heat stress in *Arabidopsis thaliana*

SUN XuWu[1], WANG LiYuan[2] & ZHANG LiXin[2†]

[1] Key Laboratory of Arid and Grassland Ecology, School of Life Sciences, Lanzhou University, Lanzhou 730000, China;
[2] Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Photosynthesis Research Center, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China

***Deg5*, *deg8* and the double mutant, *deg5deg8* of *Arabidopsis thaliana* were used to study the physiological role of the DEG proteases in the repair cycle of photosystem II (PSII) under heat stress. PSII activity in *deg* mutants showed increased sensitivity to heat stress, and the extent of this effect was greater in the double mutant, *deg5deg8,* than in the single mutants, *deg5* and *deg8*. Degradation of the D1 protein was slower in the mutants than in the WT plants. Furthermore, the levels of other PSII reaction center proteins tested remained relatively stable in the mutant and WT plants following high-temperature treatment. Thus, our results indicate that DEG5 and DEG8 may have synergistic function in degradation of D1 protein under heat stress.**

DEG proteases, D1 protein, degradation, PSII reaction center, *Arabidopsis thaliana*

Photosystem II (PS II) is the most sensitive to heat stress among the thylakoid membrane protein complexes[1,2]. Heat stress results in the inactivation of oxygen-evolving complexes of PSII, along with the release of functional manganese[3]. The PSII reaction center and light harvesting complex II (LHC II) are also perturbed during heat stress. These alterations include separation of LHCII from PSII[4], conformational changes of PSII[5], heat-induced dephosphorylation of PSII proteins[6] and heat-induced aggregation of LHCII[7]. Plants have evolved various mechanisms to cope with high temperatures so as to protect PSII. Indeed, heat shock proteins are induced within a few hours during heat stress[8]. Small, methionine-rich chloroplast heat shock proteins have been shown to be involved in protection of PSII against heat stress[9]. In addition to protective mechanisms that have evolved, the damaged, aggregated and misfolded proteins must be removed efficiently during heat stress. However, the proteases involved in the degradation of damaged PSII under heat stress remain largely unknown to date.

Here we report the identification of DEG5 and DEG8 proteases that are responsible for the degradation of PSII reaction center D1 protein after heat stress and that are involved in protection against the damaging effects of heat stress.

1 Materials and methods

1.1 Plant materials

Both wild type (WT) plants and *deg5*, *deg8* and *deg5deg8* mutant plants, of a Columbian ecotype (Col) background, were grown in soil under short day conditions (10-h-light/14-h-dark cycle) with a photon flux density of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at a constant temperature of 22℃. The T-DNA insertion lines, SALK-099162

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†Corresponding author (email: zhanglixin@ibcas.ac.cn)
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(*DEG5*, AT4G18370) and SALK-004770 (*DEG8*, AT5G39830), were obtained from the SALK stock center. Double mutant *deg5deg8* was obtained by crossing single mutants, *deg5* and *deg8*, and screening the F2 population by PCR[10].

1.2 Measurements of chlorophyll fluorescence

Fluorescence measurements were performed using a portable fluorometer (PAM-2000, Walz, Fffeltrich, Germany) connected by a leaf-clip holder (2030-B, Walz) with tri-fucated fiberoptics (2010-F, Walz). Before measurement, leaves were dark-adapted for 15 min. The maximum photochemical efficiency of PSII was determined from the ratio of variable (F_v) to maximum (F_m) fluorescence ($F_v/F_m=(F_m-F_o)/F_m$)[11].

1.3 Chlorophyll fluorescence images analysis

Chlorophyll fluorescence images were captured at room temperature using a commercially available modulated imaging fluorometer (FluorCam; Photon System instruments, Brno, Czech Republic), as described by Ma et al[12]. Values of 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, with extremes that were arbitrarily assigned values of 0.35 (lowest) and 0.85 (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.

1.4 Heat stress and recovery treatment

Five-week-old whole plants were placed in an incubator at 45℃ for 1, 2, and 4 h. To examine the effects of chloroplast protein synthesis inhibitor, detached leaves were incubated with their petioles submersed in 1 mmol/L lincomycin solution at an irradiance of 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 3 h prior to heat treatment.

1.5 Thylakoid membrane preparation and immunoblot analysis

Thylakoid membranes were prepared according to standard methods[13]. For immunoblot analysis, the thylakoid membrane protein was subjected to electrophoresis. After electrophoresis, proteins were transferred to nitrocellulose membranes, probed with specific antibodies, and visualized by the enhanced chemiluminescence method. X-ray films were scanned and analyzed using an AlphaImager 2200 documentation and analysis system (Alpha Innotech).

2 Results

2.1 PSII activity in WT and *deg5*, *deg8* and *deg5-deg8* mutant plants under heat stress

Under optimal growth conditions, there were no apparent differences in the growth and photosynthetic performance among the WT and *deg5*, *deg8* and *deg5deg8* mutant plants[10]. Chlorophyll fluorescence imaging analysis showed that the maximum efficiency of PSII photochemistry decreased in WT and mutant plants under heat stress and distinct color differences were observed in the responses of WT and mutant plants to heat stress (Figure 1). Based on the above observation, we further examined changes in the F_v/F_m ratio, reflecting the maximum PSII photochemical efficiency, in whole plants under heat stress. In the absence of lincomycin, in WT F_v/F_m declined to about 75% of the over-night dark-adapted values within 4 h of heat stress (Figure 2(a)). The extent of this decrease in the mutants was considerably stronger: F_v/F_m declined to about 50% and 40% in the single and double mutants, respectively, following 4-h heat stress (Figure 2(a)). These results clearly demonstrated the increased sensitivity of the mutants to heat stress. In the presence of lincomycin, the decline in WT leaves was rapid, and continued until F_v/F_m values approached approximately 35% after 4-h heat stress (Figure 2(b)). Since lincomycin blocks the repair of PSII by inhibiting *de novo* protein synthesis in the chloroplast, the decline in F_v/F_m suggested that WT and mutant plants have similar rates of PSII photoinhibition.

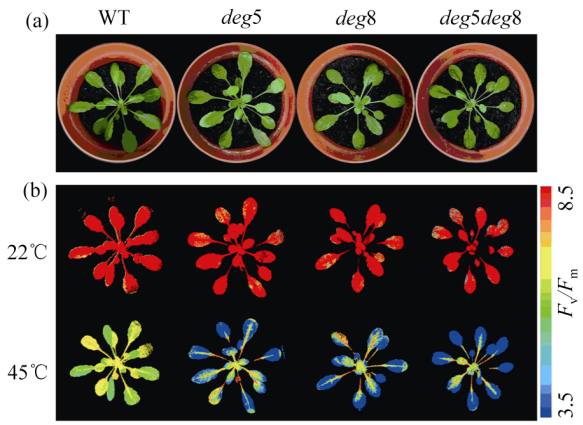

Figure 1 Chlorophyll fluorescence imaging analyses of WT and *deg5*, *deg8* and *deg5deg8* mutants. (a) 5-week-old plants grown in the growth chamber; (b) 5-week-old plants were treated at 45℃ for 4 h.

2.2 Degradation of PSII proteins under heat stress

To address the question of whether heat stress-in-

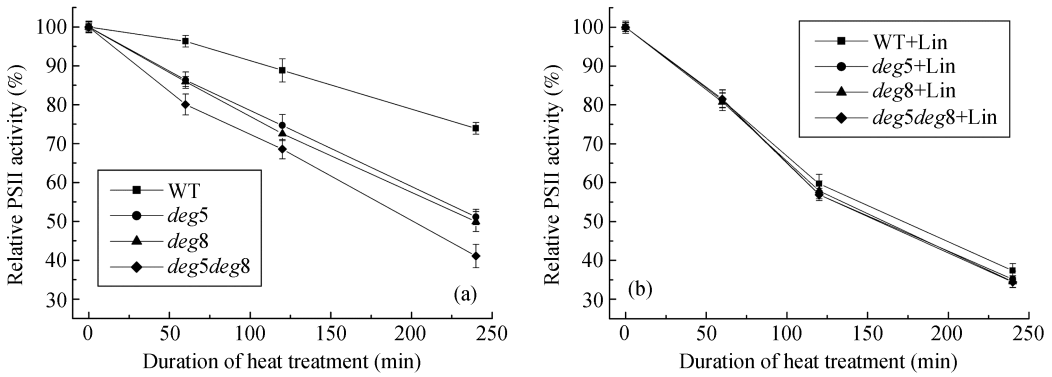

Figure 2 PSII activity under heat stress. (a) F_v/F_m was measured for whole plants from WT (squares), *deg5* (circles), *deg8* (triangles) and *deg5deg8* (diamonds) plants under heat stress at 45℃; (b) F_v/F_m was measured for detached leaves from WT (squares), *deg5* (circles), *deg8* (triangles) and *deg5deg8* (diamonds) plants during exposure to 45℃ in the presence of lincomycin.

duced PSII inactivation results from a decrease in the steady-state level of PSII complexes, we analyzed the content of PSII proteins in mutant and WT plants after heat treatment. Four hours of heat stress resulted in losses of about 50% of D1 protein in WT plants, 30% in both *deg5* and *deg8* mutants and about 10% in *deg5deg8* mutants (Figure 3). The contents of other PSII proteins, such as LHCII, CP43 and PsbO were immunodetected with the same treatment and were found to be relatively stable in both the WT and mutant plants (Figure 4).

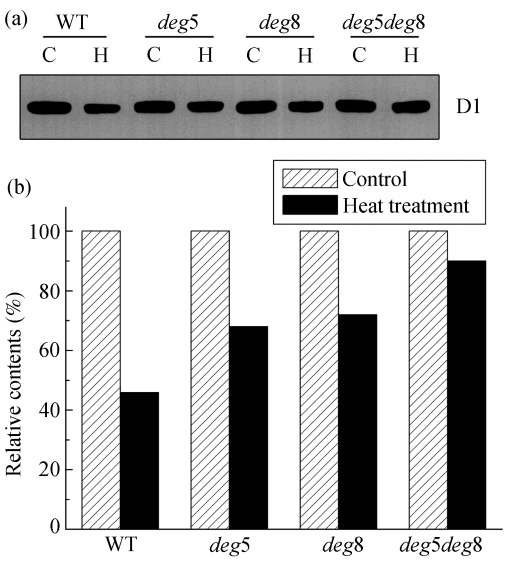

Figure 3 Immunoblot analysis (a) and quantification (b) of D1 protein levels in WT and *deg5*, *deg8* and *deg5deg8* mutant plants under heat stress.

3 Discussion

DEG/HTR family proteases are ATP-independent serine endopeptidases, and are widely distributed in living or

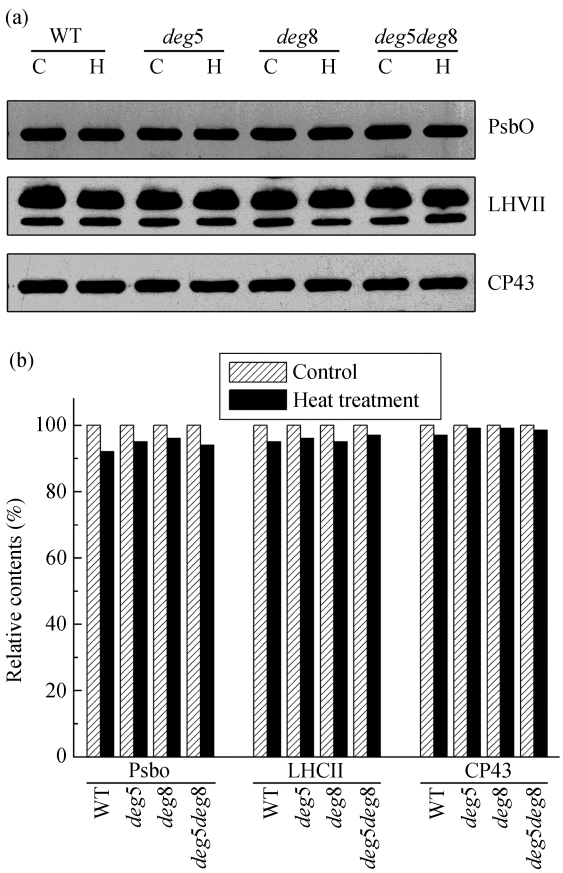

Figure 4 Immunoblot analysis (a) and quantification (b) of PsbO, LHCII and CP43 protein levels of PSII in WT and *deg5*, *deg8* and *deg5deg8* mutant plants under heat stress.

ganisms[14−19]. In the genome of *Arabidopsis thaliana*, 16 DegP-like protease genes have been identified. Out of these Deg proteases, Deg1, Deg5 and Deg8 were found in the thylakoid lumen and Deg2 was reported to be associated with the stromal side of the thylakoid membrane[20,21]. The function of these Deg proteases in

the thylakoid lumen, may be similar to those described from *E. coli*, as general-purpose proteases in the thylakoid lumen, degrading both soluble lumenal and membrane-bound proteins with lumen-exposed regions[16,17].

We have used *deg5*, *deg8* and *deg5deg8* double mutants of *Arabidopsis* to study the physiological role of the DEG proteases in the repair cycle of PSII under heat stress. PSII activity in these *deg* mutants showed increased sensitivity to heat stress and the extent of this effect was greater in the double mutant than in the single mutants (Figures 1 and 2). Inhibition of chloroplast protein synthesis resulted in similar rates of sensitivity of PSII to heat stress in the mutant and WT plants (Figure 2(b)). Thus, impairment of the PSII repair cycle may account for the increased sensitivity of PSII to heat stress observed in the mutants.

The repair of PSII is triggered by the degradation of the photo-damaged D1 protein, which is subsequently replaced with newly synthesized protein[22]. Impaired D1 synthesis or degradation may be responsible for the dysfunction of the repair cycle. Our results clearly showed a slower degradation rate of D1 protein in the mutants following high-temperature treatment than in the WT plants (Figure 3). Furthermore, the levels of other PSII reaction center proteins tested here remained relatively stable in the mutant and WT plants following the same treatments (Figure 4). These results demonstrate the function that DEG5 and DEG8 play in the degradation of PSII reaction center D1 protein.

Our results also demonstrated a higher sensitivity of PSII to heat stress and slower degradation rate in the *deg5deg8* double mutant than in the single mutants, *deg5* and *deg8*. Thus, DEG5 and DEG8 may have a synergistic function in the degradation of D1 protein.

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