Plastid Signals Confer *Arabidopsis* Tolerance to Water Stress

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Plastid-to-nucleus retrograde signalling coordinates nuclear gene expression with chloroplast function and is essential for the photoautotrophic life-style of plants. The relationship between plastid signalling and water stress response was investigated with genome uncoupled (*gun*) mutants, *gun1*, *gun3*, and *gun5*, and an abscisic acid (ABA)-responsible transcription factor mutant, *abi4*. The results showed that *gun1*, *gun3*, *gun5*, and *abi4* mutants suffered from more oxidative damages than the wild-type plants under the water stress and the water stress + herbicide (norflurazon, NF) co-treatment. Superoxide dismutase (SOD), peroxidase (POD), and ascorbate peroxidase (APX) activities could not be prompted in the plastid-signalling defective mutants under the stress conditions. At the same time, *Lhcb* expression was not repressed in the plastid-signalling defective mutants by the NF treatment or water stress. Therefore, the photosynthetic apparatus in the mutant cells could not be closed during the stresses and the excessive light caused more photodamages on the mutant leaves. The roles of GUN1, GUN3, GUN5 and ABI4 proteins in environmental stress adaptation have been discussed.

Key words: Norflurazon, Reactive Oxygen Species, Water Stress

Introduction

Plastid biogenesis involves precise coordination of both plastid and nuclear gene expression. Developmentally arrested or damaged plastids can regulate nuclear gene expression *via* retrograde signalling pathways (Koussevitzky *et al*., 2007). Five plastid-to-nucleus retrograde signalling pathways have been described (Beck, 2005; Koussevitzky *et al*., 2007). One of these pathways is related to some chlorophyll-biosynthetic-intermediates (called tetrapyrroles), possibly including Mg-protoporphyrin (Mg-Proto IX). Another signal is induced by inhibition of plastid gene expression (PGE) (Zhang *et al*., 2010). The third may correlate with sugar signals. The fourth signal is controlled by the redox state of the photosynthetic electron transport (PET) chain, and the fifth employs reactive oxygen species (ROS). These five pathways may be part of a complex signalling network that links the functional and physiological state of the plastids to the nucleus (Pesaresi *et al*., 2007; Fernández and Strand, 2008; Pogson *et al*., 2008; Woodson and Chory, 2008; Kleine *et al*., 2009; López-Juez, 2009).

To elucidate this retrograde plastid-to-nucleus signalling pathway, *Arabidopsis* genome uncoupled (*gun*) mutants, *gun1*–*gun5*, were isolated that showed a de-repression of *Lhcb* (encoding a light-harvesting chlorophyll-binding protein) expression in the presence of norflurazon (NF). *GUN5* encodes the H-subunit of Mg-chelatase (CHLH), and in the *gun5* mutant *Lhcb* transcript levels could not be reduced by NF (Strand *et al*., 2003). GUN4 is a Mg-chelatase co-factor (Larkin *et al*., 2003; Adhikari *et al*., 2009; Peter and Grimm, 2009). *gun2* (encoding heme oxygenase) and *gun3* (encoding phytochromobilin synthase) mutants accumulate high levels of heme (Nott *et al*., 2006), while heme accumulation leads to negative feedback regulation of chlorophyll biosynthesis including Mg-Proto IX (Nott *et al*., 2006;

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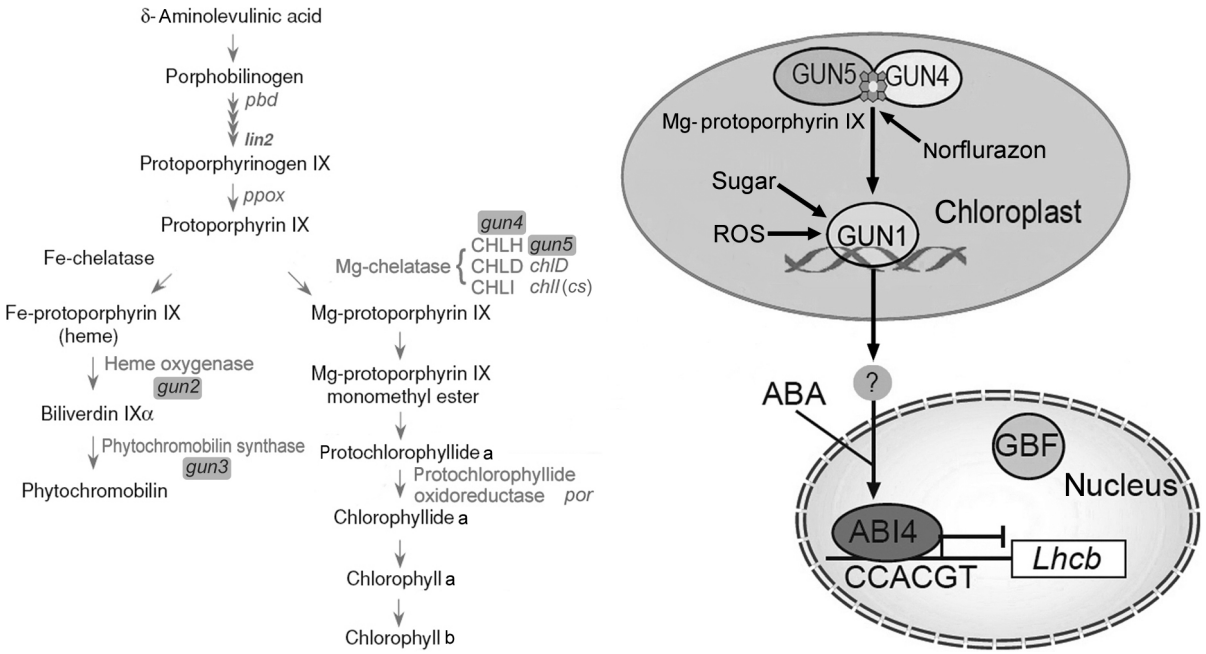

Fig. 1. Tetrapyrrole biosynthetic pathway and model of retrograde signalling pathways from chloroplasts to nuclear *Lhcb* genes. Key enzymes and their mutants are shown. Mg-chelatase has 3 subunits, CHLH, CHLD, and CHLI. The mutant *gun5* has a point mutation in the *ChlH* gene, while *GUN4* encodes a Mg-Proto IX-binding protein that can stimulate the Mg-chelatase activity *in vitro*, and plays a role in signalling. *gun2* and *gun3* have mutations in the heme oxygenase and phytochromobilin synthase, respectively. NADPH-Pchlide oxidoreductase (POR) functions dependent on light. Norflurazon (NF) treatment generates some plastid signals depending on GUN5 and its cofactor GUN4. NF, sugar, and ROS generate a common plastid signal *via* plastid GUN1 protein. GUN1 is a DNA-binding protein transmitting the signals to unknown cytosol factors (question mark). In response to the GUN1-derived signal, ABI4 binds the promoter of *Lhcb* preventing GBF from binding. ABI4 also mediates ABA signals.

Rockwell *et al.*, 2006). GUN1 is a plastid protein and integrates multiple plastid signals (Koussevitzky *et al.*, 2007). In response to the GUN1-derived signal ABI4 [an Apetala 2-type abscisic acid (ABA)-responsible transcription factor] binds the promoter of *Lhcb* preventing GBF (a G-box binding factor required for tissue-specific, light-induced expression of *Lhcb*) from binding (Fig. 1).

Considering that ROS signals tightly correlate with other plastid signals (Koussevitzky *et al.*, 2007; Kleine *et al.*, 2009) and plastid signal-inducible genes are mostly involved in anti-stress responses (Strand *et al.*, 2003), plastid signalling should play an important role in plant adaptation to environmental stresses. A previous study showed that some plastid-signalling defective mutants have impaired basal thermotolerance (Miller *et al.*, 2007), which supports this point of view. However, whether plastid-signalling defective mutants adapt worse to other environmental stresses has not been elucidated before. Drought being an important limitation for plants impairs severely growth, crop yield, and various morphological, anatomical, physiological, and biochemical processes (Liu *et al.*, 2006). During water stress, plants experience a number of metabolic changes, including changes in protein synthesis, alterations in gene expression, decline of protein and chlorophyll contents, and production of ROS (Sun *et al.*, 2006; Yuan *et al.*, 2007). In the present study, we found that *gun1*, *gun3*, *gun5*, and *abi4* mutants suffered from more oxidative damages than the wild-type plants under water stress. Their possible physiological mechanisms are also discussed.

Material and Methods

Plant growth and stress treatments

Arabidopsis thaliana seeds of *gun1* and *gun5* mutants were gifts from Dr. E. López-Juez (Royal

Holloway University of London, UK) and Prof. Joanne Chory (The Salk Institute, La Jolla, CA, USA). Other mutants were obtained from the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University (Columbus, OH, USA). All mutants were in the Col-0 background. Plants homozygous for the T-DNA insertion were identified based on PCR analysis. The seedlings were grown under an 8 h/16 h light/dark cycle of medium light intensity (100 μmol m^{-2} s^{-1}) for 3 weeks. Then the seedlings were removed from the sand, washed with tap water, and dried briefly with paper towels to remove surface water. Water stress was initiated by submerging the roots of the seedlings into polyethylene glycol (PEG) 6000 solution with an osmotic potential of −0.5 MPa. Control plants were grown in water, and all samples were treated for 0, 24, 48, and 72 h under the above conditions. The degrees of water stress were characterized by the absolute water content. The water solo-stress did not lead to large physiological differences between *gun1*, *gun3*, *gun5*, and *abi4* mutants and the wild-type plants. For inducing more severe oxidative stress, plants were pretreated with 50 μM norflurazon (NF, an herbicide and plastid-signalling initiator) for 48 h, and then subjected to water stress (PEG 6000 supplied with 50 μM NF) for 1–3 d (NF + water co-stress).

Measurement of absolute water contents

Approx. 0.5 g fresh leaves, determined as fresh weight (FW), were dried in an oven at 105 °C to constant weight (DW). The absolute water content in leaves was determined as the ratio of [(FW – DW)/FW] · 100% (Duan *et al.*, 2010).

Oxidative damage estimation

The thiobarbituric acid-reactive substances (TBARS) content was determined according to Sun *et al.* (2006) with some modifications. Approx. 0.5 g fresh leaves were cut into small pieces and homogenized by addition of 5 mL 5% trichloroacetic acid (TCA) in an ice bath. The homogenate was transferred into a tube and centrifuged at 1,000 × *g* for 10 min at 4 °C. Aliquots of supernatant and 0.5% thiobarbituric acid (TBA) in 20% TCA were added into a new tube. This mixture was incubated at 98 °C for 40 min, then cooled to room temperature, and centrifuged at 8,000 × *g* for 10 min. The absorbance of the supernatant was measured spectrophotometrically at 532 nm, 600 nm, and 450 nm.

Electrolyte leakage was measured according to Cao *et al.* (2009). After measuring the conductivity, the *Arabidopsis* leaves samples were boiled for 15 min to achieve 100% electrolyte leakage.

Superoxide and H_2O_2 staining

In situ superoxide and H_2O_2 were detected with nitroblue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB), respectively, as described previously (Yang *et al.*, 2004; Shang *et al.*, 2010). *Arabidopsis* leaves were excised at the base with a razor blade and supplied through the cut ends with NBT (1 mg mL^{-1}) or DAB (0.5 mg mL^{-1}) solutions for 8 h. Leaves were then decolourized in boiling ethanol (95%) for 15 min.

Lhcb transcript analysis

RNA was extracted from the frozen tissue as described previously (Zhang *et al.*, 2004). Representative *Lhcb* gene (*Lhcb1.3*, At1g29930) was amplified by PCR using the following primers: forward, CGGAGACTACGGATGGGACA, and reverse, CGGGAACAAAGTTGGTGGC.

Determination of antioxidant enzymes

For superoxide dismutase (SOD) and ascorbate peroxidase (APX) enzyme assays, 0.3 g of leaf was ground with 3 mL ice-cold 25 mM HEPES buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM ascorbate, and 2% polyvinyl pyrrolidone (PVP). The homogenates were centrifuged at 4 °C for 20 min at 12,000 × *g*, and the resulting supernatants were used for determination of the enzymatic activity (Zhu *et al.,* 2000). SOD activity was assayed by measuring the ability to inhibit the photochemical reduction of NBT following the method of Stewart and Bewley (1980). APX activity was measured as the decrease in absorbance at 290 nm because ascorbate was oxidized (Nakano and Asada, 1981).

For peroxidase (POD) enzyme assays, the seedlings (200–500 mg) were ground in liquid nitrogen, homogenized in sodium phosphate buffer, pH 6.5, and centrifuged for 20 min. 1.5 mL of the supernatant was combined with 0.05 mL of 1 mM guaiacol solution in ethanol. 0.05 mL of 1 mM H_2O_2 in redistilled water was added before the spectrophotometric measurements. The absorp-

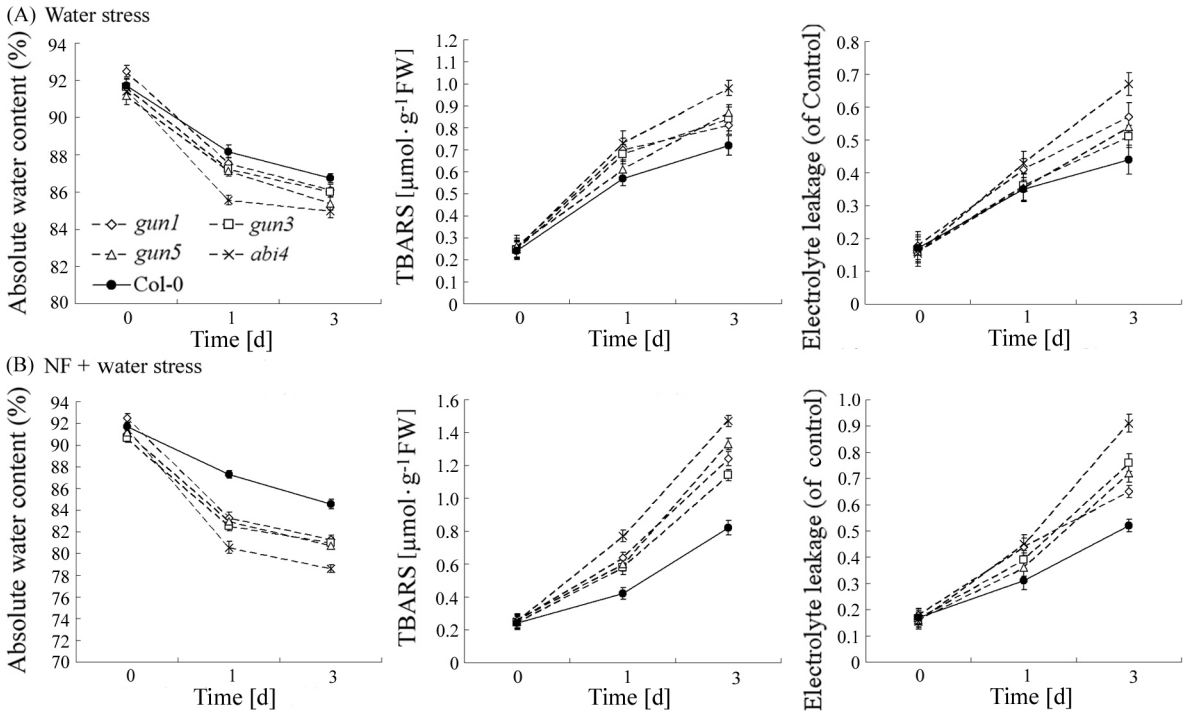

Fig. 2. Effects of (A) water stress and (B) NF + water co-stress on leaf absolute water content, TBARS level, and electrolyte leakage of *gun1*, *gun3*, *gun5*, and *abi4* mutants and the wild-type (Col-0) *Arabidopsis* seedlings. The plants were stressed for 0, 1, and 3 days. Bars represent standard deviations of 3 independent replicates.

tion was measured at 470 nm, and the POD activity was expressed as the change in absorbance (Salame and Zieslin, 1994).

Statistical analysis

Means of 3 triplicates were measured. Student's *t* test was used for comparison between different treatments. A difference was considered to be statistically significant when $P < 0.05$.

Results

More oxidative damages to the plastid-signalling defective mutants

The water status was measured in leaves of *gun1*, *gun3*, *gun5*, and *abi4* mutants and the wild-type plants (Fig. 2). Three days after the water stress, the absolute water content decreased dramatically. However, the faster declines were showed in plastid-signalling defective mutants, especially in the *abi4* mutant, compared to the wild-type plants.

To further confirm the effects of water stress on membrane integrity, TBARS content and electrolyte leakage were determined (Fig. 2). Water stress resulted in an accumulation of lipid peroxidation products and an increase of electrolyte leakage in leaves, especially in the *abi4* mutant, whose TBARS and electrolyte leakage values were about four times those of the control, while the oxidative damages in the wild-type plants were not as serious as those in the plastid-signalling defective mutants.

However, the differences were not very significant under the water stress ($P = 0.07$). For inducing more severe oxidative stress, plants were pretreated with 50 μM NF for 48 h, and then subjected to water stress (PEG 6000 supplied with 50 μM NF) for 1–3 days. At this stress condition, plastid-signalling defective mutants suffered from more severe oxidative damages, the absolute water contents decreased more rapidly and the TBARS and electrolyte leakage values increased more rapidly, compared to the wild-type plants (Fig. 2, $P = 0.03$).

DAB and NBT staining

DAB and NBT staining confirmed the result that higher H_2O_2 and superoxide accumulation occurred in the plastid-signalling defective mutants, especially in the *abi4* mutant, compared to the wild-type plants, under both the water stress and the NF + water co-stress treatment (Fig. 3).

Lhcb was not repressed in the plastid-signalling defective mutants under the stresses

The *Lhcb* gene expression was largely repressed in the wild-type plants under the water stress and the NF + water co-stress. However, similarly large repressions were not observed for *gun1*, *gun3*, *gun5*, and *abi4* mutants. Even in *gun1* and *abi4* mutants, the expression of *Lhcb* was almost unchanged during all stress treatments (Fig. 4).

Antioxidant enzyme activities were not dramatically prompted in the plastid-signalling defective mutants

Plants are capable of removing ROS using several antioxidant enzymes. Previous studies suggested that most plastid signal-inducible genes were anti-stress genes, such as antioxidant enzyme genes encoding SOD, POD, and APX (Strand *et al.*, 2003). Therefore, we measured these three antioxidant enzyme activities. As shown in Fig. 5, all antioxidant enzyme activities increased after the stresses. It is notable that the enzyme activities in *gun1* and *abi4* seedlings almost were not increased under all these stress conditions. The increases of SOD, POD, and APX activities in *gun2* and *gun3* mutants (*P* values ranged from 0.03 to 0.07) were not as significant as those in the wild-type plants (*P* values ranged from 0.01 to 0.02).

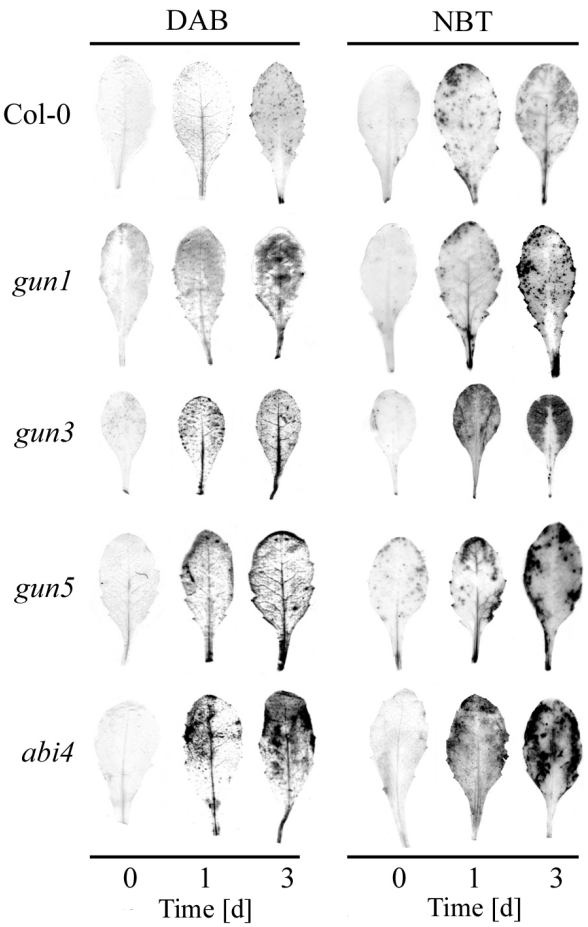

Fig. 3. DAB staining of water-stressed and NBT staining of NF + water-stressed *gun1*, *gun3*, *gun5*, and *abi4* mutants and the wild-type (Col-0) *Arabidopsis* leaves. DAB and NBT stain show the H_2O_2 and superoxide levels, respectively.

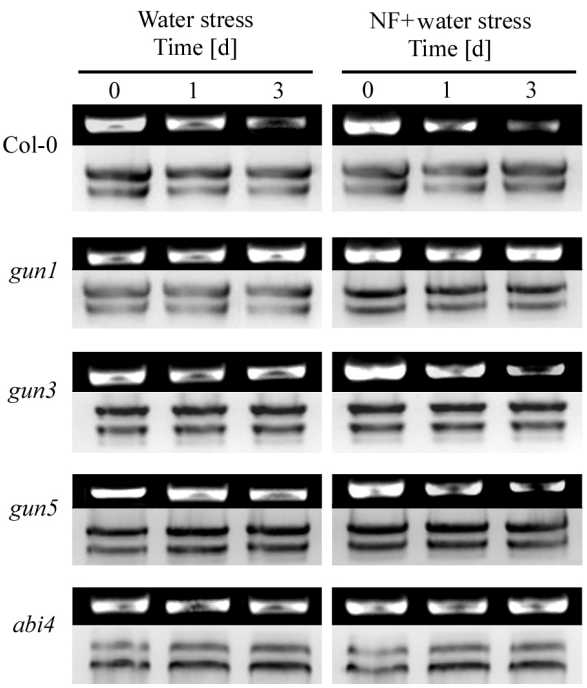

Fig. 4. Changes in steady-state levels of *Lhcb* transcripts from *gun1*, *gun3*, *gun5*, and *abi4* mutants and the wild-type (Col-0) *Arabidopsis* seedlings stressed for 0, 1, and 3 days. 10 μg of total RNA from the seedlings were loaded per lane.

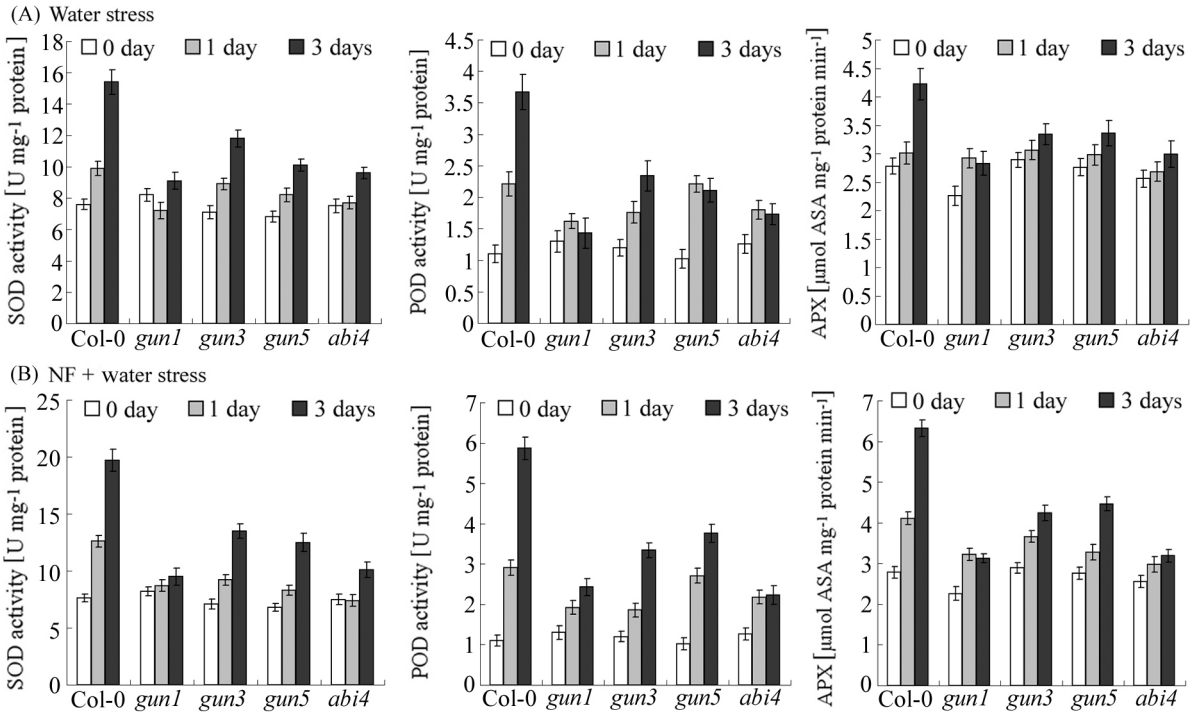

Fig. 5. Effects of (A) water stress and (B) NF + water co-stress on activities of SOD, POD, and APX in *gun1*, *gun3*, *gun5*, and *abi4* mutants and the wild-type (Col-0) *Arabidopsis* seedlings. The plants were stressed for 0, 1, and 3 days. Bars represent standard deviations of 3 independent replicates.

Lower activities of several antioxidant enzymes in plastid-signalling defective mutants may partially explain their lower tolerance to water stress.

Discussion

Plastid signals induce antioxidant gene expression and closure of the photosynthetic apparatus by repressing photosynthesis-associated nuclear gene expression (Strand *et al*., 2003; Koussevitzky *et al*., 2007). Antioxidant enzymes could not be prompted in the plastid-signalling defective mutants under the stress conditions. At the same time, *Lhcb* expression was not repressed in *gun1*, *gun3*, *gun5*, and *abi4* mutants by the NF treatment or the water stress. Thus, the photosynthetic apparatus in the mutant cells could not be closed during the stresses, and the excessive light caused more severe photodamages on the mutant leaves, because the light efficiency decreases under water stress (Chen *et al*., 2009; Liu *et al*., 2009).

GUN1 was proposed to function as a key factor responsible for multiple plastid signalling, including ROS signalling, while the down-streaming factor ABI4 functions in nuclei and binds *Lhcb* promoter directly. Thus, the worse adaptation of *gun1* and *abi4* mutants to water stress can be explained. However, plastid GUN2, GUN3, and GUN5 proteins may be not related with plastid signalling directly, but responsible for the NF-derived gene regulation (Strand *et al*., 2003; Nott *et al*., 2006; Woodson and Chory, 2008). The worse adaptation of *gun2*, *gun3*, and *gun5* mutants to the sole water stress may not be attributed to their hampered NF-derived plastid signalling. Tetrapyrroles themselves may generate ROS in light, such as Mg-Proto IX (declined in *gun5* mutant) and heme (accumulated in *gun2* and *gun3* mutants). Therefore, GUN2, GUN3, and GUN5 may simply interfere with ROS production and, in turn, interfere with the redox state of plastids (Kleine *et al*., 2009) and water stress tolerance. An alternative explanation is the signalling role of Mg-Proto IX. Although the Mg-Proto IX model has been greatly challenged by several reports (Kleine *et al*., 2009), its rationality has also been testified (our data supporting this point of view will be published elsewhere). The Mg-chelatase

complex (including GUN5 protein) is postulated to change its location between the stroma and an association with the inner envelope of the plastid depending on the concentration of Mg^{2+} inside the plastid (Nakayama *et al*., 1998). GUN1 may interact with GUN5 and translocate with GUN5, resulting in the transduction of a signal (Woodson and Chory, 2008; Jung and Chory, 2010). The inability of Mg-Proto IX accumulation in *gun2*, *gun3*, and *gun5* mutants might hamper the co-translocation of GUN1 and GUN5 proteins, and therefore affect the ROS signalling and the stress tolerance indirectly.

Although it is true that the chloroplast is dependent on the nucleus to supply much of the genetic information necessary for its function, it is also becoming clear that the plastids produce multiple signals in response to changes in the environment that orchestrate major changes in nuclear gene expression. Plastid signals assist the cell during stress responses and help the plant to respond optimally to environmental stress; information must be integrated from both cytosolic and plastid-signalling networks (Fernández and Strand, 2008; Jung and Chory, 2010). The sources of many plastid signals and *cis*-elements with transacting factors through which the signals are mediated have been identified (Nott *et al*., 2006; Woodson and Chory, 2008; Jung and Chory, 2010). However, the cytosolic components transducing the signal to the nucleus remain elusive and identifying these players is an exciting task for the future. Also the relationship between plastid signalling and plant stress response needs further investigations.

Acknowledgements

Arabidopsis thaliana seeds of *gun1* and *gun5* mutants were gifts from Dr. E. López-Juez (University of London, UK) and Prof. Joanne Chory (The Salk Institute, La Jolla, CA, USA). Other mutants were acquired from *Arabidopsis* Biological Resource Center (ABRC, Columbus, OH, USA). This research was supported by the National Key Basic Research '973' Program of China (2009CB118500), National Nature Science Foundation of China (30970214 and 30800071), and Project of Chinese Ministry of Education (108110).

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