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Overexpression of maize phospho*enol*pyruvate carboxylase improves drought tolerance in rice by stabilization the function and structure of thylakoid membrane

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Abstract

Drought impacts severely crop photosynthesis and productivity. Development of transgenic rice overexpressing maize phospho*enol*pyruvate carboxylase (PEPC) is a promising strategy for improving crop production under drought stress. However, the molecular mechanisms of protection from PEPC are not yet clear. The objective of this study was: first, to characterize the response of individual photosynthetic components to drought stress; second, to study the physiological and molecular mechanisms underlying the drought tolerance of transgenic rice (cv. *Kitaake*) over-expressing maize PEPC. Our results showed that PEPC overexpressing improved the ability of transgenic rice to conserve water and pigments during drying as compared to wild type. Despite the fact that drought induced reactive oxygen species and damaged photosystems (especially, PSI) in both lines, higher intercellular CO_2 concentration protected the photosynthetic complexes, peptides, and also ultrastructure of thylakoid membranes against the oxidative damage in transgenic rice. In conclusion, although photosynthetic apparatus suffered an inevitable and asymmetric impairment during drought conditions, PEPC effectively alleviated the oxidative damage on photosystems and enhanced the drought tolerance by increasing intercellular CO_2 concentration. Our investigation provided critical clues for exploring the feasibility of using C_4 photosynthesis to increase the yield of rice under the aggravated global warming.

Additional key words: drought stress; phospho*enol*pyruvate carboxylase; transgenic rice; oxidative stress.

Introduction

Global environmental changes impose severe challenges on ecosystems, hydrological systems, land degradation, and agricultural systems. Especially, water scarcity due to global environmental changes significantly limits crop yields (Goltsev *et al*. 2012). As the worldwide most important cereal crop, rice is widely consumed by a large population in the world, especially in Asia (Kajala *et al*. 2011). However, the yield potential of rice can be reduced significantly by drought across all agro-climatic zones of the globe (Gorantla *et al*. 2007).

Water deficits lead to a combination of biochemical, physiological, and metabolic changes in rice. Among these changes, photosynthesis in the chloroplast is one of the most stress-sensitive physiological processes (Tian *et al*. 2013). The effects of drought stress on photosynthesis can directly result from photorespiratory losses, due to the

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Abbreviations: BN-PAGE – blue native polyacrylamide gel electrophoresis; Chl – chlorophyll; C_i – intercellular CO_2 concentration; DM – dry mass; DS – drought stress; FM – fresh mass; g_s – stomatal conductance; F_t – fast chlorophyll *a* fluorescence transients; MDA – malondialdehyde; OEC – oxygen evolving complex; $O_2^{\bullet-}$ – superoxide anion; PEPC – phospho*enol*pyruvate carboxylase; P_N – net photosynthetic rate; RC – PSII reaction center; Rf – relative mobility; ROS – reactive oxygen species; RWC – relative water content; T-PEPC – transgenic rice overexpressing PEPC; TM – turgid mass; WT – wild type.
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decreased CO_2 availability caused by stomatal closure (Redillas *et al*. 2011). They can also cause indirect and secondary effects, such as the accumulation of reactive oxygen species (ROS) in chloroplasts. Hence, under drought stress chloroplast membranes and membrane-bound-structures are particularly susceptible to ROS damage (Bowler *et al*. 1992, Chaves and Oliveira 2004).

Although a cascade of direct or indirect impairment of photosynthetic apparatus is confirmed in plants under drought (Redillas *et al*. 2011), changes in the structure, function, and activity of the individual light-reaction apparatus are uncertain. For example, PSII was reported to be more susceptible to drought stress compared to PSI (Havaux and Strasser 1992), whereas other reports suggested that drought stress should affect relatively more PSI than PSII, and the primary photochemistry of PSII (φ_{Po}) decreased only under severe drought stress (Souza *et al*. 2004, Oukarroum *et al*. 2009, Redillas *et al*. 2011). Thus, the comparative effects of drought stress on PSI and PSII in chloroplasts are not still well understood.

The CO_2-concentrating mechanism possessed by C_4 plants (such as maize, sorghum, *etc*.) operates by sequentially prefixing CO_2 into C_4-carbon compounds *via* PEPC in mesophyll cells. Then, the bound carbon is transported into the gas-tight bundle sheath cells where it is decarboxylated. This mechanism increases the intercellular CO_2 concentration (C_i) around Rubisco enzyme for the Calvin cycle (Häusler *et al*. 2002). Hence, this mechanism makes C_4 plants competitive under conditions of C_i deficiency caused by high temperatures, high light intensities, and drought (Edwards *et al*. 2004).

However, many of important food crops, such as wheat, rice, soybean, and potato, are C_3 plants. They assimilate atmospheric CO_2 directly without the biochemical pump (Bandyopadhyay *et al*. 2007) and their crop yields are limited by environmental variables including drought stress (Raines 2006). Hence, engineering C_4 traits into C_3 plants, such as rice, is directed towards enhancing crop resistance to water deficit (Häusler *et al*. 2002). Although over-expressing PEPC in transgenic plants has not resulted in enhanced photosynthesis or growth under optimum situations (Peterhansel 2011), the theoretical advantage may be achieved under CO_2-limiting conditions, such as drought and high temperatures (Chaves and Oliveira 2004). It has been reported that PEPC-transgenic tobacco, potato (Matsuoka *et al*. 2001), and rice (Bandyopadhyay *et al*. 2007) exhibit better photosynthetic performance under high temperatures. The hypothesis underlying these results is that PEPC participates in the initial CO_2 fixation or it increases the concentration of CO_2 near Rubisco (Chaves and Oliveira 2004). However, the molecular mechanism for PEPC-transgenic plants to resist drought stress is still lacking.

As an extremely early flowering rice cultivar, Kitaake is a suitable model system for molecular studies. It is also the first successful example for developing transgenic plants with maize PEPC (Ku *et al*. 1999). The objective of this study was to investigate the sensitivity of individual photosynthetic components to drought stress and to study the physiological and molecular mechanisms underlying the drought tolerance of PEPC-transgenic rice. Seedlings of the wild type (WT) and transgenic rice were subjected to drought for five days. The functional and structural changes of the photosynthetic apparatus were detected by using chlorophyll (Chl) *a* fluorescence, blue native polyacrylamide gel electrophoresis (BN-PAGE), Western-blot, and transmission electron microscopy. Our results revealed that the site-dependent disruption of photosystems was induced by drought stress in both lines, although the PEPC-transformed rice showed better resistance to the stress than WT by increasing C_i and decreasing ROS damage.

Materials and methods

Plant material: *Oryza sativa* L. *japonica* cv. Kitaake (WT) and the strain (lines 704-18) overexpressing the complete PEPC gene of maize were produced previously by Ku *et al*. (1999) and were applied in this study.

Growth conditions and treatment: Seeds of the transgenic (T-PEPC) plants and WT were sterilized by 1% sodium hypochlorite solution for 10 min and rinsed several times with distilled water. After germination at 30 ± 2°C in the dark for 24 h on moistened filter paper, the uniform seedlings were pot-cultivated (6.5 × 6.5 cm) in commercial peat soil. Both types of plants were grown in greenhouse chambers at 28°C with 16 h light [600 μmol(photon) m^{-2} s^{-1}]/8 h dark cycle under 70% relative humidity. Plants were well watered every day until the three-leaf stage (14 days old). Drought stress (DS) was introduced by withholding water for 5 d in one group of plants, whereas the control plants were watered all days. The midsection of the third fully expanded leaf was used for subsequent experiments.

Relative water content (RWC) and chlorophyll (Chl) content: To assess the water status of the plants, RWC was evaluated by the gravimetric method (Oukarroum *et al*. 2007). Fresh mass (FM) of leaves was measured immediately after detachment. After rehydration in distilled water for 4 h, the samples were dried thoroughly with paper towels, and weighed to obtain the turgid mass (TM). They were then dried at 60°C until constant mass to obtain the dry mass (DM). RWC [%] was calculated as 100 × (FM – DM)/(TM – DM). The leaf Chl content was determined spectrophotometrically (*Cintra 1010, GBC scientific equipment*, Australia) in 80% acetone (Arnon 1949) and normalized to FM.

Fast Chl *a* fluorescence transients (JIP curve, F_t): Chl *a* fluorescence transients were measured with *Handy PEA* (*Hansatech Instruments*, Kings Lynn, UK) according to the method described by Strasser and Srivastava (1995). After 1 h dark adaptation, the leaves, still attached to the seedlings, were illuminated with a band of three red light emitting diodes (650 nm peak wavelengths). All measurements were taken using a saturating pulse of 3,000 μmol (photon) m^{-2} s^{-1} for 1 s starting from 20 μs, with data acquisition rate of 10^5, 10^4, 10^3, 10^2, and 10 readings per second in the time intervals of 20–300 μs, 0.3–3 ms, 3–30 ms, 30–300 ms, and 0.3–1 s, respectively.

We analyzed each Chl fluorescence transient by utilizing the original JIP data: maximal fluorescence intensity at the P-step (F_M); fluorescence intensity at the O-step (20 μs), considered as the first credible measurement (F_0); the complementary area (Area); fluorescence intensities at the L-step (about 150 μs, F_L), K-step (300 μs, F_K), J-step (2 ms, F_J), and I-step (30 ms, F_I). The derived parameters from the original data (Table 1) were used for calculation of biophysical performance indexes and events in or around PSI (Strasser 1981, Strasser *et al*. 2000, Strasser *et al*. 2004, Jiang *et al*. 2008, Zubek *et al*. 2009), and expressed as a fraction in relation to control WT plants (with value 100% = 1).

To further compare samples for the photosynthetic fluxes in the OK, OJ, and IP phases, we extended the analyses of average F_t. These are shown as various differential curves after double normalization between different time points (Strasser *et al*. 2007, Tsimilli-Michael and Strasser 2008, Yusuf *et al*. 2010). The detailed calculations were as follows: (*1*) the difference kinetics between F_O and F_K was calculated as $\Delta W_{Ok} = \Delta(F_t - F_O)/(F_k - F_O)$, where W_{Ok} in control WT is subtracted from that in other samples. L-bands, revealed by ΔW_{Ok}, indicate the degree of energetic disconnectivity of the PSII units, (*2*) the difference kinetics between F_O and F_J was calculated as $\Delta W_{OJ} = \Delta(F_t - F_O)/(F_J - F_O)$, where W_{OJ} in control WT is subtracted from that in other samples. K-bands, revealed by ΔW_{OJ}, indicate the degree of inactivation of oxygen evolving complex (OEC), (*3*) normalization between F_I and F_P was calculated as $W_{IP} = (F_t - F_I)/(F_P - F_I)$, and the horizontal dashed line at 0.5 indicates the half time needed to reduce the pool of the end electron acceptor with electrons donated by intermediate carriers, (*4*) normalization between F_O and F_I was calculated as W_{OI} ($\geq$1) = $(F_t - F_O)/(F_I - F_O)$; the maximum amplitude of IP phase illustrates the pool size of the end electron acceptors or the active PSI content. The P_{2G}, V_K/V_J, $t_{1/2}^{(I-P)}$, ΔV_{IP}, and $1/V_I$ parameters (Table 1) were displayed with the corresponding curves.

Isolation of thylakoid membrane complexes and total soluble protein: Thylakoid membrane complexes were isolated from the mid-portion of the rice leaves as described earlier (Kang *et al*. 2012). The Chl content was determined spectrophotometrically (*Cintra 1010, GBC scientific equipment*, Australia) in 80% acetone (Arnon 1949). Total soluble proteins were isolated from the rice leaves as described by Ku *et al*. (1999). Protein concentration was determined using the method of Lowry *et al*. (1951), with bovine serum albumin as a standard.

Blue native polyacrylamide gel electrophoresis (BN-PAGE) and immunodetection: BN-PAGE was carried out to separate thylakoid membrane complexes (Chen *et al*. 2007). For immunodetection, the isolated thylakoid membrane (for the thylakoid membrane peptide) and total soluble protein (for PEPC) were pretreated with the loading buffer (0.5 M Tris-HCl, pH 6.8, 1% SDS, 24% glycerol, 4% β-mercaptoethanol, and 0.001% w/v bromophenol blue) and denatured for 10 min at 90°C. Thylakoid membrane polypeptides [2 μg(Chl) per spot] or total soluble protein [15 μg(protein) per spot] were separated by 12% SDS-PAGE, and transferred to polyvinylidene difluoride membrane (*Bio-Rad*, Hercules, USA). After being blocked with TTBS buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.1% (v/v) Tween-20) containing 5% (w/v) nonfat dry milk, the membranes were developed with various primary antibodies directed towards PEPC, Lhcb1, Lhcb2, PsbO, PsbA/D1, PsbD/D2, Cyt *f*, Lhca1, PsaA, and AtpB polypeptides (*Agrisera,* Sweden, http://www.agrisera.com/). Then, the membranes were incubated with secondary antibody conjugated to alkaline phosphatase. Signals were visualized by using BCIP/NBT (*Roche*, Switzerland) as a substrate (Lindahl *et al*. 1996).

Quantitation of the band signal was performed using *Quantity One* (*Bio-Rad,* Hercules, USA) based on densitometric analysis. The experiments were repeated three times and the representative images were taken. The amount of the protein was expressed as the percentage of that in control WT.

Chloroplast ultrastructure was examined according to the method in Janacek *et al*. (2009). Samples were prefixed by vacuum infiltration with 4% (w/v) glutaraldehyde in the phosphate buffer (0.3 M, pH 7.4) at 4ºC for 6 h. They were post-fixed in 5% OsO_4 for 1 h and rinsed three times in H_2O for 10 min, followed by the graded dehydration in an ascending series of alcohols (20%, 50%, 70%, 90%, and three times 100% [v/v]). Then samples were embedded in the Epon812 resin and polymerized at 60°C for 24 h. Ultrathin sections were obtained with an *LKB-V* ultramicrotome (*LKB Ultrascan XL*, Bromma, Sweden) and collected on 200 mesh copper thin bar grids. Grids were then post-stained by uranyl acetate and lead citrate. The ultrastructure was visualized in a *Hitachi 600-A-2* transmission electron microscope (*Hitachi*, Tokyo, Japan) operating at 80 kV.

Gas exchange: The net photosynthetic rate (P_N), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were recorded using a portable infrared gas analyzer

(*CIRAS-2*, *PP Systems Ltd.*, Boston, USA). The middle sections of leaves still attached to plants were sealed in the leaf cuvette and the measurements were carried out under constant conditions of 1,200 μmol(photon) m^{-2} s^{-1}, 30°C, 66% of relative humidity, and 380 μmol(CO_2) mol^{-1}.

The rate of superoxide anion ($O_2^{\bullet-}$) production was determined by monitoring the nitrite formation from hydroxylamine in the presence of $O_2^{\bullet-}$ (Elstner and Heupel 1976). The absorbance was measured at 530 nm (*Cintra 1010, GBC scientific equipment*, Australia), and the formation rate of $O_2^{\bullet-}$ was calculated from the standard curve of sodium nitrite ($NaNO_2$) and expressed as μmol(nitrite) g^{-1}(FM).

Malondialdehyde (MDA), an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloroacetic acid, and the amount of MDA was determined by the thiobarbituric acid reaction (Heath and Packer 1968), and expressed as nmol g^{-1} (FM).

Statistical analyses: Statistical analyses were carried out using *SPSS 15.00* statistical package (*SPSS*, Chicago, USA). Parametric one-way analysis of variance (*ANOVA*) was used to determine statistical significance. Differences in the measured parameters were considered significant if $p<0.05$.

Results and discussion

Improvement of photosynthetic performance in PEPC-transgenic rice under drought stress: In previous studies, maize over-expressing PEPC increased water use efficiency under moderate drought conditions (Jeanneau *et al*. 2002). However, few researches exhibited that drought resistance had been improved by introducing a PEPC gene into rice. In this work, rice plants over-expressing PEPC showed a little dwarfed morphology compared to WT (Fig. 1S*A*, *supplement available online*) under normal water conditions. This could be attributed to the transformation, which causes metabolic perturbations and compensational changes in metabolic fluxes in rice (Jafarpour and Nulit 2011). Moreover, the T-PEPC rice remained green even after DS [Chl = 0.537 ± 0.019 mg g^{-1}(FM)], whereas WT plants showed more chlorosis [Chl = 0.332 ± 0.021 mg g^{-1}(FM), Fig. 1S]. Hence, overexpression of PEPC could alleviate the negative effect of DS on rice plants.

For quantifying the degree of DS experienced by plants, the water status of the plants was also measured as RWC (Fig. 1). Under normal conditions, the difference in RWC between WT and T-PEPC rice was minimal. However, under DS only T rice could maintain higher RWC (Fig. 1). It suggests a better water-preserving ability in the T-PEPC rice.

Chl *a* fluorescence kinetics (F_t) has been used as a very sensitive tool for monitoring changes in the physiological status of plants (Baker 2008); it is known to show a good correlation with RWC (Goltsev *et al*. 2012). We studied the impact of DS on photosynthetic machinery, using fluorescence analysis (Fig. 1). Both lines of rice plants showed a typical polyphasic rise, suggesting that all samples were photosynthetically active. No significant differences in fluorescence kinetics were observed between the two types under normal water conditions, whereas DS resulted in significant modification of the shape in WT with a more marked decrease in RWC (Fig. 1).

The photosynthetic performance index (PI_{total}, *see* definitions in Table 1), deduced from F_t, is the most sensitive JIP-test parameter for a wide range of stress conditions, and can be correlated with plant growth and survival rate (Yusuf *et al*. 2010, Oukarroum *et al*. 2012). It is related to individual photosynthetic components: PSII reaction center (RC)-density in the Chl bed [$\gamma_{RC}/(1-\gamma_{RC})$], the performance due to the quantum efficiency of primary photochemistry [$\varphi_{Po}/(1-\varphi_{Po})$], the performance due to the quantum efficiency of the conversion of excitation energy to electron transport [$\psi_{Eo}/(1-\psi_{Eo})$], and the performance due to the quantum efficiency of the reduction of end acceptors [$\delta_{Ro}/(1-\delta_{Ro})$] (Tsimilli-Michael and Strasser

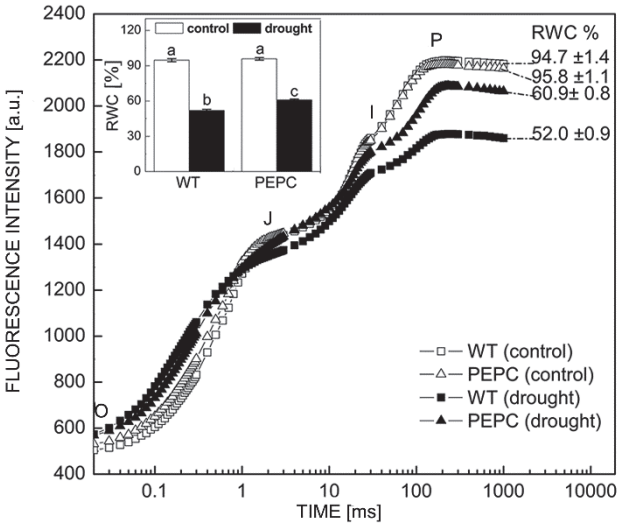

Fig. 1. The main panel shows fast chlorophyll fluorescence kinetics (OJIP, F_t) of dark-adapted leaves in wild type (WT) and PEPC-transformed (PEPC) rice under well-watered condition (control) and drought stress. Seedlings grown in commercial peat soil were irrigated regularly or withheld water for five days. The inserted panel shows the relative water content (RWC) of WT and T-PEPC rice. Data are means ± SE (n = 15). *Different letters* indicate statistically significant differences at $P<0.05$.

Table 1. Formulae and definitions of the selected JIP-test fluorescence parameters used in this study. Subscript "0" (or "o" when written after another subscript) indicates that the parameter refers to the onset of illumination, when all RCs are assumed to be open.

Fluorescence parameter	Definition
Original data extracted from the recorded fluorescence transient F_t	
$F_0 \cong F_{20\mu s}$	Minimal reliable recorded fluorescence at 20 μs, when all PSII RCs are open
$F_L \equiv F_{150\mu s}$	Fluorescence intensity at the L—step (150 μs)
$F_k \equiv F_{300\mu s}$	Fluorescence intensity at the K—step (300 μs)
$F_J \equiv F_{2ms}$	Fluorescence intensity at the J—step (2 ms)
$F_I \equiv F_{30ms}$	Fluorescence intensity at the I—step(30 ms)
F_p (= F_M)	Maximal recorded fluorescence intensity at the peak P of OJIP, when all PSII RCs are closed
t_{FM}	Time (in ms) to reach the maximal fluorescence intensity F_M
Area	Total complementary area between the fluorescence induction curve and F = F_M
Fluorescence parameters derived from the original data	
$V_t \equiv (F_t - F_0)/(F_M - F_0)$	Relative variable fluorescence at time t
$V_K = (F_K - F_0)/(F_M - F_0)$	Relative variable fluorescence at the K—step
$V_J = (F_J - F_0)/(F_M - F_0)$	Relative variable fluorescence at the J—step
$V_I = (F_I - F_0)/(F_M - F_0)$	Relative variable fluorescence at the I—step
$1/V_I = (F_M - F_0)/(F_I - F_0)$	The maximal amplitude of IP phase reflecting the relative size of the pools of final PSI electron acceptors
$\Delta V_{IP} = 1 - V_I = (F_M - F_I)/(F_M - F_0)$	The content of PSI reaction centers
V_K/V_J	A relative measure of inactivation of OEC
$t_{1/2}^{(I-P)} = (t_{FM} - 30)/2$	The time (in ms) needed for the half saturation of the final electron acceptors pools of PSI with electrons donated by intermediate carriers
$M_0 = 4\,(F_{300\mu s} - F_0)(F_M - F_0)$	Slope of the normalized curve at the origin of the fluorescence rise
$W_t = (F_t - F_0)/(F_J - F_0)$	
$W_{E,100\mu s} = 1 - (1 - W_{300\mu s})^{1/5}$	
$P_{2G} = (W_{E,100\mu s} - W_{100\mu s}) \times F_0/W_{100\mu s}\,/$ $(1 - W_{E,100\mu s})/(F_M - F_0)/V_J$	Overall grouping probability of PS II units
$\varphi_{Po} = TR_0/ABS = 1 - (F_0/F_M)$	Maximum quantum yield for primary photochemistry
$\psi_{Eo} = ET_0/TR_0 = 1 - V_J$	Probability that an electron moves an electron into the electron transport chain beyond Q_A^-
$\delta_{Ro} = RE_0/ET_0 = (1 - V_I) \times (1 - V_J)$	Probability that an electron from the reduced intersystem electron acceptors to the PSI end electron acceptors
$\gamma_{RC} = Chl_{RC}/Chl_{total} = V_J \times \varphi_{Po}/(M_0 + V_J \times \varphi_{Po})$	Probability that a PSII Chl molecule functions as RCs
$PI_{total} = [\gamma_{RC}/(1 - \gamma_{RC})] \times [\varphi_{Po}/(1 - \varphi_{Po})] \times$ $[\psi_{Eo}/(1 - \psi_{Eo})] \times [\delta_{Ro}/(1 - \delta_{Ro})]$	Performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors

2008, Yordanov *et al*. 2008). Hence, PI_{total} expresses the overall potential for energy conservation at the sequential energy bifurcations from absorption to the reduction of PSI end acceptors (Yusuf *et al*. 2010). For comparison, γ_{RC}, φ_{Po}, ψ_{Eo}, and δ_{Ro} were utilized to represent these PI_{total} components, respectively (Fig. 2). Our results showed that the PI_{total} was a little lower in T-PEPC rice (0.931 ± 0.024) than that in WT (1.164 ± 0.039) under normal water conditions. However, under DS condition, it was only reduced by 42.7% in T-PEPC rice compared with 82.3% in WT (Table 2). Similar trends were observed for individual PI_{total} components (γ_{RC}, φ_{Po}, ψ_{Eo}, and δ_{Ro}) as well (Fig. 2). These findings suggest that T-PEPC rice showed better resistance to DS due to better-operating PSII reaction center, primary photochemistry, electron transport, and the reduction of end acceptors.

Similar results can be visualized by the qualitative graphs with the self-derived numerical manifestation after double normalization of F_t (Fig. 3). Fluorescence rise during the first 0.3 ms (L-bands) indicates the energetic connectivity between PSII units (Strasser 1978), whereas rises during the first 2 ms (K-bands) reveal limitations on the donor side of PSII (Strasser *et al*. 2000). Changes in the L- and K-bands of the fluorescence transients suggest the vitality of leaves and the tolerance of plants to DS. Our results revealed a lower positive L-band in T-PEPC rice compared to WT under DS conditions (Fig. 3*A*), suggesting more energetic connectivity between independent PSII units in T-PEPC rice. This was also supported by the higher P_{2G} (overall grouping probability of PSII units, Fig. 3*A*) in T-PEPC rice under DS conditions. Results for K-bands were similar to that obtained for L-bands. Considering the dependence of K-bands on a partial uncoupling of OEC (Strasser *et al*. 2000), the inhibitory elevation of K-bands in T-PEPC rice under DS conditions (Fig. 3*B*) suggests that OEC escaped from a severe

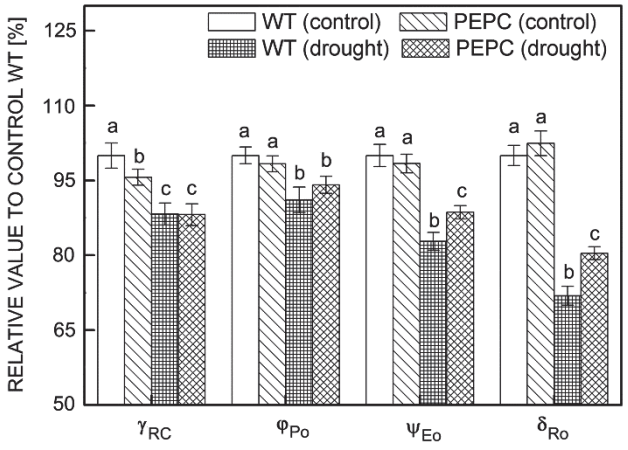

Fig. 2. The effect of drought stress on the components (γ_{RC}, φ_{Po}, ψ_{Eo}, and δ_{Ro}) of PI_{total} in wild type (WT) and PEPC-transformed (PEPC) rice under well watered (control) and drought conditions. They are expressed as relative values to the well-watered WT (with value = 100%). Data are means ± SE (n = 15), with *different letters* indicating statistically significant differences (P<0.05).

Table 2. The effect of drought stress on the total performance index (PI_{total}) in wild type (WT) and PEPC-transformed (T-PEPC) rice under well watered (control) and drought conditions. Data are means ± SE (n = 15), with *different letters* indicating statistically significant differences (P<0.05).

PI_{total}		
WT	Control	1.164 ± 0.039^{a}
	Drought	0.202 ± 0.006^{b}
T-PEPC	Control	0.931 ± 0.024^{c}
	Drought	0.533 ± 0.017^{d}

damage in the transgenic rice. The relative measure of OEC inactivation (V_k /V_J, Fig. 3*B*) was also higher in T-PEPC under DS as compared to WT. The qualitative differences in K-bands and L-bands further resulted in a higher efficiency of primary photochemistry of PSII (φ_{Po}, Fig. 2) in T-PEPC rice.

The IP phase of F_t is attributed to a bottleneck of electron flow from PSI to the final electron acceptors, *i.e.,* ferredoxin and $NADP^+$ (Schansker *et al*. 2005). W_{OI} and W_{IP} transients are used to clarify the IP phase of F_t. W_{IP} (Fig. 3*C*) indicates the relative variable fluorescence after double normalization of F_t between F_I and F_p. The rate constants of filling PSI acceptors with electrons from the intersystem carriers inverse to the time needed for the half saturation of the final electron pools of PSI, $t_{1/2}^{(I-P)}$, which is indicated by the horizontal dashed line in W_{IP} (Yusuf *et al*. 2010). A similar decline in $t_{1/2}^{(I-P)}$ was observed under DS in both lines (Fig. 3*C*), suggesting that the electron affinity of PSI was comparable in both lines. In addition, the maximum amplitude in W_{OI} (Fig. 3*D*) indicates the relative size of final PSI electron acceptor pools (Tsimilli-Michael and Strasser 2008, Jiang *et al*. 2008) or the active PSI content (Oukarroum *et al*. 2009), which are quantified by $1/V_I$ and ΔV_{IP}, respectively. The higher maximum amplitude in W_{OI} suggests that T-PEPC rice had a bigger pool size of PSI end electron acceptors or higher content of active PSI than WT under DS (Fig 4*D*).

Enhanced stability of thylakoid membrane proteins in T-PEPC rice: The stability of Chl-protein complexes in thylakoid membranes is severely affected by DS (Tian *et al*. 2013). To confirm greater stability of thylakoid membrane proteins in DS-treated T-PEPC rice, the subunit abundance and assembly status of thylakoid membrane complexes were determined by immunodetection (Fig. 4) and BN-PAGE analyses (Fig. 2S, *supplement available online*). BN-PAGE can separate thylakoid multi-subunit complexes in their native form with high resolution (Takabayashi *et al*. 2009). Although PSII supercomplexes were not observed clearly in both lines (Fig. 2S) as in other rice cultivars (Shao *et al*. 2011), we observed a lesser decrease in PsbA/D1 and PsbD/D2 (PSII core subunits) in T-PEPC rice during DS compared to WT (Fig. 4). As these subunits are responsible for the primary charge separation (Renger and Holzwarth 2005), they proved molecular basics for the strengthened quantum efficiency of primary photochemistry in PSII (φ_{Po}, Fig. 2) in the T-PEPC rice. The LHCII trimer, comprising Lhcb1, Lhcb2, and Lhcb3 subunits, can collect light energy for photosynthesis and dissipate excess excitation energy in response to environmental changes (Fan *et al*. 2011). The DS-induced loss of trimeric LHCII can be attributed to both the dissociation of the multi-subunit complexes and to the degradation of the intrinsic subunits. Our results revealed that the T-PEPC rice inhibited the loss of the functional LHCII trimer better than WT under DS (Fig. 2S). It resulted from the maintenance of Lhcb1 and Lhcb2 subunits (Fig. 4). In addition, PsbO of OEC were damaged more severely in WT after DS treatment (Fig. 4), which was consistent with the pronounced K-bands results (Fig. 3*B*). Thus, it can be concluded that PEPC endowed the transgenic rice with tightly grouped units in PSII under DS.

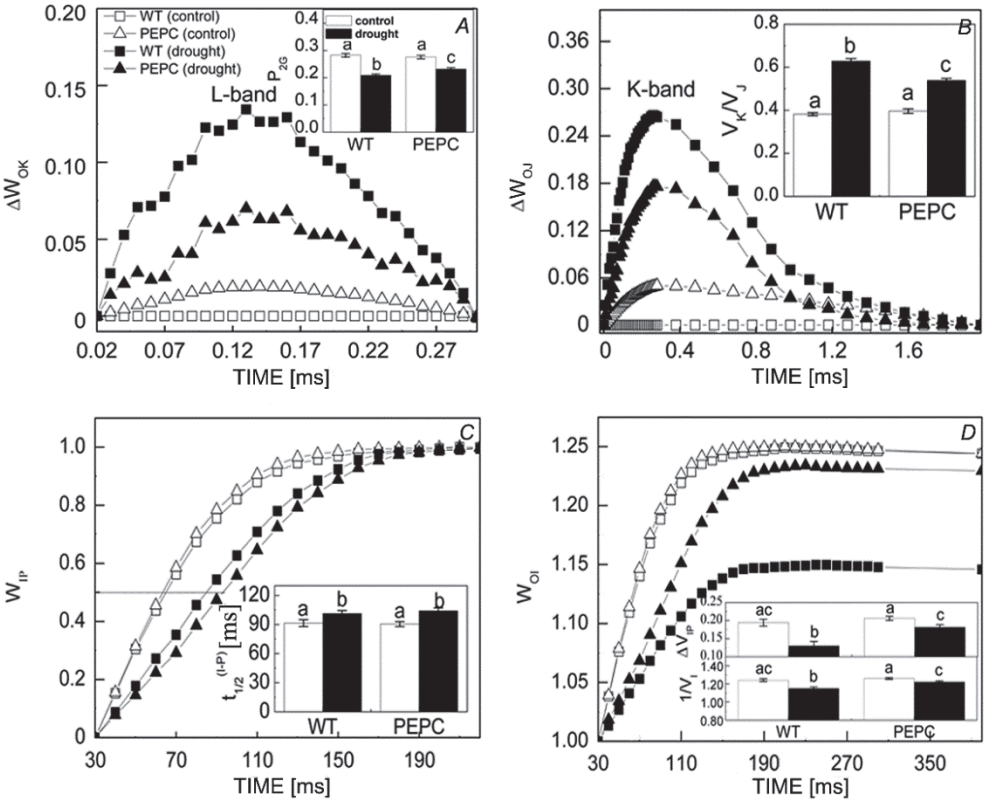

Fig. 3. The main panel shows differential curves after double normalization of mean F_t between different time points (*see* details in Materials and methods). Each curve represents the mean of 15 independent transients. (*A*) The difference kinetics between F_O and F_K to reveal L-bands: $\triangle W_{Ok}$. (*B*) The difference kinetics between F_O and F_J to reveal K-bands: $\triangle W_{OJ}$. (*C*) Normalization between F_I and F_P: W_{IP} and the horizontal *dashed line* indicates half time needed to reduce pool of the end electron acceptor with electrons donated by intermediate carriers. (*D*) Normalization between F_O and F_I: W_{OI} (≥1), and the maximum amplitude of IP phase can illustrate the differences in the pool size of the end electron acceptors or the active PSI content. In the inserted panel, numerical manifestations (P_{2G}, V_K/V_J, $t_{1/2}^{(I-P)}$, ΔV_{IP}, and $1/V_I$) for the corresponding curves were provided additionally. Data are means ± SE (n = 15). *Different letters* within each row indicate statistically significant differences (P<0.05).

The enrichment of PSI-LHCI supercomplexes and LHC-less PSI core also decreased less in T-PEPC rice under DS (Fig. 2S), in conjunction with a slower collapse of PSI subunits PsaA and Lhca1 (Fig. 4), suggesting that PSI in the transgenic rice was similarly more tolerant to DS.

Cytochrome b_6/f complex participates in electron transfer between PSI and PSII (Fan *et al*. 2011). Over-expression of PEPC in rice partly counteracted the reduction of Cyt *f* content, a cytochrome b_6/f subunit (Fig. 4). It provides an evidence for the well-preserved electron transport between PSI and PSII in transgenic rice under stress.

Overall, our results showed a good structure-function relationship between the photosynthetic proteins and F_t analyses. The integrity and components of thylakoid multi-subunit complexes involved in the light reaction remained relatively unaffected in T-PEPC rice under DS. Over-expression of PEPC in rice protected the photosynthetic apparatus from DS injury.

Oxidative damage on individual photosynthetic components under DS: The closure of stomata induced by drought limits the diffusion of CO_2 to chloroplasts (Haupt-Herting and Fock 2000). This resulted in significant reduction in the g_s and C_i in both rice lines (Fig. 5*B*,*C*). Because CO_2 deficiency can further increase ROS production by the Mehler reaction (Hoffmann *et al*. 2005, Oukarroum *et al*. 2009), the rate of $O_2^{\bullet -}$ production on the acceptor side of PSI was pronounced under DS in our study (Fig. 5*D*). The resulting oxidative stress develops as a secondary effect to potentially damage the photosynthetic machinery (Ort 2001, Chaves and Oliveira 2004). Thus, the decrease in P_N (Fig. 5*A*) under DS was a combined effect of both stomatal (a slowdown of the Calvin cycle due to CO_2 deficiency) and nonstomatal (a slowdown of photosynthetic electron transport chain due to oxidative stress) limitations.

Furthermore, ROS produced by the Mehler reaction preferentially attack PSI, and PSI is assumed to be more sensitive to stresses due to vicinal ROS (Dat *et al*. 2000, Mittler 2002). Previous studies have reported that DS has relatively little effect on PSII compared with PSI, and the ratio of PSI/PSII decreases with DS (Souza *et al*. 2004, Oukarroum *et al*. 2009).

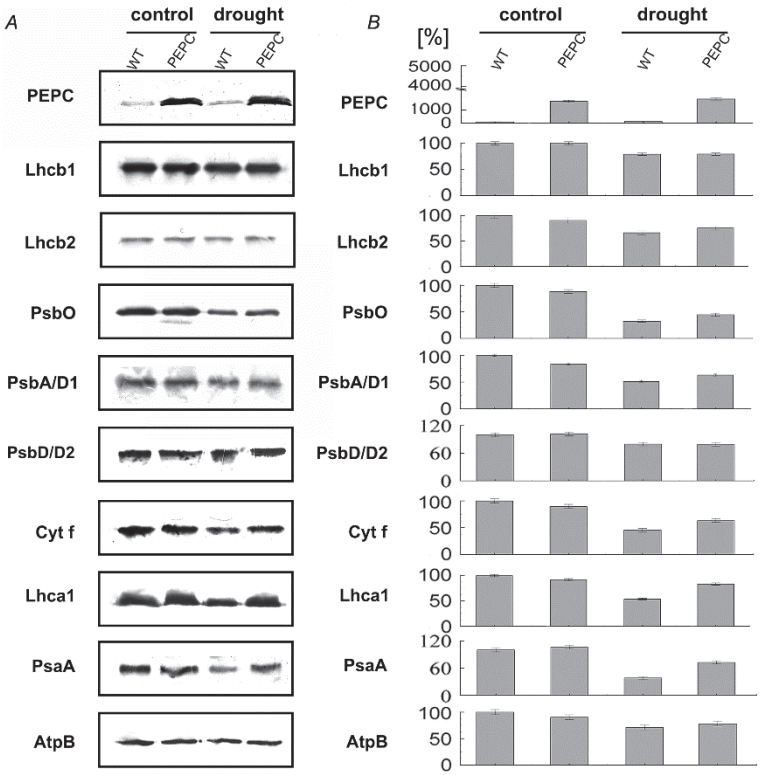

Fig. 4. Immunodetection of the PEPC protein and the peptide composition of the thylakoid membrane complexes isolated from wild type (WT) and PEPC-transformed (PEPC) rice under well-watered (control) and drought conditions. (*A*) Thylakoid membrane proteins [2 μg(Chl) per slot] or total soluble protein [15 μg(protein) per slot] were separated by SDS-PAGE, and immunodetected with the antisera against PEPC, Lhcb1, Lhcb2, PsbO, PsbA/D1, PsbD/D2, Cyt *f*, Lhca1, PsaA, and AtpB. (*B*) Quantitation of the immunoblot signal. The abundance of each peptide from three independent experiments was measured by the optical density (*Quantity One*, *Bio-Rad*, USA). Data are calibrated by the amount in well-watered WT (100%).

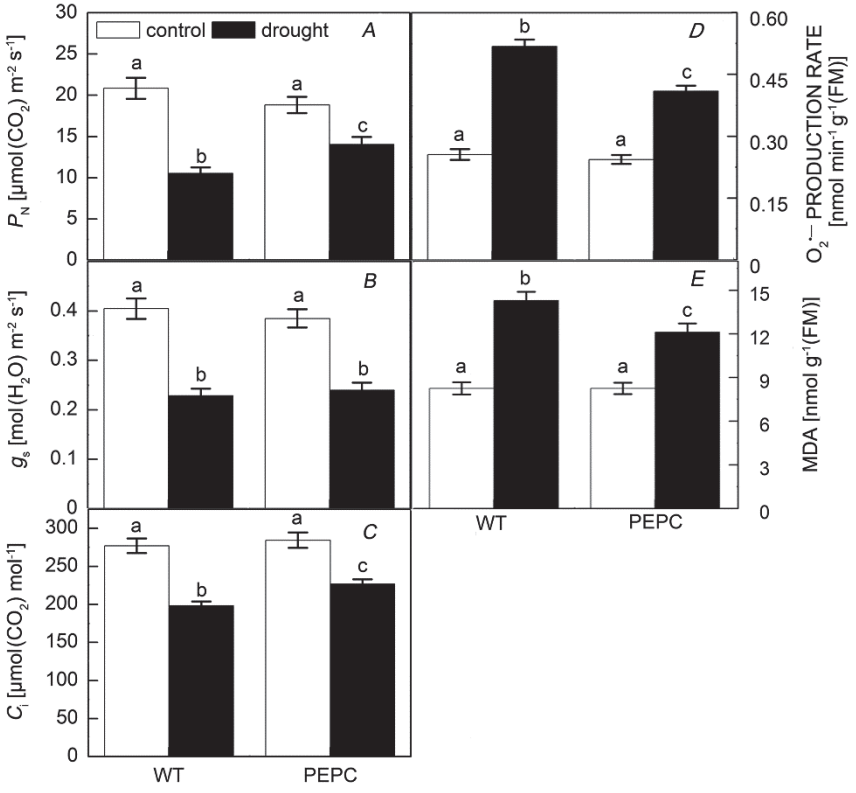

Fig. 5. The effect of drought stress on the (*A*) net photosynthetic rate (P_N), (*B*) stomatal conductance (g_s), (*C*) intercellular CO_2 concentration (C_i), (*D*) superoxide anion production rate ($O_2^{\bullet-}$), and (*E*) malondialdehyde (MDA) content of wild type (WT) and PEPC-transformed (PEPC) rice under well-watered (control) and drought conditions. Data are means ± SE (n = 5), with *different letters* indicating statistically significant differences (P<0.05).

Additional analysis of PI_{total} components (γ_{RC}, φ_{Po}, ψ_{Eo}, and δ_{Ro}) aids in further evaluating the DS sensitivity of different sections of the photosynthetic electron-transfer chain. These parameters reflect the relative concentration of the reaction center Chl (γ_{RC}) and the quantum yields or efficiencies of photo-induced electron transfer from P680

to Q_A (φ_{Po}), from Q_A^- to plastoquinone (ψ_{Eo}), and from reduced plastoquinone to the PSI electron acceptors (δ_{Ro}) (Goltsev *et al*. 2012). According to their sensitivity to water deficit, PI_{total} components were ranked in descending order as $\delta_{Ro} > \psi_{Eo} > \gamma_{RC} > \varphi_{Po}$ in both lines (Fig. 2). This suggests that light-reaction apparatus was blocked at various degrees by a "traffic jam" of electrons, and the rate-limiting step was from plastoquinone to the PSI final electron acceptor.

DS-induced degradation of the thylakoid membrane proteins also confirmed the progressive dysfunction along the photosynthetic electron transport chain in both lines. The impairment of PSII subunits (Lhcb1, Lhcb2, PsbA/D1, and PsbD/D2, Fig. 4) was within an acceptable range, in agreement with the good performance of γ_{RC} and φ_{Po} values under DS (Fig. 2). However, the PSI subunits (PsaA and Lhca1, Fig. 4) were more vulnerable to DS than other photosynthetic components (Fig. 2).

Evidence from the chloroplast ultrastructure further supported this uneven injury. It is well known that the LHCII trimer associated with PSII are responsible for the formation of grana, while PSI complexes dominate stroma lamellae (Finazzi *et al*. 2002). The thylakoid interconnections were more severely disrupted in stromal than in granal thylakoids under DS (Fig. 6), suggesting that PSII could be more tolerant to DS than PSI.

Amelioration of DS-induced oxidative damage in T-PEPC rice: Although there was no obvious improvement of P_N by introduction of PEPC into C_3 plants under normal conditions, the photosynthetic capacity of the transgenic plants seems to be more tolerant to high temperatures (Kogami *et al*. 1994) and high light intensities (Jiao *et al*. 2002). The lack of decline in the quantum yield for CO_2 assimilation with stresses involves a refixation of respired CO_2 in the PEPC-transgenic plant (Jafarpour and Nulit 2011). In our results, as illustrated by the immunoblot, a high-level expression of maize PEPC proteins was observed in the T-PEPC rice, even under DS (Fig. 4). In contrast, no obvious accumulation of PEPC proteins was found in WT. Although Taniguchi *et al*. (2008) have shown that maize PEPC is not properly phoshorylated and active in the transgenic rice during the day, and a single C_4 enzyme can not induce an effective C_4 cycle (a C_4-like CO_2 pump), the enrichment of PEPC proteins still could refix respired CO_2 into organic acids that are in turn decarboxylated in the citric acid cycle and lead to an increase in CO_2 (Jafarpour and Nulit 2011). Hence, overexpression of PEPC protein in Kitaake rice also effectively participated in the CO_2 refixation and ameliorated the extent of C_i decrease under DS (Fig. 5*C*).

The oxidative stress caused by salinity in two barley cultivars is mitigated by elevated CO_2 (Pérez-Lopéz *et al*. 2009). To testify whether the higher C_i can protect the transgenic rice from oxidative damage under DS, our studies compared the rate of $O_2^{\bullet -}$ production and the content of MDA in both rice lines. Because of the higher C_i, the rate of $O_2^{\bullet -}$ production (Fig. 5*D*) was reduced in T-PEPC rice under DS. MDA is the decomposed product of polyunsaturated fatty acids in biomembranes, and its formation is routinely used as a general indicator of the extent of lipid peroxidation resulting from oxidative stress (Singh *et al*. 2006, Monteiro *et al*. 2009). The DS-promoted accumulation of MDA was also alleviated in T-PEPC rice due to less ROS (Fig. 5*E*).

The MDA content is an indicator of oxidative injury to biological membranes, and well correlates with the degradation of thylakoid membranes due to lipid peroxidation (Fodor 2002, Monteiro *et al*. 2009). Therefore, the chloroplast ultrastructure provided an additional evidence for the protective mechanism of the T-PEPC rice against oxidative damage. Although the regular thylakoid network disintegrated in both lines as a consequence of DS, the extent of damage was lesser in T-PEPC rice (Fig. 6), which accumulated less MDA. In addition, the number of plastoglobules is known to increase in plants subjected to the environmental stress, which cause oxidative damage on the photosynthetic apparatus (Austin *et al*. 2006). In our study, we found lesser plastoglobules in T-PEPC rice under DS (Fig. 6), suggesting that overexpression of PEPC should protect the chloroplast ultrastructure from oxidative damage.

In short, the C_4 enzyme could prevent P_N (Fig. 5*A*) and photosystems from the nonstomatal limitation (oxidative damage on the light-reaction apparatus) rather than from

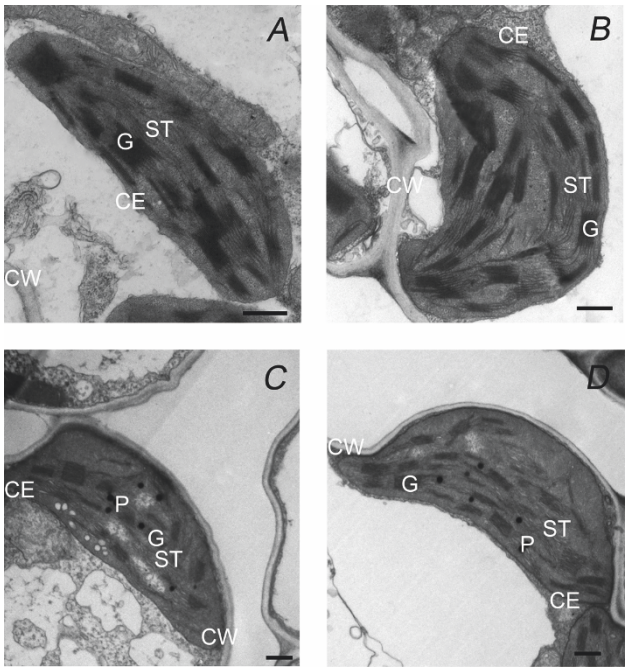

Fig. 6. Chloroplast ultrastructure of wild type (WT) under (*A*) well-watered, and (*C*) drought conditions, and PEPC-transformed (PEPC) rice under (*B*) well-watered, and (*D*) drought conditions. Scale bar = 5 μm. G – granal thylakoids; P – plastoglobule; CW – cell wall; CE – chloroplast envelope; ST – stromal thylakoids; V – vesicles.

stomatal limitation (no significant differences in g_s between both lines, Fig. 5*B*). The peptides, protein complexes, and ultrastructure of thylakoid membranes (Figs. 4, 2S, 6) suffered less oxidative damage in the T-PEPC rice under DS.

Conclusion: In summary, drought stress imposed oxidative stress on the photosynthetic apparatus in a site-dependent manner, with the down-regulation of PSI being the main factor. Over-expressed PEPC refixed internal CO_2 and reduced oxidative damage to the photosynthetic apparatus, thus improving drought tolerance in transgenic rice. However, photosynthetic responses to water deficit were complex, involving the interplay of different factors. Therefore, further studies on regulatory mechanism underlying the drought resistance of T-PEPC rice are needed. It would provide greater insights into crop improvement by genetic manipulation.

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