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Response of photosynthesis to short-term drought stress in rice seedlings overexpressing C_4 phospho*enol*pyruvate carboxylase from maize and millet

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Abstract

Abiotic stresses induce phospho*enol*pyruvate carboxylase (PEPC) expression in C_3 plants which suggests PEPC function in plant adaptation to stresses. Here, we studied the response of photosynthesis to short-term drought stress in rice seedlings overexpressing C_4 PEPC from maize and millet. The transgenic lines exhibited 1.2–5.5 fold of PEPC activities than the wild type before the treatment, while 1.5–8.5 fold after five or ten days of water deficit. Net photosynthetic rate (P_N) declined less during the water stress and recovered more after rewatering in the transgenic lines. These changes were accompanied with changes in the stomatal conductance (g_s). The lower decrease in P_N and g_s resulted in significantly higher intrinsic water use efficiency in the transgenic rice lines after ten days of water withdrawal. There were no significant differences between the wild type and transgenic lines in maximum photochemical efficiency of PSII and photochemical quenching. The nonphotochemical quenching and the quantum efficiency of PSII maintained both higher in transgenic lines than those in the wild type during drought stress. This indicated that the transgenic lines could dissipate more excess energy to heat to protect PSII. Our result suggested that the increased PEPC activities in rice could alleviate the decrease of photosynthesis during short-term drought stress.

Additional key words: chlorophyll; chlorophyll fluorescence; gas exchange; photoprotection; stomatal opening.

Introduction

Phospho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31) is an important and multifaceted enzyme that catalyzes the irreversible β-carboxylation of phospho*enol*pyruvate (PEP) to yield oxaloacetate (OAA) and inorganic phosphate (P_i). The enzyme is found in all plants, green algae, and cyanobacteria, most archaea and non-photosynthetic bacteria, but is absent in animals and fungi (O'Leary *et al.* 2011). This enzyme has been intensively studied with regards to its crucial role in catalyzing atmospheric CO_2 fixation in C_4 and CAM photosynthesis (Chollet *et al.* 1996). It also plays so called anaplerotic functions in C_3 plants or in nonphotosynthetic tissues of C_4 plants, *i.e.*, the replenishment of tricarboxylic acid cycle intermediates withdrawn for biosynthesis and N-assimilation (Miyao and Fukayama 2003, Masumoto *et al.* 2010, O'Leary *et al.* 2011). PEPC is thought to carry out various functions depending on the tissue and a stage of development. For example, PEPC plays a role in modulation of stomatal opening (Asai *et al.* 2000, Cousins *et al.* 2007). PEPC appears to play a role in the extension of cotton fibres (Li *et al.* 2010) and in the developing of various fruits (Guillet *et al.* 2002, Sweetman *et al.* 2009, Perotti *et al.* 2010, Yin *et al.* 2010) by mediating organic acid accumulation. It is proposed that PEPC activity allows malate production and therefore increased turgor that is required for fibre elongation and fruit expansion. In developing seeds, PEPC participates in converting photosynthates into fatty acids or storage proteins (Sangwan *et al.* 1992, Blonde and Plaxton 2003, Lebouteiller *et al.* 2007). In addition, PEPC helps plants to acclimatize to abiotic and biotic stresses (O'Leary *et al.* 2011). PEPC activities, although low in C_3 plants,

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Abbreviations: C_i – intercellular CO_2 concentration; E – transpiration rate; F_v/F_m – maximum photochemical efficiency of PSII; g_s – stomatal conductance; NPQ – nonphotochemical quenching; PEPC – phospho*enol*pyruvate carboxylase; P_N – net photosynthetic rate; q_P – photochemical quenching coefficient; WT – wild type; WUE – water-use efficiency (= P_N/E); Φ_{PSII} – quantum efficiency of PSII; ZM07 – rice line No. 7 transformed with the intact PEPC gene from *Zea mays*; ZM30 – rice line No. 30 transformed with the intact PEPC gene from *Zea mays*; PRM25 – rice line No. 25 transformed with PEPC gene from *Setaria italica* under the control of rice Rubisco small subunit promoter; PRM38 – rice line No. 38 transformed with PEPC gene from *Setaria italica* under the control of rice Rubisco small subunit promoter.
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were found to be related to various biotic and abiotic stresses, such as salt or drought stress, phosphorus deficiency, iron deficiency, aluminium toxicity, ozone stress, and virus infection (Doubnerová and Ryšlavá 2011).

In order to engineer one-cell C_4 photosynthesis to improve photosynthetic capacity in C_3 plants, many attempts to overexpress C_4-PEPC genes in C_3 plants have been taken (Hudspeth *et al.* 1992, Ku *et al.* 1999, Agarie *et al.* 2002, Fukayama *et al.* 2003, Bandyopadhyay *et al.* 2007, Ding *et al.* 2013, Lian *et al.* 2014). But the overexpressed PEPC did not improve the photosynthetic rate of the transgenic C_3 plants due to higher dark respiration rate and P_i limitation (Agarie *et al.* 2002, Fukayama *et al.* 2003). Although, enhanced photosynthetic rates were reported when the transgenic plants were grown under various stress conditions (Kogami *et al.* 1994, Jeanneau *et al.* 2002, Bandyopadhyay *et al.* 2007, Ding *et al.* 2007, 2013) or field conditions (Jiao *et al.* 2002, Lian *et al.* 2014). RNA inhibition of canola PEPC resulted in increased sensitivity to polyethylene glycol-induced osmotic stress (Chen *et al.* 2010). Therefore, it was supposed that the enhanced photosynthetic rate in the plants overexpressing PEPC would be the result of its function in stress adaptation but not the enhancement of the C_4 photosynthesis.

Enhanced PEPC activity under drought stress was found in some C_3 plants (Doubnerová and Ryšlavá, 2011; O'Leary *et al.* 2011). In wheat roots, drought induced the PEPC gene transcription and translation (González *et al.* 2003). Recently reported study found that protein phosphorylation was ascribed to the increased PEPC activity in tobacco leaves after drought stress (Hýsková *et al.* 2014). Plants with incrased PEPC activities would be benifit for studying the mechanism of PEPC function in stress adaptation. Here we reported the response of photosynthesis to a short-term drought stress in transgenic rice overexpressing C_4 PEPC from maize and millet. The potential mechanisms of PEPC in drought resistance of photosynthesis were discussed.

Materials and methods

Plant material and treatments: Transgenic rice lines, ZM07 and ZM30 (Ding *et al.* 2007; transformed with the intact PEPC gene from *Zea mays*), PRM25 and PRM38 (Ding *et al.* 2013; transformed with the PEPC gene from *Setaria italica* under the control of rice Rubisco small subunit promoter) and the wild type (WT) rice cv. Zhonghua 8 were used in this research. The seeds were germinated in a germination chamber at 28ºC in darkness. One week after germination, the seedlings were transplanted to large pots (20 cm diameter, 25 cm height) containing clay-calcareous soils and horticultural substrate (60:40). The seedlings were watered daily to maintain saturated water content. The plants were grown under the greenhouse condition at Institute of Crop Sciences, Chinese Academy of Agricultural Sciences (Beijing, China): a 12-h photoperiod, temperature of 28/20ºC (day/night), and photosynthetic photon flux density of 500 μmol m^{-2} s^{-1}.

The irrigation was withdrawn after three weeks. After 11 d, the seedlings were rewatered daily and the soil water content was maintained saturated. At 0, 5, 10, and 15 d after the treatment (DAT), the measurements were carried out on the last fully expanded leaves. The whole experiment lasted for 16 d.

Chlorophyll (Chl) *a* fluorescence: Prior to the gas-exchange measurements, Chl *a* fluorescence parameters were measured with a modulated fluorometer (*FMS-2, Hansatech*, Norfolk, UK) on the last fully expanded leaves with five replicas. The intensity of actinic light used in the measurements was 400 μmol(photon) m^{-2} s^{-1}. The following parameters were assessed as described by Schreiber *et al.* (1994): the quantum efficiency of PSII in the light [$\Phi_{PSII} = (F_m'-F_s')/F_m'$], the coefficients of photochemical quenching [$q_P = (F_m'-F_s)/(F_m'-F_o')$], and nonphotochemical quenching [$NPQ = (F_m-F_m')/F_m'$], where F_m and F_o are, respectively, maximum and minimum fluorescence of dark-adapted leaves, F_m' and F_s are, respectively, maximum and steady-state fluorescence in the light-adapted state, and F_o' is minimum fluorescence after far-red illumination of the previously exposed leaves.

Gas exchange: Net CO_2 assimilation rates (P_N), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) was measured at mid-morning with a gas-exchange system (*Li-6400, Li-Cor Inc.*, Nebraska, USA) equipped with a light source (*6400-02B LED*, *Li-Cor*). Environmental conditions in the leaf chamber were: light intensity of 1,500 μmol(photon) m^{-2} s^{-1}, leaf temperature of 25ºC, ambient CO_2 concentration of 390 μmol mol^{-1}. Five replicas were made for each treatment.

Protein extraction and PEPC activity: Leaves (approx. 0.1 g of fresh mass) of WT and the transgenic lines were immediately frozen in liquid nitrogen, homogenized in 1.5 mL of extraction buffer [50 mM TRIS-HCl (pH 7.5), 10 mM $MgCl_2$, 10% glycerol, 1 mM EDTA, 1 mM DTT, 14 mM β-mercaptoethanol, 10 μM leupeptine, 1% PVP]. The extract was centrifuged at 48,000 × *g* for 15 min, and the supernatant was used immediately for enzyme assays. Protein amounts were determined by the method of Bradford (1976). Total PEPC activity assay medium contained 100 mM Tris-HCl (pH 8.0), 0.3 M PEP, 5 mM $MgCl_2$, 0.2 mM NADH, 10 mM $NaHCO_3$, 10 U malate dehydrogenase (MDH). PEPC activity was measured by following the decrease of absorbance at 340 nm with a spectrophotometer (*SPECORD 200, Analytik-Jena*, Germany), interfering NADH oxidase activity was measured

in the absence of PEP and subtracted to give the PEPC activity. PEPC activity was expressed by μmol(CO_2) mg^{-1}(protein) min^{-1}.

Pigment content: Total Chl was extracted by 80% acetone and determined with spectrophotometer (*Specord 200, Analytik,* Jena, Germany) according to Arnon (1949).

Results

PEPC activity: Our previous study proved that the C_4-PEPC gene from *Zea mays* or *Sateria italica* was successfully transferred to rice plants and the PEPC activity increased 1–27 times that of untransformed plants (Ding *et al.* 2007, 2013). The expression of the introduced gene led to an increase in the PEPC activity (1.2- to 5.5-fold of WT) in the leaves of four transgenic lines used in our experiment under control conditions (Fig. 1). The PEPC activity was enhanced during the proceeding stress severity both in WT and the transgenic lines. However, the increment in the PEPC activity was higher (up to +86%) in the transgenic lines than that in WT (up to +46%) after 10 DAT. It indicated that drought stress induced the expression or post-translation regulation, such as protein phosphorylation of PEPC.

Gas exchange: Our previous study also showed that the transgenic rice plants exhibited the higher P_N during the flowering stage under upland field but not in paddy field conditions (Ding *et al.* 2007, 2013). P_N decreased significantly together with the increasing water deficit (Fig. 2*A*). WT showed the decline to 62% and 48% at 5 and 10 DAT, respectively, and back to 56% after rewatering. However, the reduction of P_N in the transgenic lines was much smaller. Their P_N were significantly higher than that of WT. The PRM38 line showed the highest resistance of P_N to drought stress. The response of g_s showed the trend similar to that of P_N during the

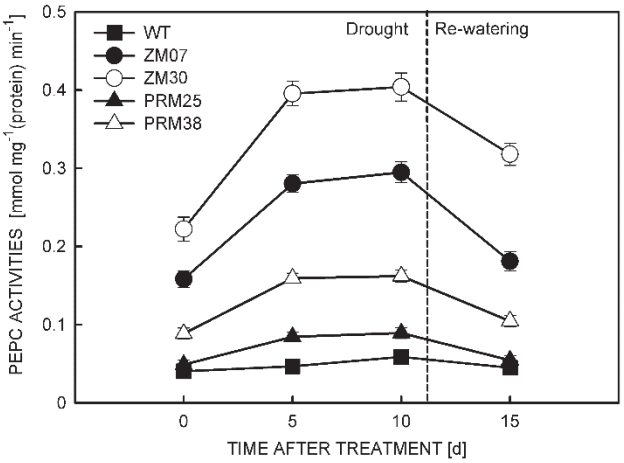

Fig. 1. Phospho*enol*pyruvate carboxylase (PEPC) activity during water withdrawal and after rewatering in PEPC transgenic plants. ZM07 and ZM30 – rice lines No. 7 and 30 which were transformed with the intact PEPC gene from *Zea mays*, PRM25 and PRM38 – rice lines No. 25 and 38, which were transformed with the PEPC gene from *Setaria italica* under the control of rice Rubisco small subunit promoter. WT – untransformed wild type rice cv. Zhonghua 8. Values represent means ± SE of three replicates.

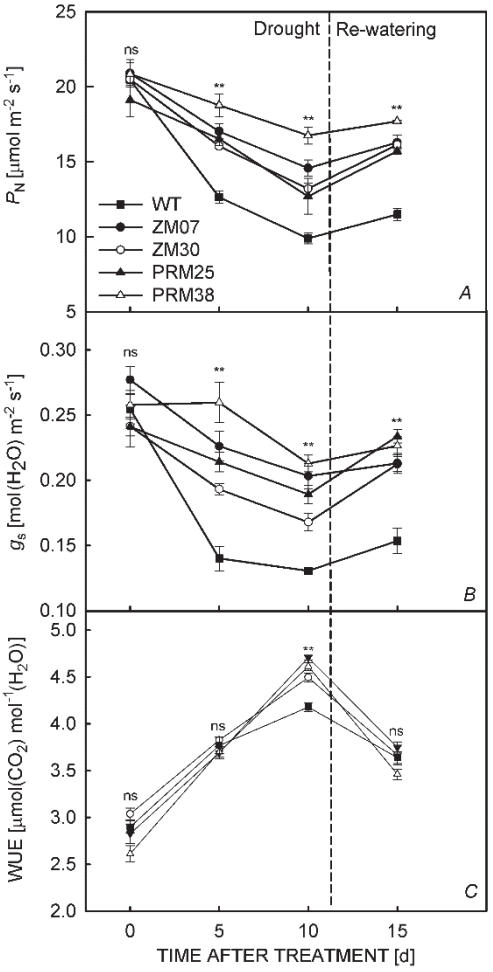

Fig. 2. Net photosynthetic rate (P_N) (*A*), stomatal conductance (g_s) (*B*), and water-use efficiency (WUE) (*C*) during water withdrawal and after rewatering in PEPC transgenic plants. Values represent means ± SE of three replicates. ns – no significant difference between the transgenic lines and the wild type (WT), ** – significant difference (P<0.01) between transgenic lines and WT, ZM07 and ZM30 – rice lines No. 7 and 30 which were transformed with the intact PEPC gene from *Zea mays*, PRM25 and PRM38 – rice lines No. 25 and 38 which were transformed with the PEPC gene from *Setaria italica* under the control of rice Rubisco small subunit promoter. WT – untransformed wild type rice cv. Zhonghua 8.

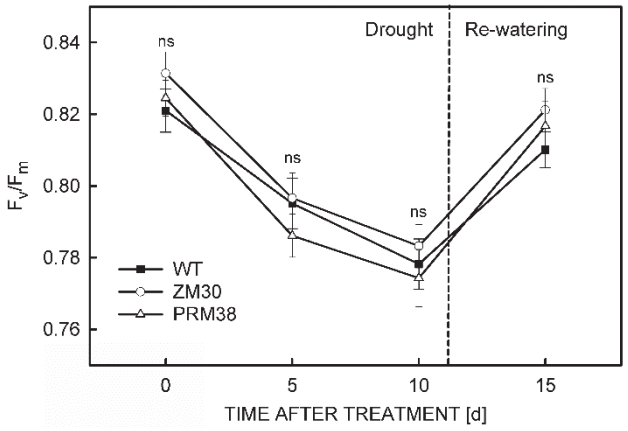

Fig. 3. Maximum photochemical efficiency of PSII (F_v/F_m) during water withdrawal and after rewatering in PEPC transgenic plants. Values represent means ± SE of five replicates. ns – no significant difference between transgenic lines and the wild type (WT). ZM30 – rice lines No. 30 which was transformed with the intact PEPC gene from *Zea mays*, PRM38 – rice line No. 38 which was transformed with the PEPC gene from *Setaria italica* under the control of rice Rubisco small subunit promoter. WT – untransformed wild type rice cv. Zhonghua 8.

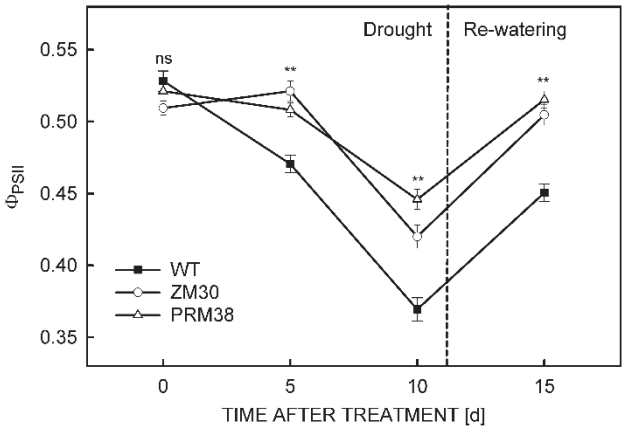

Fig. 4. Actual quantum efficiency of PSII in the light (Φ_{PSII}) during water withdrawal and after rewatering in PEPC transgenic plants. Values represent means ± SE of five replicates. ns – no significant difference between transgenic lines and the wild type (WT), ** – significant difference (P<0.01) between transgenic lines and the WT. ZM30 – rice lines No. 30 which was transformed with the intact PEPC gene from *Zea mays*, PRM38 – rice line No. 38 which was transformed with the PEPC gene from *Setaria italica* under the control of rice Rubisco small subunit promoter. WT – untransformed wild type rice cv. Zhonghua 8.

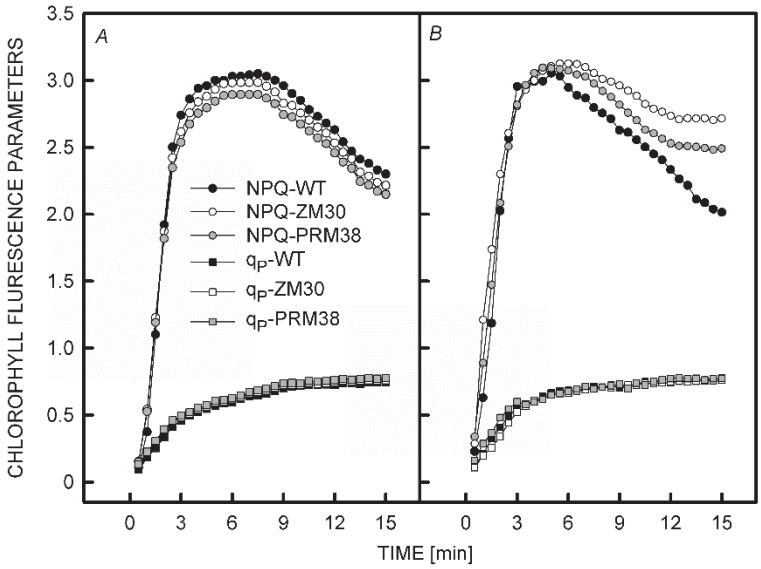

Fig. 5. Photochemical and nonphotochemical quenching coefficients during light induction (*A*) before and (*B*) after five days of water withdrawal in PEPC transgenic rice plants. ZM30 – rice lines No. 30 which was transformed with the intact PEPC gene from *Zea mays*, PRM38 – rice line No. 38 which was transformed with the PEPC gene from *Setaria italica* under the control of rice Rubisco small subunit promoter. WT – untransformed wild type rice cv. Zhonghua 8.

treatment (Fig. 2*B*). Correlation analysis showed that P_N was linearly correlated with g_s (R^2 = 0.8860, P<0.01). Water-use efficiency (WUE) was calculated as P_N/g_s. It was significantly higher in the transgenic lines only at 10 DAT.

PSII: Chl *a* fluorescence parameters were measured in WT and two transgenic lines, ZM30 and PRM38. The F_v/F_m ratio declined only by 5% in both WT and the transgenic lines even after 10 DAT (Fig. 3). Φ_{PSII} decreased significantly during the drought stress in the transgenic lines and WT, but it dropt less and recovered more in both transgenic lines (Fig. 4). Thus, the Φ_{PSII} of the transgenic lines was significantly higher than that of WT after water withdrawal and rewatering.

Quenching analysis revealed that q_P did not change due to transformation under both the normal condition and drought stress (Fig. 5*A*,*B*). NPQ showed no difference between WT and the transgenic lines under the control condition (Fig. 5*A*). However, under the drought stress, the difference of NPQ between the transgenic lines and WT became more and more obvious (Fig. 5*B*) during the 15-min light induction. At the end of the induction curve,

Table 1. Chlorophyll (Chl) content in leaves of wild type and transgenic lines during progressive drought stress and rewatering. Values represent means ± SD, n = 5. ns – not significant, * – P<0.05, ** – P<0.01. FM – fresh mass. ZM07 and ZM30 – rice lines No. 7 and 30 which were transformed with the intact PEPC gene from *Zea mays*, PRM25 and PRM38 – rice lines No. 25 and 38 which were transformed with the PEPC gene from *Setaria italica* under the control of rice Rubisco small subunit promoter. WT – untransformed wild type rice cv. Zhonghua 8.

Plant	Chl (a+b) [mg g^{-1}(FM)]			
	0	5 days	10 days	15 days (rewatering)
WT	2.62 ± 0.07	2.04 ± 0.09	1.60 ± 0.10	1.76 ± 0.05
ZM07	2.58 ± 0.04^{ns}	2.04 ± 0.08^{n}s	1.82 ± 0.02*	2.04 ± 0.11*
ZM30	2.58 ± 0.03^{ns}	2.30 ± 0.03*	1.78 ± 0.08*	2.15 ± 0.07**
PRM25	2.60 ± 0.07^{ns}	2.40 ± 0.06*	1.89 ± 0.05*	2.58 ± 0.04**
PRM38	2.59 ± 0.10^{ns}	1.97 ± 0.06^{n}s	1.82 ± 0.03*	2.30 ± 0.02**

NPQ significantly differed (+35%) in ZM30 and PRM38. It indicated that the transgenic lines with higher NPQ could dissipate more excessive energy as heat.

Chl content: Pigment content varied among plant types and under different treatments (Table 1), although there was no difference in the pigment content under the control condition. Under drought stress, the four transgenic lines exhibited a mild decrease in Chl (a+b) with the stress severity increasing, while WT reduced its Chl content more significantly (–39%) after 10 DAT. After the recovery, the Chl (a+b) content in WT remained only 67% of the control, while the transgenic line PRM25 reached 99%.

Discussion

In this study, P_N, g_s, Φ_{PSII}, and the Chl content declined during water withdrawal in the transgenic rice lines and WT. It was the normal response of photosynthesis to drought stress (Reddy *et al.* 2004). But the inhibitions were lesser in the rice lines with increased C_4-PEPC activities.

The changes in the PEPC activity did not affect apparently P_N of WT and the transgenic lines, when grown under normal conditions (Fig. 2). Similar results were reported in previous studies (Agarie *et al.* 2002, Fukayama *et al.* 2003). Increased respiration and incorrect phosphorylation patterns of PEPC in transgenic rice plant might be part of its failure to improve P_N. However, the transgenic lines with the higher leaf PEPC activity maintained higher P_N than WT, when exposed to progressive drought stress (Fig. 2*A*). Our correlation analysis showed that g_s accounted for the higher P_N in the transgenic lines under drought stress (Fig. 6*A*). Organic anions, mainly malate, compensate for the positive charge resulting from K^+ accumulation during stomatal opening. Malate synthesis is highly dependent on PEPC activity (Vavasseur and Raghavendra 2005). Under short-term water stress, abscisic acid induces downregulation of PEPC mRNA in guard cells and/or mesophyll cells (Kopka *et al*. 1997, Leonhardt *et al.* 2004), which reduces malate accumulation in vacuole and thus the stomatal opening. Here, the introduced foreign C_4-PEPC in rice leaves might alleviate this malate decreasing in the stomata. Thus the g_s in the transgenic rice leaves decreased less than that of WT during water deficit, which caused that the plants maintained higher internal CO_2 concentration and resulted in a higher carboxylation capacity.

But, the g_s before or after water-withdrawal treatment had no significant relation with the PEPC activities (Fig. 6*B*). A little more than one fold PEPC activity in line PRM25 (Fig. 1*B*) showed higher g_s than that of WT. It suggested that not the total activities but the special charcteristics of the introduced C_4-type PEPCs, such as its lesser sensitivity to inhibitors, might be more important in the maintaining of high g_s during drought stress. PEPC activities in guard cells are modulated by specific phosphorylation and drought could repress this process (Outlaw *et al.* 2002). Phosphorylation of the guard-cell enzyme in *Commelina communis* L. results in a 50% increase in the V_{max} (maximum velocity) and in a large reduction in L-malate retroinhibition (Cotelle *et al.* 1999). Phopshorylated C_4-PEPC is more tolerant to malate inhibition than C_3-PEPC (Chollet *et al.* 1996, Miyao *et al.* 2011). Also, monoubiquitination and deubiquitination followed by phosphorylation could significantly affect PEPC activity (Shane *et al.* 2013). The transcription and post-translation modifications of the overexpressed C_4-PEPC from maize or millet need further clarification.

PSII is believed to play an important role in the response of leaf photosynthesis to environmental stresses (Baker 1991). During drought stress, disturbances of the photosynthesis at the molecular level are connected to the low electron transport through PSII and/or with structural modifications of PSII (Dubey 1997). We observed that PSII was not affected by the short-term drought stress in this study; the maximum photochemical efficiency of PSII (F_v/F_m) and q_P did not decrease significantly in agreement with results reported for short-term water stress (Lu and

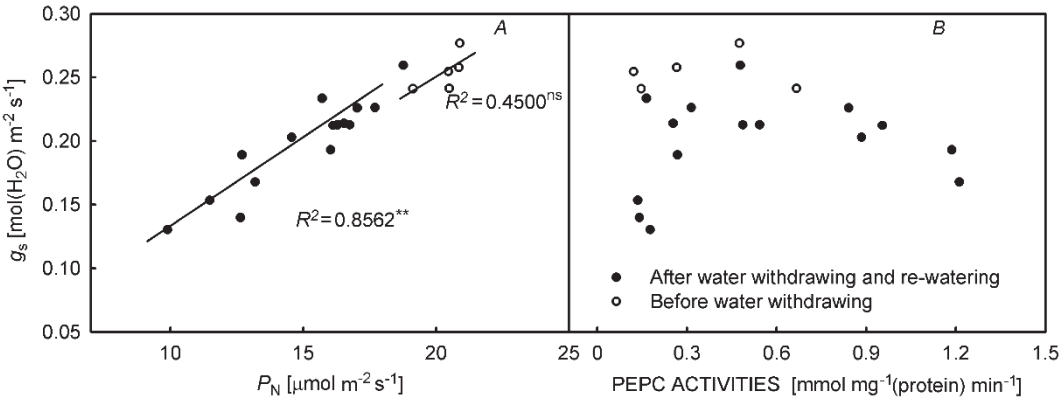

Fig. 6. Correlation analysis of stomatal conductance (g_s) with net photosynthetic rate (P_N) (*A*) and phospho*enol*pyruvate carboxylase (PEPC) activities (*B*).

Zhang 1998). Under drought stress conditions, the excessive light can result in photoinhibition (Osmond *et al.* 1999). Plants can avoid photoinhibition by either decreasing the absorption of light or increasing the dissipation of excessive energy absorbed through photochemical and nonphotochemical mechanisms (Björkman and Demmig-Adams 1994). In addition, increased thermal dissipation is known to be the major photoprotective response to avoid photoinhibition under water stress (Flexas and Medrano 2002). Our result showed that the transgenic lines maintained the higher Φ_{PSII} and NPQ during the treatment (Figs. 4, 5). Significantly higher NPQ under drought stress in the transgenic lines could dissipate more excessive energy by heat (Müller *et al.* 2001). Thermal energy dissipation *via* xanthophyll cycle is an important pathway to protect photosynthetic apparatus from damage caused by excessive light (Demmig *et al.* 1987). Violaxanthin de-epoxidase catalyzes the conversion of violaxanthin to zeaxanthin. It play important role in the cycle. Violaxanthin de-epoxidase activity can be improved by low pH value (Hager and Holocher 1994). Oxaloacetate catalyzed by PEPC can quickly convert to malic acid to regulate intracellular pH, and it ultimately alleviates intracellular alkalization caused by drought stress (Gollan *et al.* 1992, Bacon *et al.* 1998).

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