

Effects of drought on photosynthetic characteristics of flag leaves of a newly-developed super-high-yield rice hybrid

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Abstract

We investigated responses of chloroplasts from flag leaves of a newly-developed super-high-yield rice (*Oryza sativa* L.) hybrid LiangYouPeiJiu (LYPJ) to water stress (withholding irrigation) during the grain-filling period. In the early stage of water stress (0–6 d) only the activity of Hill reaction was inhibited, whereas activities of photophosphorylation and Ca²⁺-ATPase, and ATP content were increased and peaked in the day 6 of withholding irrigation. In the late stage of water stress (6–12 d), the activities of photosynthetic O₂ evolution, Hill reaction, photophosphorylation, and Ca²⁺-ATPase, and ATP content were significantly reduced. The membrane lipid content was sharply decreased, especially of sulfoquinovosyl-diacylglycerol (SQDG) and phosphatidylglycerol (PG). The changes in the ultrastructure of chloroplasts included mainly a decrease in number of grana and increase in number of osmiophilic granules.

Additional key words: chloroplast ultrastructure; Hill-reaction; *Oryza sativa*; photophosphorylation; photosynthetic O₂ evolution; membrane lipids; water stress.

Introduction

In rice, over 80 % of the sugars accumulated in grains is produced by the top two leaves, particularly the flag leaf (Li *et al.* 1998). Grain-filling duration is under genetic control (Metz *et al.* 1985, Smith and Nelson 1987) and sensitive to stress, suggesting that the length of the grain-filling period is an important determinant of yield of all grain crops (Brevedan and Egli 2003). Therefore, photosynthetic efficiency, photosynthetic function duration, and responses to environmental stresses of flag leaf are important for yield production of hybrid rice.

LiangYouPeiJiu (LYPJ) is a newly developed two-line rice hybrid (Pei-ai 64 s × 9311), called super-high-

yield hybrid rice in China. Much work has been done on its heterosis, morphology, and photosynthetic properties under normal conditions (Wang *et al.* 2000) as well as under irradiance (Wang *et al.* 2002) and Hg²⁺ (Yang *et al.* 2002) stresses. Compared with other traditional cultivars, this hybrid rice is characterized by higher water consumption and longer maturing. In the present study, we examined the effects of water stress on photochemical, biochemical, and ultrastructural properties of chloroplasts from flag leaves of LYPJ hybrid rice during the grain-filling stage.

Materials and methods

Plants and treatments: Rice plants at the grain-filling stage were collected in September 2002 from experimental fields of the Institute of Food Crop, Jiangsu Academy of Agricultural Sciences. The heads of rice were cut in order to get rid of the effects of natural senescence on the experimental results.

The rice plants collected from the field were transplanted into plastic pots, three plants per pot, and water level was maintained 2 cm above soil surface. These

plants were randomly set into two groups. In the control one, water level was maintained 2 cm above soil surface till the end of experiment. In the second group irrigation was withheld after three days to simulate natural drying of soil by evaporation. The degree of water stress was expressed as days of water withholding. During the experiment, all plants were kept in a glasshouse (transmittance of 75 %) with diurnal control of temperature (28±5 °C) and relative humidity (70±10 %). Sampling was made

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once every three days. Three replicates were made for each sampling of each group, one flag leaf from one plant.

Isolation of chloroplasts was performed according to the method of Ketcham *et al.* (1984) with slight modifications. After removal of the midrib from 20 g of flag leaves, the specimen was kept in refrigerator at 4 °C for 30 min. Then, the leaves were cut into pieces and were triturated in a *Triturator* with four times volume of cool extracting medium (50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 10 mM NaCl, 0.4 M sucrose, 0.1 % bovine serum albumin, BSA). The homogenate was filtered to remove large debris, and then placed in a *TGLL-18G* centrifuge at 1 000×g for 1 min. The upper solution was re-centrifuged at 2 000×g for 2 min. After removing the upper solution, the precipitate was supplemented with 5 cm³ of 0.06 M phosphate buffer and the tube was slightly rotated in ice blocks to make uniform chloroplast suspension, which was then kept in dark on ice for use in the subsequent procedures.

Measurement of chlorophyll (Chl) content: 1 cm³ of chloroplast suspension with 9.9 cm³ of 80 % acetone was centrifuged at 3 000×g for 5 min. The upper solution was measured with a *UV-754* spectrophotometer at 645 and 663 nm. Chl content was calculated as described by Arnon (1949).

Photochemical functions: An oxygen electrode attached with a logger was used to measure the activity of photosynthetic oxygen evolution according to Van Gorkom and Gast (1996) and the activity of Hill-reaction as described by Kutík *et al.* (1999). The photophosphorylation activity was measured according to Ketcham *et al.* (1984). ATP content was measured by the bioluminescence method described by Zhu *et al.* (2001). Ca²⁺-ATPase activity was determined according to Vallejos *et al.* (1983).

Ultrastructure of chloroplasts: The chloroplast suspension was centrifuged, the upper solution was removed,

Results

Chl content decreased significantly already after three days of water stress (Fig. 1A). Chl content in stressed plants was only 37 % of that in control plants after 6 d, less than 30 % after 9 d, and around 10 % after 12 d. The activity of photosynthetic O₂ evolution decreased slightly before the first 6 d of water stress, but it was 54.8 % of that of control plants after 9 d and 23.5 % after 12 d, respectively (Fig. 1B). Hill reaction, a measure of activity of photosystem 2 (PS2), declined during the whole period of water stress (Fig. 1C). After 6 d of water withholding, the Hill-reaction activity was 33.78 % of that of control, 21.48 % after 9 d, and 13.86 % after 12 d, respectively. Hill reaction activity was more sensitive to water stress

and the sediment was fixed in buffered 4 % glutaraldehyde at <4 °C for over 24 h. The mixture was centrifuged and a little deposit was spread on a carrier and 2 % agar was immediately added at 45 °C. The coagulated agar was cut into small blocks. The small blocks were fixed in buffered 4 % glutaraldehyde and transferred to a secondary fixative containing 1 % osmium tetroxide, then dehydrated in an acetate series and embedded in the *Epon 812* resin. Thin sections were obtained with an *LKB-V* ultramicrotome (Sweden) and double stained with uranium acetate-lead citrate before being examined with a transmission electron microscope (*Hitachi 600-A-2*, Japan).

Fatty acid composition of membrane lipids: The lipids and fatty acids were analysed according to the method of Rémy *et al.* (1984). The extract of chloroplast membrane lipids was surged several times with 1 cm³ of 0.4 M methanol solution and kept motionless for 2 h. The mixture was extracted with 8 cm³ of 10 % acetic acid and 1 cm³ of heptane. The upper solution was imbibed and evaporated to dryness under reduced pressure. The extract with some chloroform was then quantitatively analysed by gas chromatography.

The separation was performed on an *HP 5890* gas chromatograph connected to an *HP 3295* computing integrator (*Hewlett-Packard, Shanghai Analytic Apparatus*, Shanghai, China). The silica capillary column (33 m×0.25 mm) was packed with polyethylene glycol. The column temperature was programmed from 170 to 240 °C at 2 °C per min; lower and upper temperature was held for 3 and 10 min, respectively. The temperature of the hydrogen flame ionization detection was 240 °C and the pressure of H₂ was 0.75 kg cm⁻². The carrier gas was N₂ at a flow rate of 364.2 kg cm⁻² and the air pressure was 0.5 kg cm⁻². A chromatographic data processor was used to record and store chromatograms and to calculate peak areas. Peaks were identified through comparison with several external qualitative standards. Fatty acids were detectable to the nanogram level by using this procedure.

than the Chl content.

The activity of photophosphorylation responded differently to different water stress. During the first 6 d of water stress, an increasing trend of photophosphorylation activity was observed and it peaked after 6 d (133.74 % of that of control); after the sixth day of water withholding it decreased by 50 % of that of control, and after 12 d only by 20 % (Fig. 1D). Similarly, the activity of Ca²⁺-ATPase increased steadily during the first six days of water withholding. The activity of Ca²⁺-ATPase was 117.76 and 145.99 % of that of control after 3 and 6 d, respectively. The Ca²⁺-ATPase activity after 6 d dropped significantly, by 22.16 % after 9 d and by 41.59 % after

12 d (Fig. 1E). Also the ATP content first increased and then decreased when the peak was reached, similar to that of photophosphorylation and Ca^{2+} -ATPase activities. ATP content in the day 6 of water stress was 1.5 times that of control. There was a significant decrease after 6 d of water stress, and after 12 d of water stress the content was only 43.34 % of that in control (Fig. 1F).

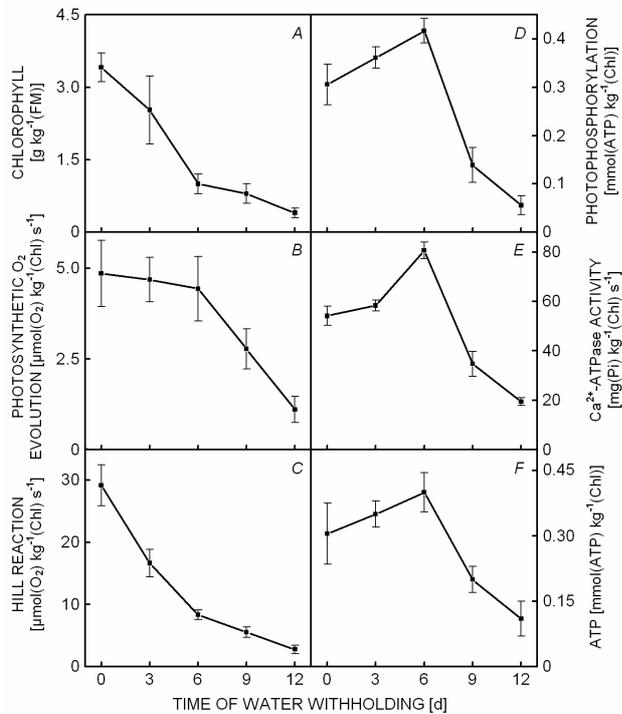


Fig. 1. Effects of water stress on chlorophyll content (A), activities of photosynthetic O₂ evolution (B), Hill-reaction (C), photophosphorylation (D), Ca²⁺-ATPase (E), and ATP content (F) in flag leaves of a new hybrid rice during grain filling. Vertical bars show standard errors ($n = 3$).

Under electron microscope, chloroplasts from control plants were elliptic or navicular, with orderly-stacked tight grana thylakoids. A small number of osmiophilic granules was observed in some chloroplasts. In the early stage of water stress (0–6 d), the structure of chloroplasts and the number of grana did not change significantly as compared to those of control plants: lamellae were still stacked orderly, and only the number of osmiophilic granules increased slightly. But with the increasing water stress, chloroplasts changed from ellipse or ship-like shape to circle, with much damage in membrane structure. A significant decline was observed in the number of grana with loose stacks of thylakoids and large increase in the number of osmiophilic granules (Fig. 2).

During early water stress, there were no significant changes in both compositions and individual contents of membrane lipids (Fig. 3). But in the day 6 of water stress, there was a decrease in monogalactosyl diglycerols (MGDG) and digalactosyl diglycerols (DGDG). The

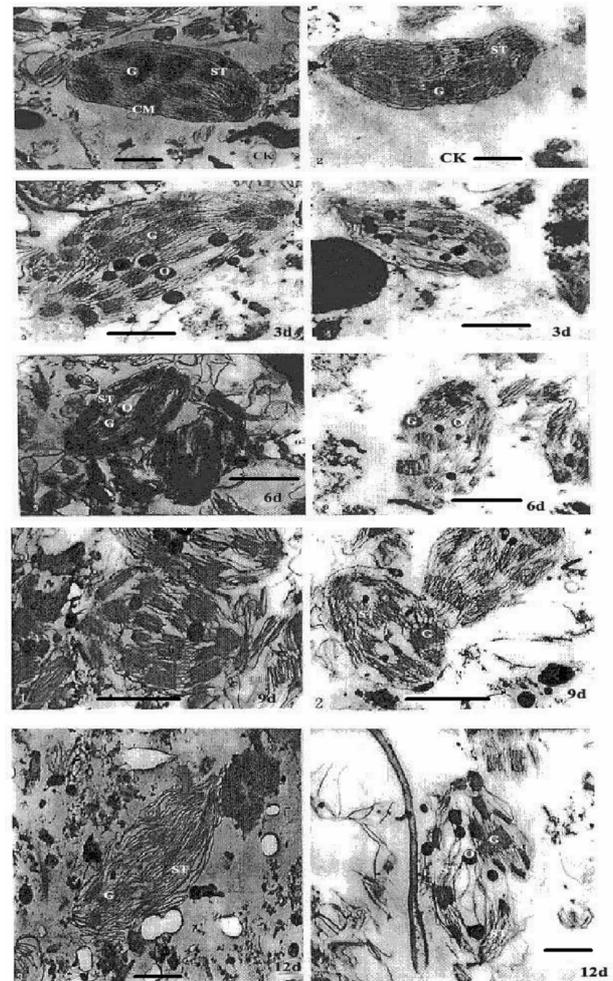


Fig. 2. Effects of water stress on the ultrastructure of chloroplasts from flag leaf in a new hybrid rice during grain filling. Control plants or plants exposed to withholding irrigation for 3, 6, 9, or 12 d. G = granum thylakoid, ST = stroma thylakoid, O = osmiophilic granule. Bar = 1 μm.

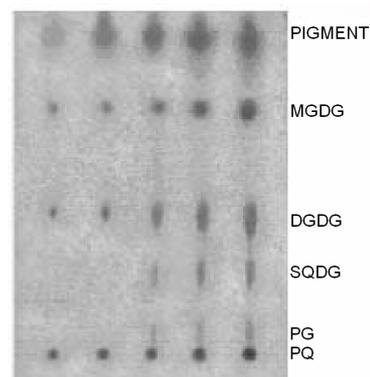


Fig. 3. Effects of water stress on the membrane lipids of chloroplast from flag leaves in a newly-developed hybrid rice during grain filling. Lanes from right to left indicate membrane lipids of chloroplast after 0, 3, 6, 9, and 12 d of water withholding.

contents of lipids markedly dropped with increasing water stress. When water stress lasted for 9 d, only trace amounts of PG and SQDG were found. After 12 d, the PC amount became trace, and PG and SQDG were immeasurable. Contents of MGDG and DGDG were less than 20 % of that of control plants.

The major fatty acids in membrane lipids of rice leaves consisted of 16:0 (palmitic acid), 16:1 (palmoleic acid), 18:0 (stearic acid), 18:1 (oleic acid), 18:2 (linoleic acid), and 18:3 (linolenic acid). Under well-watered conditions, the content of 18:3 lipids was greatest,

Discussion

In LYPJ hybrid rice, MGDG and DGDG were the major galactolipids (Fig. 3) and contained a high proportion of 18:3 (Table 1). This indicates that galactolipids have a structural and functional role in cell membrane of LYPJ hybrid rice, and that the composition of leaf lipids of LYPJ hybrid rice is similar to that of higher plants of the so-called "18:3 (α -linolenic)" group. Water stress led to a decrease in contents of all lipid classes, especially in

accounting for 52.16 % of the total fatty acids (Table 1). The decreasing trend was observed in contents of unsaturated fatty acids with water stress going on, but contents of saturated fatty acids increased. The amounts of 16:1 and 18:2 lipids were decreased more significantly, by 57.38 and 42.69 %, respectively, more than those of other lipids. Strange enough, the content of 18:1 lipids firstly decreased and then increased with water stress, but the explanation of this phenomenon is missing. The index of unsaturated fatty acid (IUFA) also decreased with increasing water stress.

MGDG and DGDG (Fig. 3). Heavy water stress (after 6 d of water withholding) also caused a significant decrease in contents of unsaturated fatty acids, an increase in the saturated fatty acid percent, and reduced ratio of unsaturated : saturated fatty acids in the leaves (Table 1), implying decreased fluidity of thylakoid membrane that in turn would adversely affect the compartmentation of cells (Ferrari-Iliou *et al.* 1984).

Table 1. Changes in the fatty acid composition of chloroplast membrane lipids in flag leaves of newly-developed hybrid rice LiaoYouPeijiu in China during the grain-filling stage when subjected to water withholding. IUFA: Index of unsaturated fatty acid, $\{[16:1\text{mol}\% \times 1] + [18:1\text{mol}\% \times 1] + [18:2\text{mol}\% \times 2] + [18:3\text{mol}\% \times 3]\} \times 100$ %. Means of three replicates of three plants. All standard deviations were lower than 10 % and are not shown for simplicity. Means of different fatty acids separated by different letters within a column for each individual are significantly different at the 5 % level.

Water stress [d]	Compositions of fatty acids [mol%]							
	16:0	16:1	18:0	18:1	18:2	18:3	20:0	IUFA
0	9.04a	4.13a	3.19a	5.31a	13.96a	52.16a	12.22a	193.84a
3	11.19a	3.75a	3.65a	4.17a	13.77a	49.42a	14.15b	183.72a
6	13.96b	3.22b	3.95b	2.69b	13.61a	44.65b	17.94c	167.08b
9	14.13b	2.13c	4.98c	3.67c	12.17b	41.53b	21.45d	154.73b
12	15.48c	1.76c	7.43d	4.06d	10.43c	29.89c	30.97e	116.35c

Changes in the lipid composition of chloroplasts are responsible for altered thylakoid membrane structures, such as disrupted chloroplast envelopes and swollen thylakoids, which in turn affect photosynthetic activities (for review see Repellin *et al.* 1997). In LYPJ hybrid rice exposed to heavy drought (6–12 d), significant changes in the lipid composition were simultaneously accompanied by serious damage to thylakoid structure (Fig. 2).

Drought-induced degradation of polyunsaturated galactolipids is no doubt responsible for the disorganization of chloroplast membrane (Vieira da Silva *et al.* 1974) and free fatty acids inhibit electron transport during photosynthesis (Yordanov *et al.* 2000). Therefore, altered thylakoid membrane structure may directly affect membrane functionality and could have deleterious effects on photosynthetic activities of chloroplast, which was supported by declines in photochemical functions of chloroplast such as activities of photosynthetic O_2 evolution (Fig. 1B), Hill reaction (Fig. 1C), photophosphorylation

(Fig. 1D), and Ca^{2+} -ATPase (Fig. 1E).

The Hill-reaction activity responded to water stress with a similar changing pattern as the Chl content (Figs. 1A and 3C). Bao *et al.* (2001) attributed the reduction in Hill-reaction activity to membrane lipid peroxidation in the study on photosynthetic adaptation of *Vicia faba* to water stress. But we did not find any noticeable change in peroxidation of membrane lipids in the early stage of water stress. Similar results were observed in two turf-grasses (*Festuca arundinacea* and *Poa pratensis*) during the first 18 d of water stress (Jiang and Huang 2001). Based on the analysis of correlation ($r = 0.983$, $p < 0.05$), the reduced Hill-reaction activity could be attributed to the breakdown of Chl, at least under mild drought.

With leaf discs of epidermis, Tang *et al.* (2002) found that under mild drought stomata closure contributed to the inhibition of the activity of photosynthetic O_2 evolution measured by gas-phase oxygen electrode, while the inhibition became entirely metabolic when subject to

severe drought. In the present work, during 0–6 d of water stress, a constant value was observed in the activity of photosynthetic O₂ evolution and a significant decrease during 6–12 d of water stress (Fig. 2B). The inhibition of stomata patchy closure was excluded since we measured the activity of photosynthetic O₂ evolution with a liquid-phase oxygen electrode. Therefore, the reduction in the activity of photosynthetic O₂ evolution could be due entirely to metabolic inhibition (Boyer 1976, Tang *et al.* 2002) rather than to stomatal limitation.

Ca²⁺-ATPase activity increased with water withholding (0–6 d) but decreased significantly with further water withholding (6–12 d) (Fig. 1E). The increased Ca²⁺-ATPase activity under mild drought was also observed in wheat (Zhou *et al.* 2003) and maize (Wang and Li 1985). In these two reports, the increased Ca²⁺-ATPase activity was attributed to chloroplast membrane protein denaturation which inhibited the activity of photophosphorylation and in turn resulted in the increased activity of ATPase (Wang and Li 1985). This explanation is in conflict with the idea that reduced ATPase activity can inhibit photophosphorylation activity, and also contrary to the increased photophosphorylation activity under mild drought (0–6 d) (Fig. 1D). Therefore, the increase in Ca²⁺-ATPase activity under mild drought is not attributable to chloroplast membrane denaturation. Abscisic acid (ABA) accumulation, a typical response in many plants subjected to drought, stimulated the increase in cytosolic Ca²⁺ by inducing both Ca²⁺ influx from the extracellular space and Ca²⁺ release from intracellular stores (Pei *et al.* 2000, Murata *et al.* 2001). Cytosolic Ca²⁺ efflux is regulated by Ca²⁺-ATPase (Lam *et al.* 1998), implying that Ca²⁺-ATPase activity must be increased to pump excess cytosolic Ca²⁺ into extracellular space and/or vacuole. In the present work, therefore, the increased Ca²⁺-ATPase activity under mild drought (0–6 d) may result from the stimulation of excess cytosolic Ca²⁺ concentration.

There was a similar changing pattern in Ca²⁺-ATPase and photophosphorylation activities (Fig. 1D,E) suggest-

ing that both activities are closely related (Salisbury and Ross 1969, Gregory 1989). Guo and Li (1997) found a close positive correlation between activities of Hill-reaction and photophosphorylation in rice. But there was no positive correlation between them in the early stage of water stress in the present study (Fig. 1C,D). Therefore, the change in photophosphorylation activity was not explainable by the Hill-reaction activity, but resulted from the increased Ca²⁺-ATPase activity (Fig. 1E), which in turn caused a similar increase in ATP content (Fig. 1F).

However, further water withholding (6–12 d) induced severe drought and resulted in significant decreases in activities of Ca²⁺-ATPase and photophosphorylation and of ATP content (Fig. 1D–F). As discussed above, severe drought (6–12 d) caused serious damage to thylakoid membranes and hence could increase ion leakage, implying that Ca²⁺-ATPase would be unnecessary for Ca²⁺ efflux. Additionally, strong water deficit leads to a disturbance of the association between membrane lipids and proteins as well as the enzyme activity and transport capacity of the bi-layer (Caldwell and Whitman 1987). Therefore, the reduced Ca²⁺-ATPase activity under severe drought may result from the lost Ca²⁺ gradient, namely the uncoupling effect of water stress on chloroplasts (Younis *et al.* 1979). The reduction in photophosphorylation activity under heavy drought was due to thylakoid membrane conformational changes (Fig. 2) (Fellows and Boyer 1976). It is easy to conclude that the decline in ATP content under severe drought could be due to reductions in Ca²⁺-ATPase and photophosphorylation activities (Younis *et al.* 1979, Tezara *et al.* 1999).

In summary, the increases in photosynthetic activities in the early stage of water stress suggest that mild mid-summer drainage is necessary for rice grain filling. However, water stress in the late stage caused serious damages to the ultrastructure, membrane lipid contents, and photon energy conversion of chloroplast, which may be responsible mainly for the reduction in yield of the super high-yield hybrid rice under heavy drought.

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