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BRIEF COMMUNICATION

Rice leaf heterogeneity in chlorophyll fluorescence parameters under short-term osmotic stress

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Abstract

The effects of short-term osmotic stress [a polyethylene glycol (PEG) treatment] on photosystem II (PS II) of upland and lowland rice seedlings were investigated using chlorophyll (Chl) fluorescence imaging. Spatial heterogeneity in the top fully expanded leaf for all Chl fluorescence parameters was found under stress conditions. After exposure to PEG, a decrease in the effective quantum yield of PS II photochemistry (φPS II) and photochemical quenching (qP), and an increase in non-photochemical quenching (NPQ) proceeded from the upper section to the base of the leaf. The most sensitive position in the leaf was different between the two ecotypes. Chl fluorescence parameters, net photosynthetic rates (P_N), and stomatal conductance (g_s) were more sensitive to the PEG stress in upland rice than in lowland rice. These results also indicate different leaf anatomy and development in the two rice ecotypes. Additionally, the findings suggest a more rapid stress response in upland rice.

Additional key words: chlorophyll fluorescence imaging, net photosynthetic rate, *Oryza sativa*, polyethylene glycol, stomatal conductance.

Photosynthesis is one of the main metabolic processes that determine crop production. However, photosynthesis in higher plants was long regarded as being homogeneously distributed over single leaves. This dogma was destroyed in recent years with the development and application of chlorophyll fluorescence techniques. The features of gradients, inhomogeneities, and transient growth rate fluctuations in a growing leaf have been demonstrated using image-processing techniques (Meng *et al*. 2001, Calatayud *et al*. 2006). For a leaf developing from a basal meristem, there is a clear gradation within the first few millimeters of the leaf. Strong developmental gradients are still apparent along the length for some monocotyledonous leaves that have attained full length (Shaikh *et al*. 2000). However, the base-to-tip gradient is not a general characteristics for leaf growth (Ainsworth *et al*. 2005). Furthermore, little is known about the spatial distribution of leaf photosynthesis in rice (*Oryza sativa* L.).

Drought stress is one of the most important environmental stresses inhibiting photosynthesis. Under stress conditions, net photosynthetic rate, quantum yield, and photochemical efficiency of photosystem II (PS II) decline, whereas non-photochemical quenching increases (Giardi *et al*. 1996, Lichtenthaler *et al*. 1996, Pal *et al*. 2013). Owing to the different zones of leaf anatomy and mesophyll development, photosynthesis shows spatio-temporal heterogeneity in the leaves of cotton and *Arabidopsis thaliana* under drought stress (Massacci *et al*. 2008, Sperdouli *et al*. 2012). Thanking to the

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Abbreviations: c_i - intercellular CO_2 concentration; g_s - stomatal conductance; NPQ - non-photochemical quenching; PEG - polyethylene glycol; P_N - net photosynthetic rate; PS II - photosystem II; qP - photochemical quenching; φPS II - effective quantum yield of PS II photochemistry.

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remarkable progress in the Chl fluorescence imaging technique, the heterogeneity in photosynthetic function in/across a leaf, and the rapid visualization of spatio-temporal variations in plant responses to stress can be demonstrated (Pietrini *et al.* 2010, Song *et al.* 2013).

Upland rice and lowland rice are two different ecotypes generated by a long period of natural and human selections under drought conditions. Upland rice is more tolerant to water stress than lowland rice, usually showing a strong root system, a high osmotic adjustment, and a stable net photosynthetic rate under a long time drought stress (Gao *et al.* 2009, Hu *et al.* 2009). Although the Chl fluorescence imaging technique has been widely used, little is known about photosynthesis heterogeneity in a single rice leaf under a short-term drought stress. In this research, we used a Chl fluorescence imaging system to detect spatial heterogeneities of individual leaves of upland rice and lowland rice and to analyze the differential responses of the photosynthetic apparatus to a short-term osmotic stress. The gas exchange parameters have been also observed to analyze the whole-plant response to this stress.

Oryza sativa L., an upland tropical *japonica* cultivar IRAT109 and a lowland temperate *japonica* cultivar YueFu, were used in this study. Seeds were surface-sterilized in 10 % (v/v) H_2O_2, and germinated at 36 °C for 64 h. Uniformly sized germinated seedlings were transplanted into plastic foam frames suspended in plastic boxes containing an aerated Hoagland nutrient solution (pH = 5.5). The rice seedlings were grown in a greenhouse at a 13-h photoperiod, an irradiance of 300 μmol m^{-2} s^{-1}, a temperature of 27 ± 1 °C, and an air humidity of 60 - 70 % . Four-week-old seedlings were used for the experiments. Dehydration stress was imposed by immersing the roots in a 15 % (m/v) polyethylene glycol (PEG 6000) solution (an osmotic potential of -0.8 MPa). The Chl fluorescence parameters and images were recorded at 0 min (control), 30, 60, 90, and 120 min of the treatment. Leaf gas exchange parameters were investigated at 0, 60, and 120 min of the treatment. Three replicates with five plants for each variety in each replicate were used.

An *Imaging-PAM* fluorometer (*Walz*, Effeltrich, Germany) connected to a computer with a data acquisition software (*ImagingWin v2.0 m*, *Walz*) was used. An array of blue radiation-emitting diodes (a total of 96 LEDs; 470 nm) was applied by this measuring system for fluorescence excitation, actinic irradiation, and saturating radiation pulses. A CCD camera had a resolution of 640 × 480 pixels. All measurements were carried out on an imaged sample area of 10 × 13 cm. The images of the fluorescence parameters were displayed by means of a colour code ranging from black (0.0) to purple (1.0). For all treatments, the leaves were dark-acclimated for 20 min. Measurements were performed in a closed chamber under controlled growth conditions. The top fully expanded leaves were selected from each plant for measurement. The average length of each leaf was 17 cm. Nine different areas of interest, 3, 4.5, 6, 7.5, 9, 10.5, 12, 13.5, and 15 cm from the leaf tip, were selected for data recording. Minimum fluorescence (F_0) was measured under a weak modulating radiation (0.5 μmol m^{-2} s^{-1}), and maximum fluorescence (F_m) was induced by a saturating pulse of radiation (2 400 μmol m^{-2} s^{-1}). The minimum fluorescence in a light-acclimated state (F_0') was calculated as $F_0' = F_0/(F_v/F_m + F_0/F_m')$ (Oxborough and Baker 1997). The efficiency of photosystem (PS) II photochemistry, φPS II, was measured and calculated as $(F_m' - F_s)/F_m'$ according to Genty *et al.* (1989) at a photosynthetic photon flux density of 500 μmol m^{-2} s^{-1}. Non-photochemical quenching (NPQ) is the quantum yield of regulated energy dissipation of PS II and was calculated as $(F_m - F_m')/F_m'$. Photochemical quenching (qP) is fluorescence quenching caused by photosynthesis and was calculated as $(F_m' - F_s)/(F_m' - F_0')$.

Leaf gas exchange parameters, stomatal conductance (g_s), net photosynthetic rate (P_N), and intercellular CO_2 concentration (c_i) were measured at the midpoint of the uppermost fully expanded leaf using a photosynthesis system *LI-6400* (*LI-COR*, Lincoln, NE, USA) at a leaf temperature of 27 °C, an irradiance of 500 μmol m^{-2} s^{-1}, and a CO_2 concentration of 400 μmol mol^{-1}.

Means and standard deviations (SDs) were calculated based on three biological replicates. Data were analyzed by the one-way analysis of variance (*ANOVA*). Means were tested by the least significant difference at $\alpha = 0.05$ according to the Duncan's multiple range test. The statistical analysis was performed using the *SAS/STAT* software, *v. 9.2* of the *SAS system for Windows*.

Under the control conditions, φPS II continuously decreased by 15.6 and 22.4 % from the leaf base (15.0 cm) to the tip (3.0 cm) in lowland rice and upland rice, respectively, revealing a clear developmental gradient along the leaf length (Fig. 1*A*). φPS II was greatest in the base of the leaf, with a maximum value of 0.59 and 0.53 in lowland rice and upland rice, respectively. Under the PEG stress, fluorescence images of φPS II showed heterogeneity and a temporal variation in the leaf (Fig. 2). φPS II values decreased differentially in different parts of the leaf, decreasing more in the upper section than in the leaf base. Regarding the time of treatment, 60 min or longer of the PEG stress significantly induced NPQ (Fig. 1*B*) and reduced φPS II and qP (Fig. 1*A*,*C*). However, lowland rice showed a less reduction than upland rice in both φPS II and qP (Fig. 1 Suppl.). The average value of φPS II in lowland rice decreased from 0.54 to 0.36 after 120 min of the PEG stress, but decreased from 0.46 to 0.05 in upland rice, especially in the upper section (3.0 to 7.5 cm from the tip). With regard to qP, a weaker developmental gradient and temporal-spatial variation in the leaf under the PEG stress were found as compared to φPS II (Fig. 1*A*,*C*). NPQ was almost the same in nine sections of the leaf under the control conditions (Fig. 1*B*), which implied that no gradient existed for NPQ in the fully expanded rice leaf. However, NPQ in upland rice was higher (an average value of 0.53) than in lowland rice (an average value of 0.25). After the PEG stress, NPQ increased greatly and the increase was different between different parts of the leaf, with the greatest increase in the upper section (4.5 - 6.0 cm). NPQ in upland rice was higher than in lowland rice at the same position of the leaf also after 120 min of the stress. The increase in NPQ reflected the increase in dissipation of excess excitation energy that protected the leaf from photoinhibition.

As concerns gas exchange parameters (Table 1), g_s and P_N in upland rice decreased more than in lowland rice during the first 60 min of the stress and their values in upland rice were lower than in lowland rice also after 120 min of the stress. This indicates that upland rice stomata were more sensitive to the PEG stress than lowland rice stomata. Regarding c_i, lowland rice exhibited a greater decrease than upland rice during the first 60 min of the stress, and then had a rising tendency. These results indicate that the 120 min stress reduced the photosynthetic activity of mesophyll cells in lowland rice.

Spatial heterogeneity is a feature of leaf growth in most plants, and the spatial heterogeneity of photosynthetic efficiency in single leaves has been confirmed using Chl fluorescence imaging technique (Meng *et al.* 2001, Calatayud *et al.* 2006, Sperdouli *et al.* 2012). In the present study, spatial heterogeneity was observed in single rice leaves for Chl fluorescence parameters. The reason for this heterogeneity may be the leaf anatomy

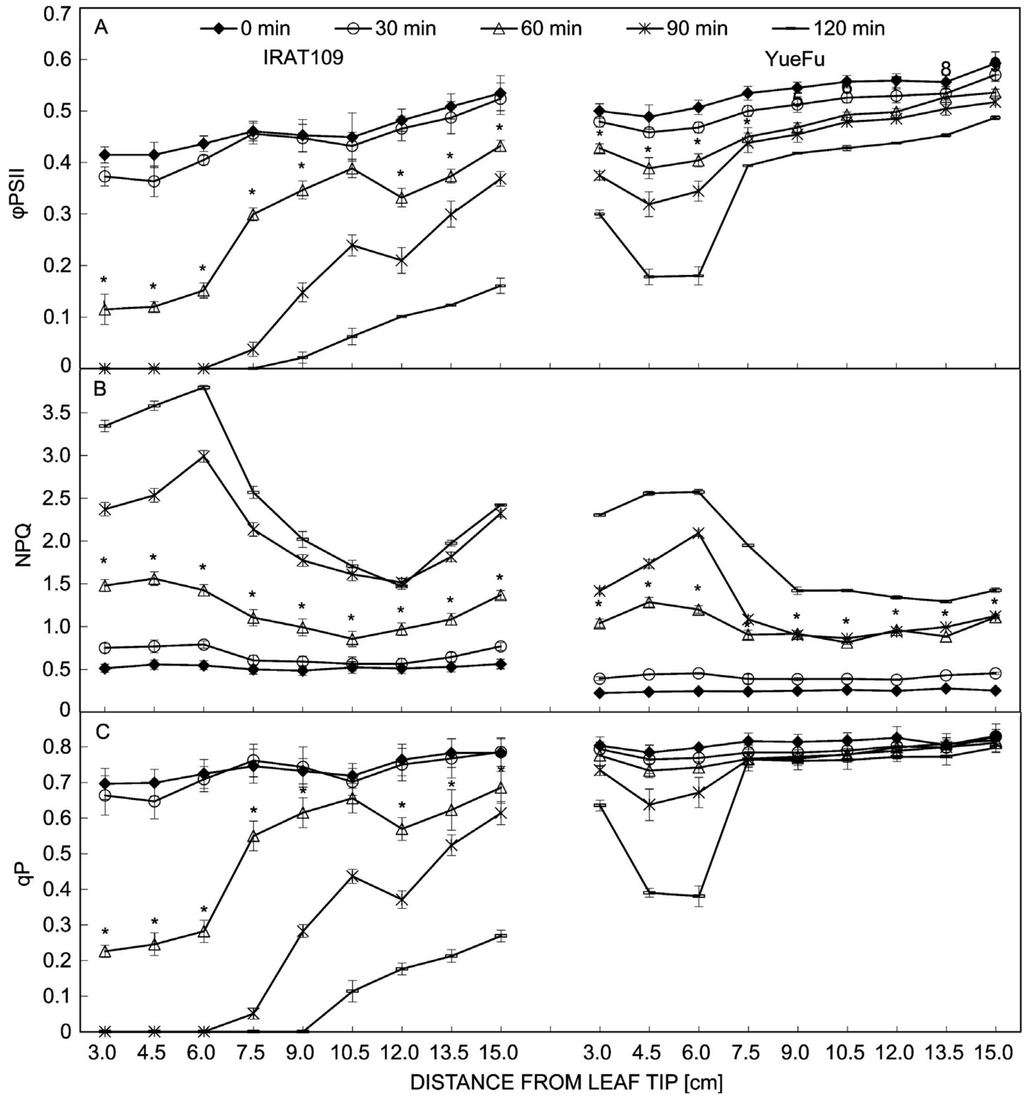

Fig. 1. Chlorophyll fluorescence parameters - the effective quantum yield of PS II photochemistry (*A*), non-photochemical quenching (*B*), and photochemical quenching (*C*) in upland rice (IRAT109) and lowland rice (YueFu) exposed to the PEG stress for 0 min (control), 30, 60, 90, and 120 min. Four-week-old seedlings were used. Nine different areas of interest (3, 4.5, 6, 7.5, 9, 10.5, 12, 13.5, and 15 cm from the leaf tip) were selected for images and data recording. The *asterisks* (*) represent significant differences ($P \leq 0.05$) between the control and 60 min PEG stress. Values are means ± SD ($n = 3$).

Table 1. Stomatal conductance (g_s), net photosynthetic rate (P_N), and intercellular CO_2 concentration (c_i) in the control and after 60 or 120 min of the PEG stress. Means ± SD (*n* = 3). Means within a column of the same variety followed by a different letter are significantly different ($P \leq 0.05$).

Genotype	Time [min]	g_s [mmol(H_2O) m^{-2} s^{-1}]	P_N [µmol(CO_2) m^{-2} s^{-1}]	c_i [µmol(CO_2) mol^{-1}]
IRAT109	control	518.62 ± 32.42^a	23.70 ± 1.02^a	352.29 ± 16.05^a
	60	165.03 ± 23.64^b	9.32 ± 0.83^b	234.07 ± 13.63^b
	120	72.86 ± 5.53^c	3.28 ± 0.25^c	176.23 ± 6.69^c
YueFu	control	582.57 ± 69.37^a	22.13 ± 1.52^a	341.80 ± 14.47^a
	60	362.04 ± 13.53^b	15.86 ± 0.81^b	181.12 ± 11.42^c
	120	82.67 ± 4.14^c	5.73 ± 0.19^c	223.73 ± 18.51^b

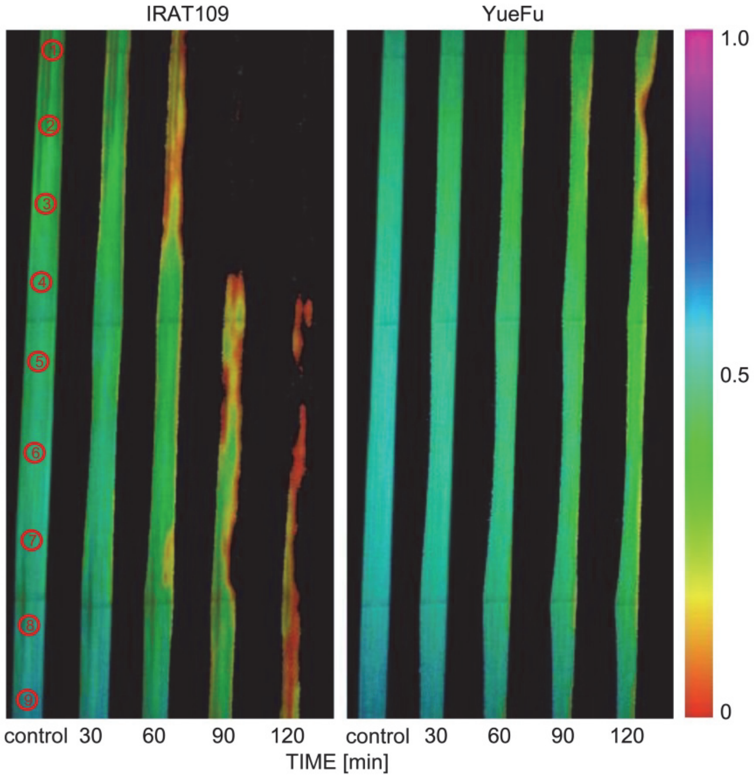

Fig. 2. A representative chlorophyll fluorescence image of the effective quantum yield of PS II photochemistry (φPS II) in upland rice and lowland rice exposed to the PEG stress. Four-week-old seedlings were used. The average length of the leaves was 17 cm. Nine different areas of interest, 3, 4.5, 6, 7.5, 9, 10.5, 12, 13.5, and 15 cm from the leaf tip, were selected for data recording. Images were obtained at 0 (control), 30, 60, 90, and 120 min after the PEG application. The color code depicts the value of φPS II on the right side of the image and ranges from black (0) to purple (1). The imaged area is 10 cm × 13 cm.

and growth processes, such as patchy stomatal distribution, phloem loading conditions, and leaf variegation (Siebke *et al.* 1995a,b, Osmond *et al.* 1999). Environmental factors, for example, abiotic stresses and plant pathogens, can also cause heterogeneity in leaf photosynthesis (Calatayud *et al.* 2006, Iqbal *et al.* 2012, Sperdouli *et al.* 2012). In our study, decreases in φPS II and qP and an increase in NPQ proceeded from the upper section to the base of the leaf under the stress. Such an obvious gradient of heterogeneity in Chl fluorescence in response to abiotic stress has seldom been reported in rice (Pereira *et al.* 2013) but it is common in some other plants (Wingler *et al.* 2004, Massacci *et al.* 2008). The pattern of non-uniform photosynthesis under water stress varies distinctly between species and is likely associated with leaf anatomy, mesophyll development, and treatments (Osmond *et al.* 1999, Sperdouli *et al.* 2012, Li *et al.* 2013).

Upland rice IRAT109 is more drought tolerant than lowland rice YueFu due to its better osmotic adjustment and a stronger root system (Qu *et al.* 2008, Gao *et al.* 2009). However, in the present study, φPS II, qP, NPQ,

g_s, and P_N in upland rice were more sensitive to the short-term PEG-induced drought stress than in lowland rice, which is not the same as results obtained under a long-term stress (Hu *et al*. 2009). The reason may be that effects of the long-term stress were more significant than of the short-term stress. Under the mild and short-term drought stresses, upland rice might be more sensitive and respond faster to the stress than lowland rice. Upland rice has a more rapid ABA accumulation in response to PEG stress (Lian *et al*. 2006). Gao (2009) found a more rapid regulation mechanism to reach metabolic balance under stress in upland rice IRAT109 than in lowland rice YueFu. A rapid increase in NPQ is helpful for protecting the photosynthetic apparatus against photoinhibition. The rapid stomata closure is helpful for avoiding an excessive water loss causing detrimental effects. Furthermore, a strong osmotic adjustment in upland rice could protect the photosynthetic apparatus, cell membranes, proteins, *etc.* from damage during stress progression. Although in lowland rice, non-stomatal limitation of photosynthesis might occur based on an increase of c_i after the 120 min stress, the same was not observed in upland rice.

In conclusion, the spatial heterogeneity of Chl fluorescence parameters was observed in the fully expanded leaves of the rice seedlings under the control conditions and under the short-term osmotic stress. Chl fluorescence parameters, net photosynthetic rate, and stomatal conductance in upland rice were more sensitive to the stress than in lowland rice. The spatial heterogeneity of Chl fluorescence parameters and different responses of photosynthesis to osmotic stress are helpful for understanding short-term drought tolerance mechanisms of upland and lowland rice cultivars.

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