A Chlorophyll-Less Barley Mutant "*NYB*" Is Insensitive to Water Stress

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"*NYB*" is a chlorophyll-less barley mutant, which grows relatively slow and unhealthily. The effects of water stress on photosystem II (PSII) of *NYB* and its wild type (WT) were investigated. Unexpected results indicated that the mutant was more resistant to water stress, because: PSII core proteins D1, D2 and LHCII declined more in WT than in *NYB* under water stress, and the corresponding *psbA*, *psbD* and *cab* mRNAs also decreased more dramatically in WT; CO_2 assimilation, stomatal conductance, maximum efficiency of PSII photochemistry (*F*v/*F*m), efficiency of excitation energy capture by open PSII reaction centres (*F*v'/*F*m'), quantum yield of PSII electron transport (Φ_{PSII}) and DCIP photoreduction in *NYB* were less sensitive to water stress than in WT, although the non-photochemical quenching coefficient (q_N) and the photochemical quenching coefficient (q_P) were almost the same in *NYB* and WT. Effective chlorophyll utilization and improved PSII protein formation in the mutant may be the reason for the enhanced stress resistance. Other possible mechanisms are also discussed.

Key words: Chlorophyll-Less Barley, Photosystem II, Water Stress

Introduction

Water deficit in leaves results in reduction of the net CO_2 assimilation rate and net photosynthetic rate (Lu *et al*., 1998; Yuan *et al*., 2005; Duan *et al*., 2006), which to some extent can be attributed to impairment of the primary photosynthetic machinery (Yuan *et al*., 2005). Photosystems, the pigment-protein complexes in the thylakoid membranes, play a key role in the responses of photosynthesis in higher plants to environment stresses. Previous studies showed that the two photosystems I and II (PSI and PSII), particularly PSII, are affected by water stress and this leads to lowered electron transport through them (Yuan *et al*., 2005; Liu *et al*., 2006). The steady-state levels of the PSII proteins D1, D2 and LHCII and the corresponding genes *psbA*, *psbD* and *cab* mRNAs also decrease dramatically under water stress (Yuan *et al*., 2005; Duan *et al*., 2006; Liu *et al*., 2006). Furthermore, LHCII could protect PSII core against various stresses by altering its conformation and migrating between PSII and PSI (Allen, 1992, 2003).

However, there are few reports about the relationship between deficits in chlorophyll (chl) content and effects of water stress on PSII. Therefore, here we chose a chlorophyll-less barley mutant, "*NYB*", to study the changes of PSII in water stress.

NYB is obtained by ^{60}Co-γ-ray radiation (Lin *et al*., 1998, 1999). Its phenotype is stable and not affected by light or temperature. The chloroplast of *NYB* contains fewer thylakoids and grana, with a lower total chl content (Lin *et al*., 1999). There is an apparent decrease of LHCII in *NYB*, but other PSII antennae are little affected (Lin *et al*., 1998). As compared with WT, *NYB* has a lower net photosynthetic rate and apparent quantum yield, which may result from lower chl content (Tan *et al*., 1996). A study by Tan *et al*. (1997) demonstrated that the *NYB* has a higher PSII photochemical efficiency (*F*v/*F*m), which may be due to the lesser excitation energy transfer from PSII to PSI.

When *NYB* hybridizes with WT, the ratio of character segregation is 3:1, and the ratio of testcross is 1:1 (Cheng *et al*., 2001). Therefore, the yellowish leaves of *NYB* are most likely controlled by a recessive nuclear gene. We further confirmed that the mutation occurres at protochlorophyllide oxidoreductase (POR), which is a key enzyme for chlorophyll biosynthesis (our unpublished data).

In this study, we compared the effects of water stress on PSII in *NYB* and WT. The results indicated that although the mutant is yellowish and grows slowly, it adapted better to water stress.

Materials and Methods

Plant growth and stress treatments

Nanchong Yellow barley (*NYB*) and its wild type (*Hordeum vulgare* L.) were grown in sand at (25 ± 1) °C under a 12-h photoperiod and the photosynthetic photon flux of 100 μmol m^{-2} s^{-1}. When grown to the third-leaf stage (14 d after germination), 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 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 results of water stress were characterized by the relative water content (RWC). Chlorophyll (chl) contents were determined by using the equations of Lichtenthaler and Wellburn (1983).

Isolation of proteins and Western blot analysis

Isolation of leaf total proteins was carried out according to Sun *et al.* (2006). Thylakoid membranes were isolated as described by Lin *et al.* (1998) and pigment-proteins were fractionated by SDS-PAGE. For the comparison of one sample with another, gel lanes were loaded on an equal protein basis and not on an equal chl basis, because the amount of chl per unit of thylakoid varies largely during water stress. For analyzing the changes in the polypeptide composition of the PSII complex, gels were stained with Coomassie blue.

For Western blotting, the proteins were electron-transferred onto a nitrocellulose film according to Sambrook *et al.* (1989). The antisera used were kind gifts from Dr. Eva-Mari Aro (anti-D1 and anti-D2) and Dr. Naoki Yamamoto (anti-LHCII). Alkaline phosphatase-conjugated antibodies were used as the secondary antibodies. The intensity of the signals of Western blotting was analyzed densitometrically by a thin-layer scanner.

Isolation of RNA and Northern blot analysis

Leaf total RNAs were isolated according to Zhang *et al.* (2004). For Northern blotting, 20 μg RNAs were resolved on a 1.2% (w/v) formaldehyde-denaturing agarose gel and transferred onto a nylon membrane. Respective DNA fragments corresponding to the *psbA* gene (provided by Dr. Nai-Hu Wu), *psbD* gene (provided by Dr. Long-Fei Yan), *cab* gene (provided by Dr. Yu-Sheng Zhu), 16S ribosome RNA (rRNA) gene and 18S rRNA gene (Zhang *et al.*, 2004) were labeled with [α]-^{32}P and used as probes for RNA blot analysis. The hybridization was carried out according to the standard procedures of Sambrook *et al.* (1989). The intensity of the signals was analyzed densitometrically by a thin-layer scanner. The amount of chloroplast 16S rRNA was used as an internal standard for *psbA* and *psbD* mRNAs and the level of *cab* mRNA was normalized to nuclear 18S rRNA for each treatment.

Analysis of gas exchange

Gas exchange analysis was made using an open system (TPS-1, PP system, UK). Net CO_2 assimilation rate was determined at a CO_2 content of 360 cm^3 m^{-3}, 80% relative humidity and 180 μmol m^{-2} s^{-1} light intensity. Leaf stomatal conductance (*G*s) was measured under the same conditions with a steady-state porometer (TPS-1, PP system).

Determination of DCIP photoreduction

DCIP (2,6-dichlorophenol indophenol) photoreduction was determined spectrophotometrically according to Tang and Satoh (1985). The components of the reaction mixture were 50 mM Mes [2-(*N*-morpholino)ethane-sulfonic acid]-NaOH (pH 7.5), 10 mM NaCl, 60 μM DCIP, 2 mM $MgCl_2$, and 20 μg chl ml^{-1} intact chloroplasts.

Measurements of chlorophyll fluorescence

Chlorophyll fluorescence quenching analysis was carried out at room temperature (25 °C) with a portable fluorometer (PAM-2100, Walz, Germany). The minimal fluorescence level in the dark-adapted state (*F*o) was measured using the modulation light, which was sufficiently low (< 0.1 μmol m^{-2} s^{-1}) not to induce any significant variable fluorescence. The minimal fluorescence level in the light-adapted state (*F*o') was measured after turning off 50 μmol m^{-2} s^{-1} of the actinic light, which was equivalent to the growth light intensity and illuminating with far-red light for 3 s. The maximal fluorescence levels in the dark-adapted (*F*m) and light-adapted (*F*m') states were

determined before and after addition of the actinic light by 0.8 s saturating white light (8000 μmol m^{-2} s^{-1}) to close all reaction centres and drive photochemical quenching to zero. The steady-state value of fluorescence (*F*s) was also recorded after 5 min illumination at 50 μmol m^{-2} s^{-1} using the actinic light. Using both light and dark fluorescence parameters, we calculated: (1) the maximum efficiency of PSII photochemistry in the dark-adapted state (*F*v/*F*m); (2) the photochemical quenching coefficient, q_P = (*F*m' – *F*s)/(*F*m' – *F*o'), which measures the proportion of open PSII reaction centres; (3) the non-photochemical quenching coefficient, $q_N = 1 - (Fm' - Fo')/(Fm - Fo)$; (4) the quantum yield of PSII electron transport, Φ_{PSII} = (*F*m' – *F*s)/*F*m'; (5) the efficiency of excitation energy capture by open PSII reaction centres, *F*v'/*F*m' = (*F*m' – *F*o')/*F*m'. All samples were dark-adapted for 10 min before chlorophyll fluorescence was determined.

Statistical analysis

Means of 5 triplicates were measured. Student's t test was used for comparison between *NYB* and WT. A difference was considered to be statistically significant when $p < 0.05$.

Results

Leaf water status and protein, RNA and chlorophyll changes

When barley seedlings were somatically stressed in PEG solution with −0.5 MPa osmotic potential for 0, 24, 48 and 72 h, leaf water status expressed as relative water content (RWC) deteriorated gradually. At 24 h after stressing, only a minor de-

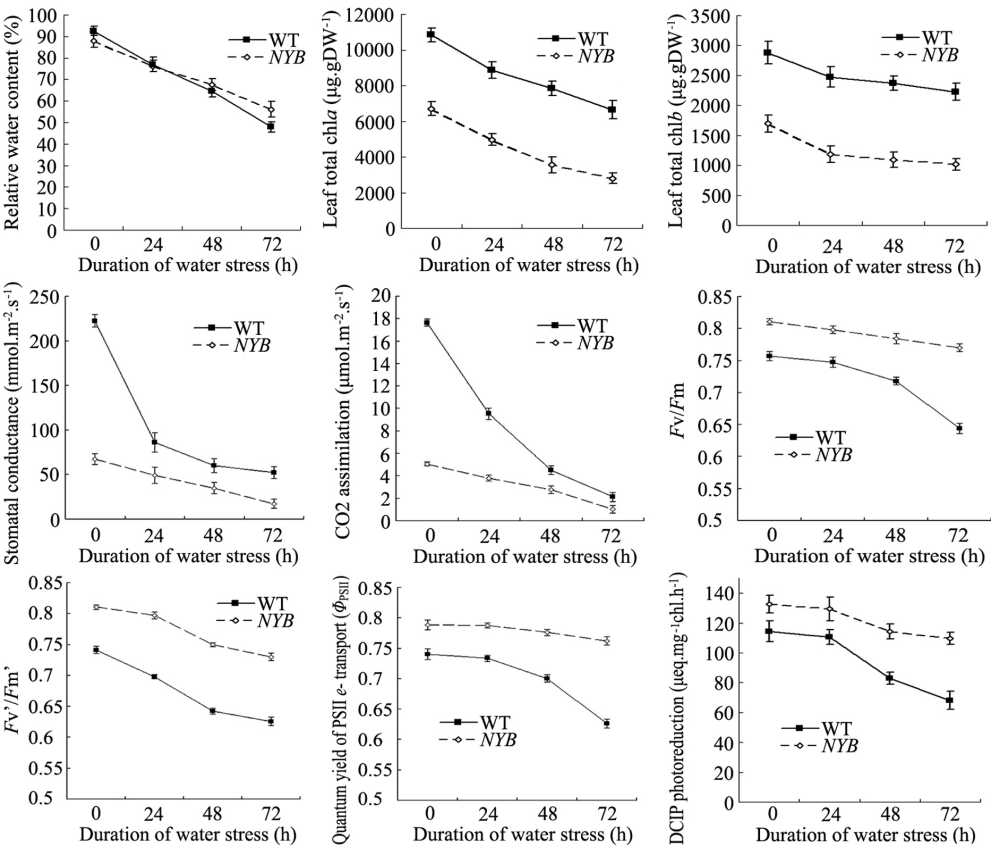

Fig. 1. Effects of water stress on leaf relative water content (RWC), chl *a* and chl *b*, leaf stomatal conductance, CO_2 assimilation rate, and PSII photochemical functions (indicated by *F*v/*F*m, *F*v'/*F*m', Φ_{PSII} and DCIP photoreduction activity) under water stress. Bars represent standard deviations of 5–8 independent replicates.

crease in RWC of WT and *NYB* was observed. However, at 72 h, RWC of WT reduced by 52%, and that of *NYB* reduced by 44% (Fig. 1), indicating that the plants were seriously dehydrated. Stress of 24, 48 and 72 h were assessed as mild, moderate and severe stress, respectively.

To further confirm the water stress severity that plants experienced and to understand the physiological conditions of plants during the stress regime, leaf total RNAs, total proteins and thylakoid proteins were monitored. At 72 h of severe stress, leaf total RNA, total protein and thylakoid protein

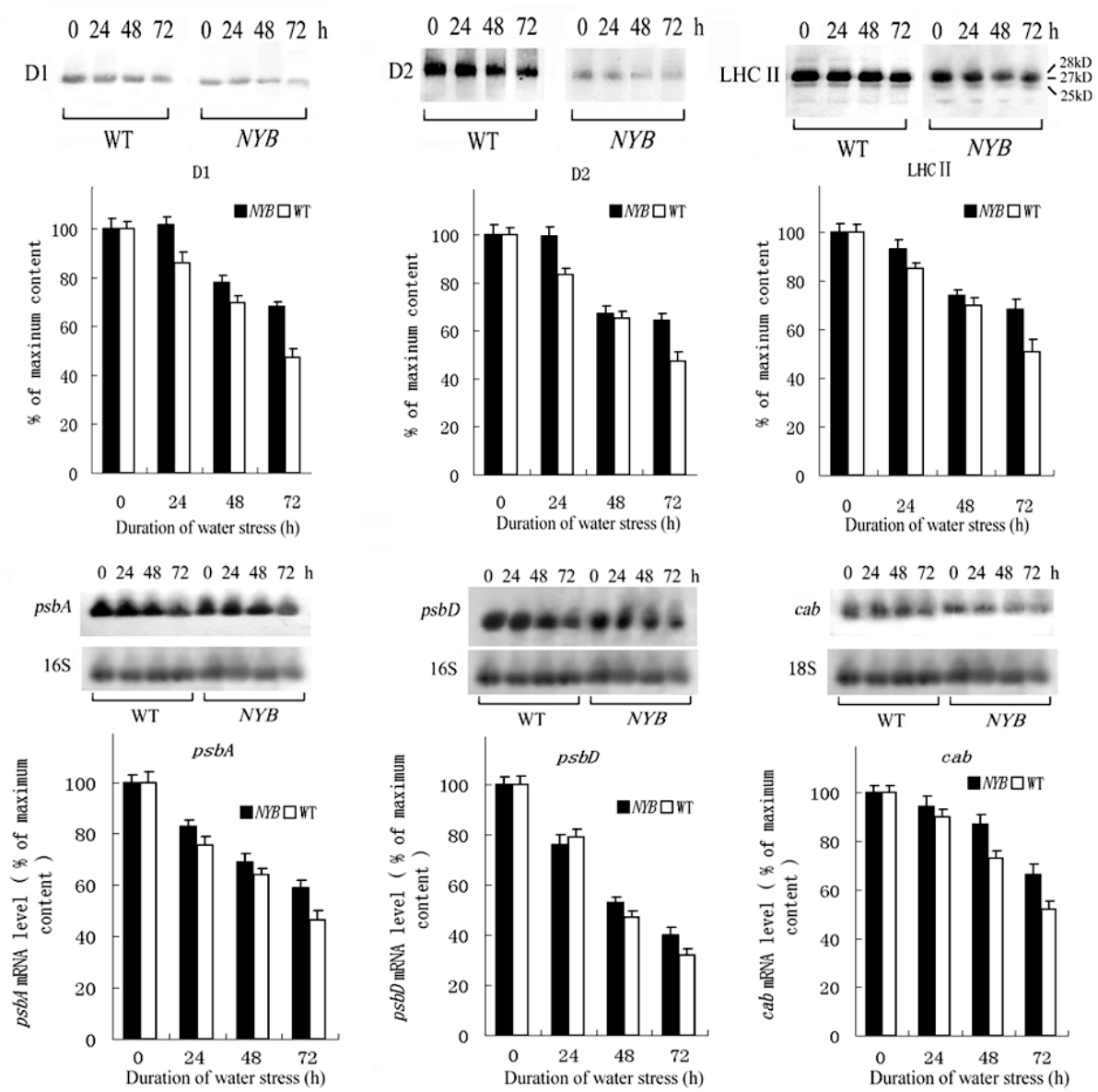

Fig. 2. Changes in steady state levels of D1, D2, LHCII proteins and corresponding gene *psbA*, *psbD*, *cab* transcripts from barley leaves stressed for 0, 24, 48, 72 h. The contents of proteins were detected by Western blotting and the contents of mRNAs were detected by Northern blotting. The amount of *psbA* or *psbD* mRNA was normalized to 16S rRNA for each treatment, and the level of *cab* mRNA was normalized to18S rRNA. The results are expressed as % of control (0 h). Quantities of control seedlings (stressed for 0 h) were normalized to 100%. Bars represent standard deviations of 5 independent replicates.

contents reduced to 39%, 54%, 38% in WT and 46%, 48% and 37% in *NYB*, respectively (data not shown), suggesting that water stress damages RNAs and proteins. Chlorophyll *a* and *b* was much lower in *NYB*, but they decreased accompanying with WT under water stress (Fig. 1).

Changes in steady-state levels of PSII polypeptides in NYB and WT

Immunoblotting with specific antibodies to PSII proteins showed a progressive decline in the light-harvesting complex (LHCII) and PSII core proteins D1/D2 with advancing water stress (Fig. 2). As far as WT was concerned, at 72 h of severe stress, D1, D2 and LHCII protein contents reduced to 47%, 46% and 51%, respectively, and the corresponding data were 68%, 64% and 67% for *NYB* (significant higher than WT).

Different changes in steady-state levels of PSII mRNAs in NYB and WT

The Northern blotting (Fig. 2) revealed that the transcript levels of corresponding genes also decrease markedly and progressively as water stress developed. The result indicated that during the whole period of water stress, the transcript levels of *psbA*, *psbD* and *cab* genes in WT decreased to 46%, 32% and 52%, respectively, and these of *NYB* decreased to 59%, 40% and 67% individually (also significant higher than WT). Obviously, *NYB* has slower decreases in transcript levels than WT. All the data about the PSII proteins, mRNAs and activities indicated that *NYB* was more resistant to water deficit.

Different changes in PSII photochemical activities in NYB and WT

Chlorophyll fluorescence quenching analysis has been proven a non-invasive, powerful and reliable method to assess the changes of the functions of PS II in the steady-state of photosynthesis in response to different environmental stresses (Lu *et al.*, 1998). We thus examined the effects of water stress on the fluorescence characteristics under the steady-state of photosynthesis. Fig. 1 shows the changes in the maximum efficiency of PSII photochemistry in the dark-adapted state (F_v/F_m), the efficiency of excitation energy capture by open PSII reaction centres (F_v'/F_m'), and the quantum yield of PSII electron transport (Φ_{PSII}) after *NYB* and WT seedlings were exposed to different times of water stress. The results showed that water stress caused a decrease in F_v/F_m, F_v'/F_m' and Φ_{PSII}. The photochemical quenching (q_P) and non-photochemical quenching (q_N) were almost the same in *NYB* and WT, and they changed similarly (q_P decreased little and q_N was unchanged) in *NYB* and WT during water stress (data not shown). These results were consistent with data previously reported by Lu *et al.* (1998). DCIP photoreduction was also employed as an indicator to PSII electron transport activities. Similar with Φ_{PSII}, DCIP photoreduction also decreased progressively during water stress. Limitation of the photosynthesis apparatus is one of the reasons for the decreased net photosynthesis rate (CO_2 assimilation). Another reason is decline of stomatal conductance, as shown in Fig. 1.

PSII photochemical activities were higher in *NYB* than in WT, which is different from many chlorophyll-less mutants. Furthermore, *NYB* had a relative large PQ pool, and a higher PSII electron transport rate, but a lower PSI electron transport rate (Tan *et al.*, 1996). Therefore, it is deduced that the higher PSII photochemical efficiencies in *NYB* result from the less excitation energy transfer from PSII to PSI, which may compensate the lower light harvest capacity due to chl deficiency (Tan *et al.*, 1996, 1997). However, photosystems per thylakoid membrane decreased severely in *NYB* (Lin *et al.*, 1999). Consequently, net photosynthesis rate (CO_2 assimilation) is lower in *NYB* than in WT.

Discussion

The effects of water stress on PSII proteins, RNAs and photochemical activities have been well documented previously (Lu *et al.*, 1998; Yuan *et al.*, 2005). However, reports on these in chlorophyll-less mutants are limited. Our results indicated that PSII mRNAs and proteins in the WT mutant were less affected by the water stress than *NYB*, and PSII activities were milder hampered in the chlorophyll-less mutant under water stress, suggesting a certain relationship between deficits in chlorophylls and responses of PSII to water stress.

Early studies suggested that transcripts of major PSII proteins declined during water stress. However, there is no research about whether the chlorophyll content is involved in this decrease or not. Through Western blotting, we confirmed that D1, D2 and LHCII proteins decreased more quickly

under water stress in WT than in *NYB*. It can be attribute to three possible reasons: (1) More decreased mRNA levels in WT; therefore, the template level for translation was decreased (Yuan and Lin, 2004). (2) A compensatory mechanism to maintain PSII proteins by altering the formation of the PSII protein complex or utilizing limited chlorophylls more effectively (Chen *et al*., 2006). (3) Altered cellular environment. The decrease of chlorophyll and LHCII could change the redox state and the content of active oxygen, which subsequently regulates mRNA and protein abundance (Maxwell *et al*., 1995; Allen and Nilsson, 1997; Pfannschmidt, 2003). Thus, *NYB* and WT response to water stress differently.

Similar to proteins, PSII major mRNAs also declined more rapidly in *NYB*. Altered cellular environment (as mentioned above) may be the main reason for the different changes of PSII mRNAs in *NYB* and WT. Besides, accumulation of chlorophyll biosynthetic pathway intermediates inhibits the expression of chloroplast protein genes that reside in the nucleus, such as the *cab* gene (Strand *et al*., 2003; Nott *et al*., 2006). The chlorophyll intermediates (Mg-protoporphyrin IX and Mg-protoporphyrin IX monomethyl ester) accumulate more in *NYB* (our unpublished data). Consequently, the expression of *cab* gene may be altered in the mutant.

Lack in chlorophyll could hamper plant growth and development, but alleviate declines of PSII activities during water stress. Why do these irrelevant changes happen? Both chl *a* and chl *b* play a key role in maintaining PSII protein stability and function. However, it is not always necessarily the case. In a transgenetic cyanobacterium, *Synechocystis* sp. PCC 6803, with over-accumulated chl *b*, chlorophyll *b* could functionally substitute for chlorophyll *a* and subsequently incorporate into photosystem II complexes to keep its normal function (Xu *et al*., 2001). On the other hand, not all chlorophyll-deficient mutants have lower photochemical efficiencies. Besides our *NYB* mutant, Simpson *et al*. (1985) also reported some chlorophyll-less barley mutants with higher PSII activities. Meurer *et al*. (1996) reported some chlorophyll-less *Arabidopsis* mutants producing higher chlorophyll fluorescence. Similar mutants have been identified too in maize (Pasini *et al*., 2005) and rice (Chen *et al*., 2006). Just like substitution of chl *b* by chl *a*, some mutant plants adopt a compensatory mechanism to maintain its PSII activities by altering the formation of the PSII protein complex or utilizing limited chlorophylls more effectively (Tan *et al*., 1996, 1997; Chen *et al*., 2006). Consequently, PSII activities on a per-chlorophyll basis were higher in some mutants, but the net photosynthetic rate and apparent quantum yield were lower due to the decreased photosynthetic apparatus per thylakoid membrane. Although chlorophylls and PSII proteins were less in the mutant (Fig. 2), the remaining chlorophylls in *NYB* bound apoproteins more effectively, and PSII in the mutant adjusted its formation to contain less light-harvesting complexes (Lin *et al.*, 1999) but converted light energy and transport electrons more efficiently (Fig. 1). As a result, PSII in *NYB* has a better resistance to water stress.

Chlorophyll-less mutants usually are less sensitive to high-light-stress (photoinhibition), due to the less light harvesting (Tan *et al*., 1996, 1997). Furthermore, some chlorophyll-less mutants were much resistant to cold stress, because the non-photochemical quenching is stronger in these mutants, which may produce heat (Pasini *et al*., 2005). Here, we first report a chlorophyll-less mutant insensitive to water stress. It is neither due to the decreased sensitivity to photoinhibition, nor the increased non-photochemical quenching (q_N and q_P are almost the same in *NYB* and WT). Effective chlorophyll utilization and improved PSII protein formation may be counted as the reason. Future biophysical studies of the photosynthetic apparatus of the mutant may uncover the details for different responses of PSII to water stress in *NYB* and WT.

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