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Research article

The roles of *Arabidopsis* proteins of Lhcb4, Lhcb5 and Lhcb6 in oxidative stress under natural light conditions

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ABSTRACT

Under light conditions, highly reactive oxygen species (ROS) can be generated in the antenna systems and the reaction center of photosystems (PS). The protective roles of Lhcb4 (CP29), Lhcb5 (CP26) and Lhcb6 (CP24), three minor chlorophyll binding antenna proteins during photoinhibition have been well studied. However, their regulatory mechanisms against oxidative damages under natural light conditions remain unknown. Here we investigated their specific roles in oxidative stress responses and photosynthetic adaptation by using the *Arabidopsis thaliana* knockout lines grown in the field condition. All three mutant lines exhibited decreased energy-transfer efficiency from the LHCII (light-harvesting complex II) to the PSII reaction center. Oxygen evolution capacity decreased slightly in the plants lacking Lhcb4 (*koLHCB4*) and Lhcb6 (*koLHCB6*). Photosynthetic rates and fitness for the plants lacking Lhcb5 (*koLHCB5*) or *koLHCB6* grown in the field were affected, but not in the plants lacking Lhcb4. Antioxidant analysis indicated the lowest antioxidant enzyme activities and the lowest levels of non-enzymatic antioxidants in *koLHCB6* plants. In addition, *koLHCB6* plants accumulated much higher levels of superoxide and hydrogen, and suffered more severe oxidative-damages in the field. Our results clearly demonstrate that Lhcb6 may be involved in alleviating oxidative stress and photoprotection under natural conditions.

1. Introduction

Photosystems II (PSII) is a large multi-subunit protein-complex located in the thylakoid membranes of cyanobacteria, algae and terrestrial plants that uses light energy for H_2O oxidation and plastoquinone reduction. In vascular plants, the PSII outer antenna systems are composed of the major antenna (LHCII) and the minor antenna complexes Lhcb4 (CP29), Lhcb5 (CP26) and Lhcb6 (CP24). LHCII comprises different heterotrimers of LHCb1, LHCb2 and LHCb3 proteins (Jansson, 1994, 1999). In *Arabidopsis thaliana*, Lhcb5 and Lhcb6 are encoded by the single gene respectively, while Lhcb4 is encoded by three highly-conserved genes, called *LHCB4.1*, *LHCB4.2* and *LHCB4.3*. Many studies have strongly suggested that each protein-complex has specific functions under natural environment conditions (Jansson, 1999; Ganeteg et al., 2004).

Rapid changes in temperature, light quality and intensity, and water status easily yield into the over-excitation of photosystems when the photosynthetic capacity is insufficient to utilize the absorbed light energy. However, incomplete photochemical quenching results in an increased Chl singlet (^{1}Chl*) excited state lifetime, thus enhancing Chl triplet (^{3}Chl*) generation through intersystem crossing (Melis, 1999). Then Chl triplet excited state easily reacts with oxygen molecules and produce harmful reactive oxygen species (ROS) (Barber and Andersson, 1992). Powerful ROS contains the highly reactive singlet oxygen, the superoxide anion radical, and hydrogen peroxide. The excessiveness of ROS usually causes oxidative damage to proteins and lipids. To prevent deleterious effects of ROS on photosystems in the variable stress conditions, photoprotection mechanisms are activated (Niyogi, 2000) by (1) down-regulating ^{1}Chl* lifetime via the non-photochemical quenching (NPQ) of chlorophyll fluorescence that the over-load energy absorbed by the pigment-binding proteins is dissipated as heat (Horton, 1996); (2) quenching ^{3}Chl*, thus avoiding ROS formation; and (3)

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scavenging ROS through the enzymatic and non-enzymatic antioxidant systems (Pospisil, 2012).

As three important minor antenna proteins, the specific roles of Lhcb4, Lhcb5 and Lhcb6 in light-harvesting and energy-dissipation in the photosystems have been well studied in *Arabidopsis* plants (Andersson et al., 2001; Kovacs et al., 2006; de Bianchi et al., 2008, 2011; Betterle et al., 2009). Lhcb4 and Lhcb5 have been shown to be involved in conformation changes through the violaxanthin exchange for zeaxanthin and carry protonatable sites (Pesaresi et al., 1997; Morosinotto et al., 2002). Lhcb6 consistently participates in function and organization of PSII, while the slower relaxing part of NPQ (qI) is altered in *koLHCB5* mutant (Kovacs et al., 2006; de Bianchi et al., 2008). In addition, *koLHCB4* mutant is more sensitive to photo-inhibition and that Lhcb4 is determinant for PSII macroorganization and the photoprotective dissipation of excess excitation energy (de Bianchi et al., 2011). More recently, our studies have indicated that the levels of Lhcb4 and Lhcb5 are influenced by environmental stress (Chen et al., 2016b, 2017). Although the roles of these proteins in NPQ and PSII structures have been demonstrated, the relationship between these three proteins and oxidative damage remains unclear.

In this work, we isolated and characterized *LHCB4*, *LHCB5* and *LHCB6* knockout (*koLHCB4*, *koLHCB5* and *koLHCB6*) plants. Their performance in photosynthesis, photoprotection, antioxidant systems, cell death, and ROS was analyzed in *Arabidopsis* seedlings grown in the field. We further confirmed their functions as described in previous work on *koLHCB4*, *koLHCB5* and *koLHCB6* (Kovacs et al., 2006; de Bianchi et al., 2008, 2011). Upon comparison of corresponding photosynthetic parameters, we discovered that *koLHCB6* plants exhibited the worst photosynthetic capacity and fitness in the field. Surprisingly, low antioxidant enzyme activity, severe cell death, and high levels of ROS were observed in the mutant of Lhcb6, suggesting that this mutant is more sensitive to the light changes under natural environmental conditions. These results suggest a possible mechanism connecting the Lhcb6 protein to ROS and NPQ.

2. Materials and methods

2.1. Plant materials and growth chamber experiments

Arabidopsis thaliana T-DNA insertion mutants (Columbia ecotype) *koLHCB4* (de Bianchi et al., 2011), *koLHCB5* (SALK_014869) and *koLHCB6* (SALK_077953) (de Bianchi et al., 2008) were kindly provided by Dr Malgorzata Pietrzykowska (Umea University, Umea, Sweden) from Stefan Jansson's Lab. Homozygous plants were identified by immunoblot ananlysis using mono-specific Lhcb4, Lhcb5 and Lhcb6 antibodies, respectively. All seedlings were grown for two weeks in a growth chamber with 120 μmol photons m^{-2} s^{-1}, 16/8 h light/dark cycles and humidity of 70%. Short term high light (HL) treatment was applied at 1200 μmol photons m^{-2} s^{-1} for two h (22 °C) because the highest photon flux density (PPFD) in the field is approximately 2000 μmol photons m^{-2} s^{-1} at noon (11:00–13:00) on a clear day. A light source was provided by metal halide lamps and passed through a 3 cm recirculation water system in HL treatments.

2.2. Field experiments

The field experiment was performed in the experimental garden of Sichuan Agricultural University in Ya'an (29°58′N, 103°01′E). The seeds were sown on April 20, 2015 and the plants were transferred to their original pots ten days later (April 30) in the growth chamber. *Arabidopsis* seedlings had three to four leaves after transferring to the experimental garden on May 10. The seedlings were shaded on the first day after the transfer to get some acclimation. PPFD was measured in the field and ranged from very low levels to 2000 μmol photons m^{-2} s^{-1} during the photoperiods. And the mid-day temperature varied from 18 °C to 31 °C, and the relative humidity varied from 40% to 90%. The vegetative growth rate was monitored during the first month outside by measuring the rosette size of 5 randomly chose plants. After 4 weeks in the field, the plants were brought back to laboratory for the measurements of various parameters in the morning. After 8 weeks in the field, the plants were transferred to the laboratory to dry and for later seed and silique counting. The total number of siliques per plant was calculated on 50 individual plants of each genotype. Then the number of seeds in five siliques was calculated from 50 biological replicates within each genotype. This number was also used to determine the total amount of seeds per plant (Mishra et al., 2012).

2.3. Pigment analysis

Chlorophyll (Chl) was extracted from whole leaves in 80% (v/v) acetone and its content was measured with a spectrophotometer (Hitachi-U2000, Hitachi, Ltd., Tokyo, Japan) at wave-lengths of 646.6 nm and 663.6 nm according to Porra et al. (1989). The levels of carotenoids were determined by high performance liquid chromatography (HPLC) (Gilmore and Yamamoto, 1991).

For anthocyanin assay, seedlings were ground with a plastic pestle in a 1.5 mL tube in 300 μl of 1% HCl in methanol. Anthocyanin content was calculated from A530 corrected for the background A657 (Cottage et al., 2010).

2.4. Thylakoid isolation, gel electrophoresis and immunodetection

Functional thylakoids were isolated under a safe green light as previously described (Chen et al., 2016a) with 10 mM NaF. Standard SDS-PAGE analysis with 6M urea was used with the Tris-Gly buffer system (Laemmli, 1970). Then thylakoid samples were electro-blotted on a PVDF membrane (Immobilone, Millipore, Darmstadt, Germany). Proteins were detected by specific antibodies raised against D1, D2, Lhca1-4, PsaD, PsbO, PsbS, AtpB, PetB, Lhcb1-3, Lhcb4 (CP29), Lhcb5 (CP26), and Lhcb6 (CP24) and CP43. All antibodies have been purchased from Agrisera Comp. (Umea, Sweden). For detection to phosphoproteins, the anti-phospho/threonine antibody (Cell Signaling, Ipswich, MA, USA) was used. The GE Healthcare ECL reagent was used for detection of the immunoblots. Signal amplitudes of the immunoblots were quantified with the Quantity-One software (Bio-Rad Comp. Hercules, CA, USA).

2.5. Blue native gel and 2D electrophoresis

Blue native gel electrophoresis was performed as described previously (Chen et al., 2016a). Thylakoid membranes concentrated to 1 mg/mL Chl were solubilized by a final 1% *n*-dodecyl-β-$_D$-maltoside (Sigma Chemical Co. St. Louis, MO, USA), and 20 μg of Chl was loaded on each lane. For the second-dimension separation, each strip was excised from the first-dimension gel and incubated in Laemmli (1970) buffer for 1 h at 25 °C prior to SDS-PAGE on 15% (v/v) PAGE gels containing 6 M urea. After electrophoresis, the bands were visualized through staining of Coomassie Brilliant-Blue R.

2.6. Chl fluorescence analysis and NPQ measurements

Chlorophyll fluorescence images was obtained using a modulated imaging fluorometer (the Imaging PAM M-Series Chlorophyll Fluorescence System, Heinz-Walz Instruments, Effeltrich, Germany) according to the manufacturer's instruction. Seedlings were dark-adapted for a minimum of 30 min before fluorescence measurements. A saturated pulse intensity was set to 8000 μmol m^{-2} s^{-1} and the actinic light was at 1200 μmol m^{-2} s^{-1}. The image data obtained were normalized to the false color scales. Fv/Fm, ΦPSII, qP, and NPQ were calculated according to the methods of Maxwell and Johnson (2000).

PSI absorbance changes and Chl *a* fluorescence in wild-type and mutant plant leaves were detected with the dual PAM-100 fluorometer