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Adaptation of photosynthesis to water deficit in the reproductive phase of a maize (*Zea mays* L.) inbred line

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Abstract

Photosynthesis is sensitive to water deficit (WD) stress. Maize (*Zea mays* L.) yield is vulnerable to water stress, especially if it occurs during the reproductive stage. In this study, the expression patterns of photosynthesis-related genes, together with photosynthetic gas-exchange and fluorescence parameters were investigated in a maize inbred line exposed to 50% of field water capacity (moderate WD) for 15 d after tassel emergence. The results demonstrated that WD down-regulated expression of *psbA, psbB, psbC, psbP, psaA, psaB*, and *cab*, especially at later periods of WD stress. Besides, with the increased WD stress, the steady decline in the value of photosynthesis performance index, maximum quantum yield of primary photochemistry, quantum yield for electron transport, quantum yield for the reduction of end acceptors of PSI per photon absorbed, and the efficiency of an electron beyond Q_A^- that reduced PSI acceptors, and a clear increase in the J-step and I-step, K-band as well as L-band were observed. The results suggested that WD might restrict light-harvesting and electron transport. Interestingly, leaf transcript levels of *rbcL* and *rbcS* were up-regulated at the later stage of water stress in maize inbred line, which helped repair injury to PSII centers and maintain PSII activity (increased quantum yield of dissipation and effective antenna size of an active reaction center) under 15-d lasting WD.

Additional key words: biomass; OJIP transients; photosynthetic capacity; tassel emergence; withholding water.

Introduction

Water deficit (WD) is one of the world's most widespread climatic disasters, affecting agricultural production and therefore influencing world food security (Liu *et al.* 2012). Maize (*Zea mays* L.) is one of the most important food crops, however, it is vulnerable to WD stress. WD stress has been reported to reduce maize yield approximately by 40% or more, especially during its reproductive phase. With climate change, WD is predicted to reduce the maize yield more significantly in the future (Daryanto *et al.* 2016).

Photosynthesis is a complex process that involves light energy, light absorption, energy conversion, electron transfer, ATP synthesis, and CO_2 fixation. Reduced photosynthetic ability decreases a yield potential (Gilbert and Medina 2016) in crops under WD. Therefore, it is important to study photosynthesis in crops under stress to meet future global food demand (Long *et al.* 2015). Many studies have shown that WD could cause changes in photosynthesis at physiological and molecular levels. In addition to stomatal limitations, other photosynthetic processes may also reflect co-limited photosynthetic rate (P_N). Under moderate or severe WD stress, nonstomatal

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Abbreviations: : ABS/RC – effective antenna size of an active reaction center; Chl – chlorophyll; C_i – intercellular CO_2 concentration; CK – control; DI_O/RC – trapped energy flux per reaction center; FC – field capacity; F_0 – minimal fluorescence yield of the dark-adapted state; F_M – maximal fluorescence yield of the dark-adapted state; g_s – stomatal conductance; OEC – oxygen-evolving complex; P_N – net photosynthetic rate; PI_{ABS} – performance index for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors; RCs – reaction centers; T – days of treatment; TR_0/RC – quantum yield of dissipation; WD – water deficit; W_{OK} – relative variable fluorescence for the normalization between F_O and $F_{300\mu s}$; W_{OJ} – relative variable fluorescence for the normalization between F_O and F_J; V_{OP} – relative variable fluorescence at any phase of the fluorescence induction curve; φ_{Po} – maximum quantum yield of primary photochemistry; φ_{Eo} – quantum yield for electron transport; φ_{Ro} – quantum yield for the reduction of end acceptors of PSI per photon absorbed; ψ_O – the efficiency of an electron beyond Q_A- that reduced PSI acceptors.
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limitations became crucial (Ye *et al.* 2008, Mo *et al.* 2016), the activity of enzymes related to the Calvin cycle were down-regulated, and the photosynthetic rate was restricted. After long-term WD stress, the performance of PSII is reduced and electron transport is blocked, resulting in down-regulation of photosynthesis in mulberry and wheat leaves (Guha *et al.* 2013, Živčák *et al.* 2013, Ye *et al.* 2013) in response to the adverse environment. Furthermore, at the molecular level, WD affects the levels of some mRNAs and proteins of PSII and PSI (Yuan *et al.* 2005). For example, transcription rates of *psbA* and *cab,* which encode the D1 and the LHCII of PSII, respectively, are reduced dramatically under osmotic stress in barley and wheat cultivars, further reducing electron transport (Yuan *et al.* 2005, Liu *et al.* 2006). The light-harvesting proteins, CP47 and CP43, as well as *psaB* of PSI are down-regulated under WD stress, thereby reducing the activities of the PSII electron transport and light-harvesting complexes (Muhammad Salman *et al.* 2016). The *psbP* protein (23 kDa) is essential for the regulation and stabilization of PSII (Ifuku *et al.* 2005), decreasing amounts of expressed *psbP* protein led to the progressive loss of variable fluorescence and a marked decrease of F_V/F_M (Yi *et al.* 2007). Although WD could result in damage to the photosynthetic apparatus, plants have evolved several mechanisms to protect it from injury. For example, up-regulation of *rbcL* and *rbcS* to maintain photosynthesis capacity has been reported (Fu *et al.* 2007, Xu *et al.* 2013). However, down-regulation of *rbcL* and *rbcS* (Seki *et al.* 2002, Yuan *et al.* 2005, Hansen *et al.* 2013), Rubisco, and other enzymes involved in photosynthesis (Parry *et al.* 2002) have been reported. These contrasting results may reflect the fact that photosynthetic responses to WD stress are influenced by species, stress intensity, and stress duration (Živčák *et al.* 2013).

Research has indicated that the PSII photochemical efficiency of maize is inhibited by WD during the reproductive phase, resulting in a higher rate of kernel and ear abortion (Kakumanu *et al*. 2012). Furthermore, under long-term WD conditions, a dramatic decrease in photosynthetic activity led to a more significant decline of growth and yield (Hayano-Kanashiro *et al.* 2009, Guha *et al.* 2013, Anjum *et al*. 2016). Photosynthesis genes play an important role in adaptation of photosynthesis to WD in the reproductive phase. But there are few studies on expression of photosynthesis-related genes in response to long-term WD stress. Therefore, in this study, we assessed chlorophyll (Chl) *a* fluorescence measurements combined with leaf transcript levels of genes related to photosynthesis processes (*i.e.*, light-harvesting, electron transport, and carbon assimilation) involved in the WD-stress response. The results of this study provide insight into photosynthetic regulatory mechanisms that act in response to WD stress in the reproductive stage of maize.

Materials and methods

Plant materials and growth conditions: This study was conducted as a potted plant experiment under an auto-rain shelter from June 24 to October 3, 2015 at Henan Agricultural University in Zhengzhou, China. Experiments were performed using maize (*Zea mays* L.) inbred line HZ4, which was germinated in plastic trays containing a soil:compost (2:3) mixture. One week after germination, uniform seedlings with a single stem were selected and transplanted into plastic pots (35 cm diameter × 30 cm high) filled with 20 kg air-dried clay loam [soil water content of 36.4%; 8.1 g(organic matter) kg^{-1}; 61.8 mg(N) kg^{-1}; 22.4 mg(P) kg^{-1}; 134.1 mg(K) kg^{-1}]. These plants were treated with 12 g of compound N–P_2O_5–K_2O fertilizer (N, 25%; P_2O_5, 18%; K_2O, 12%). Finally, 3 g of urea was added to each pot at the bell stage (Zhao *et al.* 2013).

WD stress: Maize plants were watered daily to 75% field capacity (FC) before stress application. After tassel emergence, plants were either watered at 75% FC (control, CK) or subjected to moderate WD stress at 50% FC (Zhang *et al.* 2015) for 15 d. Each pot was weighted at about 18:00 h every day to determine whether the water was needed for maintaining the required water stress levels (Zheng and Yan 2006). Maize plants were then sampled after 0, 5, 7, 9, 11, 13, and 15 d (T) following initiation of WD stress to examine leaf gas exchange and Chl *a* fluorescence parameters. Healthy and undamaged ear leaves were excised from sample plants, frozen in liquid N_2, and stored at –80°C for further analysis. After 15 d, WD-treated maize plants were restored to the control level of watering. The shoot biomass of CK and WD plants were measured after maize plants were harvested. Four biological replicates of each treatment were measured for physiological parameters, and three biological replicates were collected for measuring the expression patterns of photosynthesis-related genes at T0, T5, T7, T9, T11, T13, and T15.

Leaf gas-exchange parameters: Photosynthetic rates (P_N), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) of the ear leaves of maize plants were measured between 11:00–13:00 h with a hand-held leaf photosynthesis system (*CI-340, CID,* Camas*,* WA*,* USA) at T0, T5, T7, T9, T11, T13, and T15 according to the manufacturer's instructions (Syuhada *et al.* 2014).

OJIP transient analysis: Chl *a* fluorescence OJIP transients were measured using a plant efficiency analyzer (*Handy-PEA, Hansatech Instruments Ltd*., UK) according to the methods reported by (Zhang *et al*. 2011) at 11:00–11:30 h at T0, T5, T7, T9, T11, T13, and T15 using the same leaves used for leaf gas-exchange parameters. Clips were attached to maize plants, allowed to adapt under dark conditions for 25 min, and then used for 5-min measurements (Zhang *et al*. 2011). The rise in OJIP transients was analyzed using the JIP-test (Panda *et al*. 2006, Strasser *et al*. 2010). Chl *a* fluorescence transients were double-normalized (between F_O and F_M) and presented as the kinetics of relative variable fluorescence at any time, $V_{OP} = (F_T - F_O)/(F_M - F_O)$. Subsequent normalizations and subtractions were carried out to determine the different kinetic profiles, using the following formula: $\triangle V_{OP} = V_{OP}$ (treatment) – V_{OP} (control) (Strasser *et al*. 2007). The relative fluorescence between steps O and K [W_{OK} =

Gene	Gene description	Sequence [5'-3']	Tm [ºC]
actin		F: CTGAACCCCAAGGCAAACA	59.0
		R: ACTGGCGTACAGGGAAAGAA	57.3
psbA	PSII protein D1	F: GGTATTCGTGAGCCTGTTTCTG	59.1
		R: GACCGCCATTGTATAACCATTC	58.9
psbB	PSII CP47 chlorophyll apoprotein	F: AGGCGTAACGGTGGAGTTCTAT	59.9
		R: GCAAAGGTAGCATGACCAAAAG	59.5
psbC	PSII CP43 chlorophyll apoprotein	F: TTTTGGGACCTTCGTGCTC	58.2
		R: ACAAAATGGGAGGTCGCTAA	57.4
psbP	Chloroplast oxygen-evolving complex/thylakoid lumenal protein	F: TCAGGCTCCAAACTACACCAG	57.7
		R: CTATCCTTCATCTTTTCCCACC	57.4
psaA	PSI P700 apoprotein A1	F: TCACCACTTAGCGGGATTATTA	57.3
		R: TTAGGATCAACCCCAGCGTC	59.8
psaB	PSI P700 apoprotein A2	F: ACGCCGAATCTCGTCTGAAT	59.6
		R: CCGGGAATAGCAACATGAACT	59.3
rbcL	Rubisco large subunit	F: CCGTTTCGTCTTTTGTGCC	58.9
		R: TGCGGTGAATCCTCCTGTT	58.3
rbcS	Rubisco small subunit	F: CGCTACTGGACCATGTGGAA	59.0
		R: ACTGCGTCTGCTTGATGTTGT	58.1
cab	Light harvesting chlorophyll *a*/*b* binding protein	F: CAACATGATGGATGGCTTCTACA	59.8
		R: GCTCGCATTGGAACGATTTT	60.0
rca	Ribulose-1,5-bisphosphate carboxylase/oxygenase	F: CAACATGATGGATGGCTTCTACA	60.1
		R: GCTCGCATTGGAACGATTTT	60.3

Note: The primers were designed according to sequences homology among published sequences of various photosynthetic genes in maize in *GeneBank* in our study. F – forward primer; R – reverse primer.

$(F_T - F_O)/(F_K - F_O)$], and between steps O and J [W_{OJ} = $(F_T - F_O)/(F_J - F_O)$] were normalized and displayed as $\triangle W_{OK} = W_{OK}(WD) - W_{OK}(CK)$ and $\triangle W_{OJ} = W_{OJ}(WD) - W_{OJ}(CK)$ at different treatment times, which made the L-band [$(F_{100\mu s} - F_O)/ (F_{300\mu s} - F_O)$] and K-band [$(F_{300\mu s} - F_O)/(F_J - F_O)$] visible, respectively (Jiang *et al.* 2006, Meng *et al*. 2016, Sarkar and Ray 2016). The phases of OJIP curves reflect the different reduction processes of the electron transport chain (Strasser *et al*. 2004, Schansker *et al.* 2005). The J-step, I-step, and IP phase of OJIP transients correlate with the redox states of the primary quinone acceptor of PSII (Q_A), the redox states of plastoquinone, and the redox states of end acceptors of PSI, respectively (Lin *et al.* 2009). The OJIP Chl fluorescence transient is not only due Q_A reduction and the JIP test, other processes also contribute to the transients (Lazár 2006). In order to gain further insights into the mechanisms underlying WD-induced changes, we further quantified OJIP fluorescence transients in PSII to determine structural and functional information. Descriptions and equations for all parameters (Strasser *et al.* 2010, Stirbet 2011, Goltsev *et al*. 2012, Gomes *et al.* 2012) are shown in Table 1S (*supplement*).

RNA extraction and real-time RT-PCR: Real-time RT-PCR was used to determine changes in gene expression. Total RNA and cDNA synthesis were conducted using *RNAiso Plus* and the *PrimeScript™ RT Reagent* kit with *gDNA Eraser* (*AMV First Strand cDNA Synthesis* kit) (*Sangon Biotech Co., Ltd,* Shanghai, China) according to the manufacturer's protocols. Quantitative real-time (qRT)-PCR was performed using the *SG Fast qPCR Master Mix* (2×) kit (BBI) on a *Light Cycler 480* machine (*Roche Diagnostics,* Germany) according to the manufacturer's instructions. The qRT-PCR amplification mixture (20 µl) contained 2 µl of cDNA, 10 µl of *SYBR Premix Ex Taq II* (*Tli RNaseH Plus*), and 0.4 µl of forward and reverse primers. Actin was used as a reference gene for data normalization. The gene ID of actin was 100281811. Relative transcript levels were calculated using the $2^{-\triangle\triangle Ct}$ method. Each data point is expressed as the average ± SD of three independent replicates. Special primers were designed using *Primer Premier 5.0*. Primers used to determine the transcript levels of *psbA, psbB, psbC, psbP, psaA, psaB, rbcL, rbcS, cab,* and *rca* are shown in the text table (on the top of the page).

Statistical analysis: Data was analyzed using one-way analysis of variance (*ANOVA*) in *SPSS* (*version 19.0*) and *Duncan*'s multiple range test to determine significant (p<0.05 or p<0.01) differences between treatments. Data were expressed as averages ± standard deviation (SD) of independent replicates.

Results

Effect of WD stress on leaf gas-exchange parameters: Continuous WD stress resulted in loss of leaf water and a 23% decline of shoot biomass compared with CK (Table 1), simultaneously, the maize yield decreased to 18.93 g per plant compared with the CK conditions. In addition, we

Table 1. The maize yield and shoot dry mass after postharvest under the control conditions (CK) and continuous water deficit (WD). Data are the means ± standard deviation (SD) of four replicates. * and ** represent significant differences between CK and WD treatments according to *Duncan*'s multiple range test at $p<0.05$ and $p<0.01$, respectively.

Treatment	Yield [g per plant]	Shoot dry mass [g per plant]
CK	71.2 ± 0.37	189.9 ± 17.5
WD	50.3 ± 0.34**	146.0 ± 12.4*

found that continuous WD stress conditions could result in plant phenotypic alterations (Fig. 1S, *supplement*), including reduced plant height and inhibited growth compared to corresponding controls under continuous WD stress conditions. This might be why the postharvest shoot biomass after the WD treatment was significantly lower than that of CK. In addition, the exposure of maize to progressive drought stress resulted in a decrease in relative water contents compared with the CK conditions, and the decrease was enhanced with increased drought duration. The relative water contents of maize ear leaf dropped at T15 from 92.3 (T0) to 77.5% (Fig. 1*A*).

There were no significant differences in P_N between treatments under the CK conditions. The gs was improved with increasing time under CK conditions; at T9, T11, T13, and T15, the values were all significantly higher than that of T0 and T5. However, under the WD, P_N and g_s at T5 were not significantly different from T7, but they were significantly different from T9, T11, T13, and T15. Compared with corresponding CK, WD samples at T5, T7, T9, T11, T13, and T15 exhibited reductions of 3.7, 9.2, 15.4, 24.5, 33.8, and 41.7% in P_N, respectively, with concurrent reductions of 10.4, 12.7, 27.6, 39.5, 53.7, and 57.9% in g_s, respectively (Fig. 1*B,C*). The C_i values of WD-stressed maize exhibited less severe reductions compared with the corresponding CK, with values declining by 8.2, 9.4, 3.7, 5.6, 6.5, and 6% at T5, T7, T9, T11, T13, and T15, respectively (Fig. 1*D*).

Effect of WD on OJIP transients and PSII parameters: In order to analyze further the photosynthesis adaptation, OJIP transients were measured. Chl *a* fluorescence transient curves (V_{OP} curves) exhibited a typical polyphasic rise during the basic steps of O-J-I-P (Fig. 2). Heterogeneity in the OJIP transients significantly increased with WD treatment (Fig. 2). However, the fluorescence intensity showed no significant changes at different treatment times in the CK group (Fig. 2). Under the WD, the relative fluorescence of L (0.15 ms) and K (0.3 ms) bands as well as the value of the $\triangle V_{OK}$ and $\triangle V_{OJ}$, increased with prolonged WD duration,

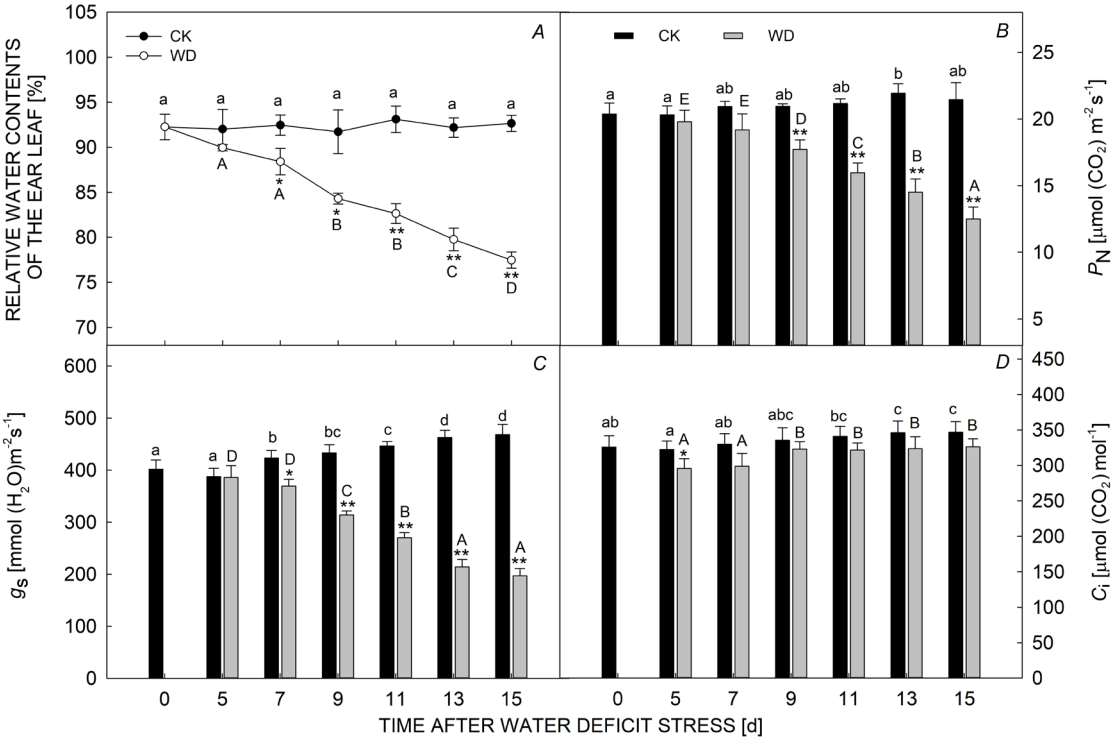

Fig. 1. Effects of continuous water-deficit stress on (*A*) ear leaf relative water content (*B*) photosynthetic rate (P_N), (*C*) stomatal conductance (g_s), and (*D*) intercellular CO_2 concentration (C_i) of reproductive phase of maize. Maize was treated as follows: the control conditions (CK) and continuous water deficit (WD). T0, T5, T7, T9, T11, T13, and T15 represent 0, 5, 7, 9, 11, 13, and 15 d of WD stress, respectively. Data are the mean ± standard deviation (SD) of four replicates. * and ** represent significant differences between CK and WD stress treatments according to *Duncan*'s multiple range test at $p<0.05$ and $p<0.01$, respectively, the *capital letters* and *lowercase letters* represent significant differences between water deficit groups and control groups, respectively, according to *Duncan*'s multiple range test at $p<0.05$.

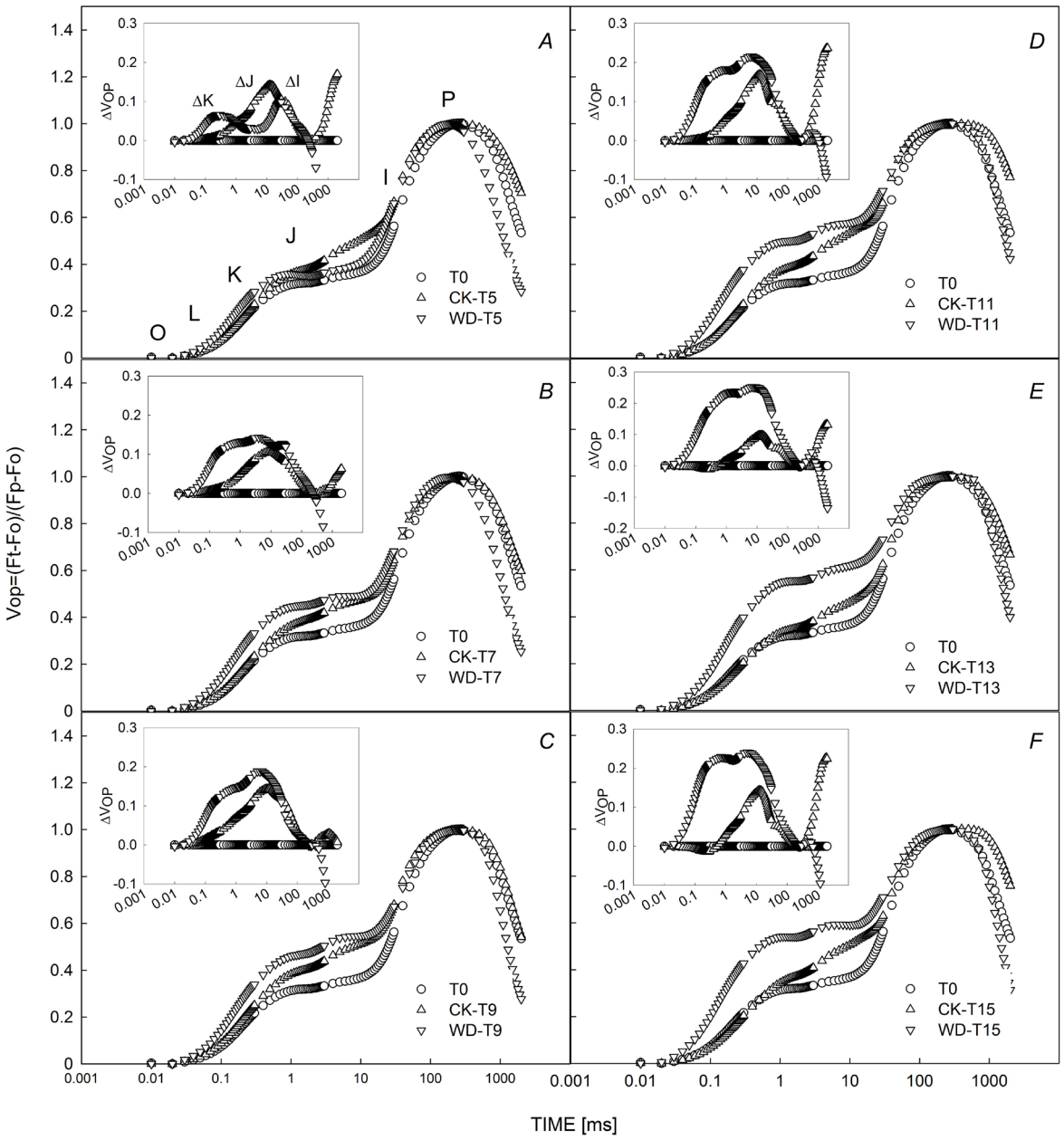

Fig. 2. Changes in the shape of the chlorophyll (Chl) *a* fluorescence transient curves of maize leaves under continuous WD stress. The large figures *A*, *B*, *C*, *D*, *E*, and *F* represent the changes of V_{OP} values after 5, 7, 9, 11, 13, and 15 d of WD stress, respectively. Small inner plots within each graph represent $\triangle V_{OP}$ of the corresponding treatment day. V_{OP} represents Chl *a* fluorescence transient curves double normalized between the two fluorescence extremes, O (F_O) and P (F_M) phase: $V_{OP} = (F_T - F_O)/(F_M - F_O)$; based on V_{OP}, $\triangle V_{OP} = V_{OP}$(treatment) − V_{OP}(control), showing the steps of L (0.15 ms), K (0.3 ms), J (0.45 ms), and I (30 ms) were obtained as periodic differences. L, K, J, and I represent the L (0.15 ms)-step, K (0.3 ms)-step, J (0.45 ms)-step, and I (30 ms)-step of the Chl *a* fluorescence transient curves, respectively. CK – control; WD – water-deficit stress. Data are the means of four replicates.

especially from the T7 (Fig. 3*A,B*).

Changes in fluorescence parameters are presented in Table 2. Compared to corresponding CK, the φ_{Po}, φ_{Eo}, φ_{Ro}, and ψ_O decreased in the WD group. In contrast, the TR_o/RC and ABS/RC increased, and were still higher than that corresponding to CK treatments. Among these parameters, the biggest change was found in PI_{ABS}, the performance index based on absorption of light energy, which decreased 5.7-fold at T15 of WD stress. However, the parameters did not change significantly in the CK group (Table 2).

Effect of WD stress on transcript levels of photosynthesis genes: Changes in the transcript levels of *psbA, psbB, psbC, psbP, psaA, psaB, rbcL, rbcS, cab,* and *rca* became more obvious with increasing WD duration (Fig. 4). The expression of *psbB, psbP, psaA, psaB,* and *cab*

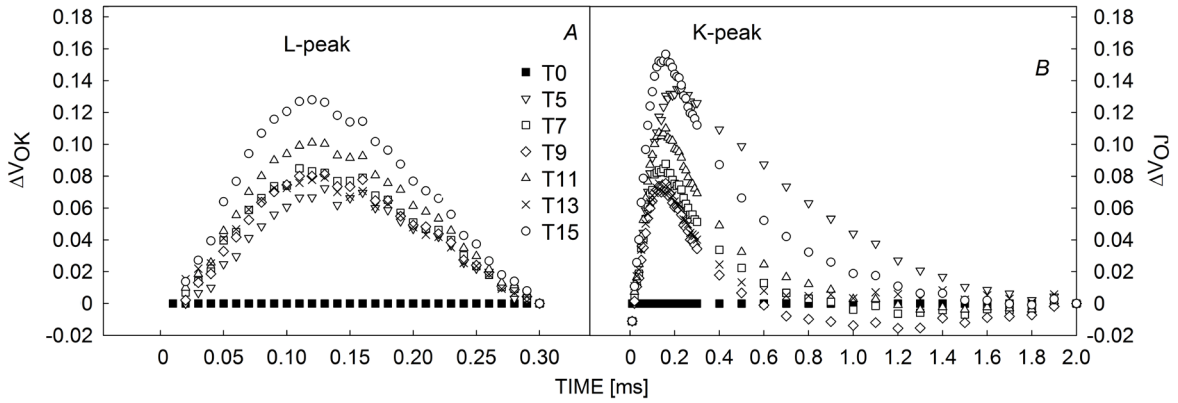

Fig. 3. $\triangle V_{OK}$ (*A*) and $\triangle V_{OJ}$ (*B*) in water-deficit stress group at different treatment times. The relative fluorescence between step F_O and $F_{300\mu s}$: $W_{OK} = (F_T - F_O)/(F_K - F_O)$, and between F_O and F_J: $W_{OJ} = (F_T - F_O)/(F_J - F_O)$ were normalized and displayed as $\triangle W_{OK} = W_{OK}(WD) - W_{OK}(CK)$ and $\triangle W_{OJ} = W_{OJ}(WD) - W_{OJ}(CK)$ at different treatment times, which made the L-band (at about 120–150 μs) and K-band (at about 200–300 μs) visible, respectively. CK – control; WD – water-deficit stress. Data are the means of four replicates.

Table 2. Changes in Chl *a* fluorescence transient parameters in dark-adapted maize plants under continuous water deficit conditions in maize leaves. All parameters are deduced from the OJIP-test analysis conducted on potted plants in maize ear leaves at 11:00–11:30 h. *See* Table 1S for the meaning of the symbols and the parameters. Data are the means ± standard deviation (SD) of four replicates. * and ** represent significant differences between control and water-deficit stress treatments according to *Duncan*'s multiple range test at $p<0.05$ and $p<0.01$, respectively.

Treatment		φ_{Po}	φ_{Eo}	φ_{Ro}	ψ_O	ABS/RC	TR_o/RC	PI_{ABS}	DI_o/RC
T0	CK	0.72 ± 0.01	0.48 ± 0.02	0.31 ± 0.02	0.66 ± 0.03	3.23 ± 0.47	2.31 ± 0.31	1.54 ± 0.18	0.92 ± 0.17
T5	CK	0.76 ± 0.02	0.45 ± 0.02	0.25 ± 0.02	0.60 ± 0.03	2.73 ± 0.35*	2.07 ± 0.20*	1.73 ± 0.4	0.66 ± 0.15*
	WD	0.7 ± 0.01	0.44 ± 0.03	0.23 ± 0.02*	0.63 ± 0.05	3.88 ± 0.42	2.71 ± 0.27	1.01 ± 0.16*	1.17 ± 0.16
T7	CK	0.72 ± 0.02	0.43 ± 0.03	0.25 ± 0.03	0.59 ± 0T.04	2.83 ± 0.23*	2.05 ± 0.12*	1.34 ± 0.24	0.78 ± 0.12*
	WD	0.67 ± 0.04*	0.34 ± 0.05*	0.2 ± 0.03*	0.51 ± 0.05*	3.79 ± 0.5	2.54 ± 0.21	0.55 ± 0.22*	1.26 ± 0.32
T9	CK	0.74 ± 0.01	0.42 ± 0.02	0.23 ± 0.03	0.57 ± 0.02	2.86 ± 0.19*	2.11 ± 0.12*	1.33 ± 0.24	0.75 ± 0.08*
	WD	0.68 ± 0.03*	0.34 ± 0.05*	0.22 ± 0.03	0.51 ± 0.06*	3.56 ± 0.39	2.41 ± 0.21	0.60 ± 0.20**	1.15 ± 0.2
T11	CK	0.75 ± 0.02	0.44 ± 0.03	0.25 ± 0.02	0.58 ± 0.02	2.64 ± 0.11*	1.99 ± 0.06*	1.62 ± 0.29	0.65 ± 0.07**
	WD	0.67 ± 0.04*	0.32 ± 0.05*	0.19 ± 0.03*	0.48 ± 0.06*	3.76 ± 0.45	2.52 ± 0.17	0.5 ± 0.24**	1.25 ± 0.29
T13	CK	0.75 ± 0.02	0.48 ± 0.03	0.28 ± 0.02	0.63 ± 0.04	2.66 ± 0.32*	2.01 ± 0.21*	1.97 ± 0.37	0.66 ± 0.11**
	WD	0.68 ± 0.03*	0.29 ± 0.08*	0.18 ± 0.06*	0.43 ± 0.1*	3.54 ± 0.35	2.42 ± 0.15	0.46 ± 0.26**	1.12 ± 0.22
T15	CK	0.75 ± 0.01	0.46 ± 0.02	0.27 ± 0.01	0.61 ± 0.02	2.48 ± 0.20**	1.87 ± 0.13**	1.92 ± 0.42	0.61 ± 0.07**
	WD	0.64 ± 0.02*	0.28 ± 0.03*	0.18 ± 0.02*	0.45 ± 0.04*	4.15 ± 0.28	2.64 ± 0.12	0.34 ± 0.12**	1.51 ± 0.18

were not significantly different between T5 and T7 under WD compared with the corresponding CK. However, expression declined for the seven photosynthesis-related genes under WD, particularly, after T11. For example, transcript levels of *psbA, psbB, psbC, psbP, psaA, psaB,* and *cab* were lower by 23.8, 12.8, 23.5, 10.4, 0.3, 9.9, and 2.2%, respectively, compared with the corresponding CK at T11. At T13, transcript levels of these genes were also significantly reduced compared with the corresponding CK. The expression of *rbcL* and *rbcS* significantly increased by 21.2 and 14.6%, respectively, at T13; and significantly increased by 6.7 and 49.7%, respectively, at T15. However, *rbcS* was down-regulated compared with CK at early stages of WD and there were no significant changes of *rbcL* at T5, T7, and T9. Interestingly, *rca* decreased by 29.9, 22.1, and 39.6% at T5, T7, and T9, respectively, compared with the corresponding CK. However, the expression of *rca* was higher by 11.9, 1.3, and 9.3% at T11, T13, and T15, respectively, compared with the corresponding CK.

Discussion

WD stress inhibits electron transport, which in turn impacts photosynthesis. Nonstomatal mechanism may play a greater role in inhibiting P_N (Guha *et al.* 2013). In this study, the reductions in P_N under adverse conditions were mainly due to changes in g_s and C_i (Ye *et al.* 2008). When both C_i and g_s decrease simultaneously, stomatal limitations are crucial for P_N. In contrast, if C_i increases or does not change when g_s decreases, nonstomatal mechanisms become the main limiting factor in determining P_N (Dias

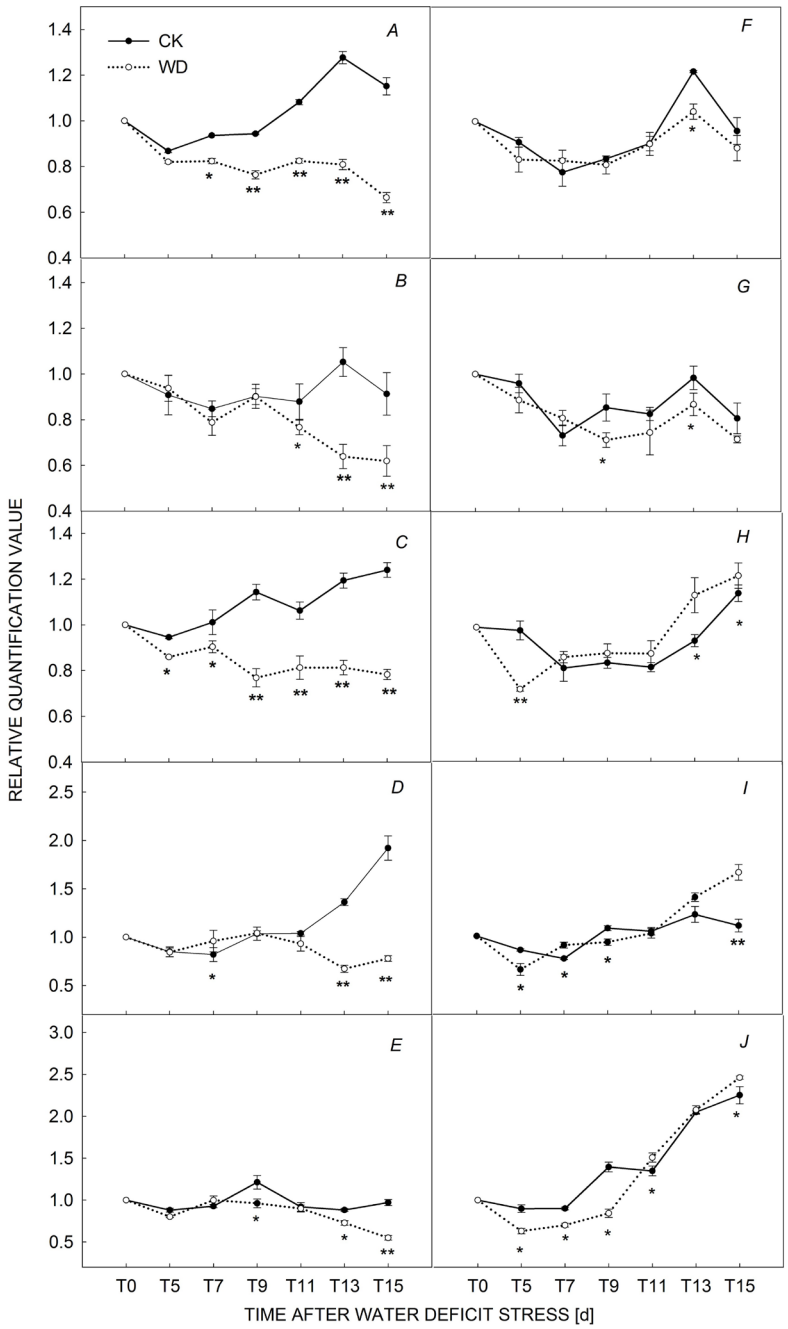

Fig. 4. The expression of the photosynthesis related genes *psbA* (*A*), *psbB* (*B*), *psbC* (*C*), *psbP* (*D*), *cab* (*E*), *psaA* (*F*), *psaB* (*G*), *rbcL* (*H*), *rbcS* (*I*), and *rca* (*J*) for maize plants after 0, 5, 7, 9, 11, 13, and 15 d of water-deficit stress. T0, T5, T7, T9, T11, T13, and T15 represent 0, 5, 7, 9, 11, 13, and 15 d after of water-deficit stress, respectively. * and ** represent significant differences between control and water-deficit stress treatments according to *Duncan*'s multiple range test at $p<0.05$ and $p< 0.01$, respectively. CK – control; WD – water-deficit stress. Data are the means ± standard deviation (SD) of three replicates.

et al. 2010). In this study, under long-term WD stress, photosynthetic disturbances may be mainly attributed to nonstomatal limitations.

Chl *a* fluorescence can be used to evaluate the impact of stress factors on photosynthesis (Gomes *et al.* 2012) and it is sensitive to drought stress (Shao *et al.* 2010). Research has shown that drought stress affected OJIP transients and related parameters in many plants (Redillas *et al.* 2011, Kalaji *et al.* 2014). In the present study, the J-step and I-step gradually increased (Fig. 3) along with increasing duration of drought, which might cause the accumulation of reduced Q_A and consequently drastically decreased electron transport after Q_A, which was also confirmed by decreased φ_{Eo}, φ_{Po}, and ψ_O in our study. These OJIP-related parameters are indicators of photoinhibition and the degree of injury to PSII (Zhang *et al*. 2015, Ghotbi-Ravandi *et al*. 2014). Decreases in φ_{Eo}, φ_{Po}, and ψ_O, suggested that WD stress led to photoinhibitory impairment, which mainly occurred at the donor side of PSII of the photosynthetic electron transport chain because the reduction end acceptors of PSI were seriously damaged. Reduction of the photosynthetic electron transport chain under drought conditions resulted in a decrease of PI_{ABS}. This indicated that the potential PSII activity, photosynthesis photoinhibition, and PSII function were damaged (Oukarroum *et al.* 2009). The L peaks (120–150 μs) were sharper with increasing duration of the treatment under drought, which might indicate that the stability and structure of PSII RCs were affected

(Zhang 1999). ABS/RC, TR_o/RC, and DI_o/RC increased under WD, indicating that partial RCs were inactive and efficiency per RC was enhanced (Redillas *et al*. 2011, Guha *et al*. 2013, Meng *et al*. 2016). This result may represent a self-protection mechanism of maize leaves, which helped plants resist drought stress and maintain growth under water stress.

WD stress could also induce or increase expression of PSII proteins or genes, which are involved in various pathways of the photosynthetic process (Yuan *et al.* 2005, Duan *et al.* 2006, Guha *et al.* 2013, Chen *et al.* 2016). Therefore, we also analyzed the transcript levels of photosynthesis-related genes to verify the photosynthetic changes under WD during the reproductive phase. The primary light-driven photosynthetic reactions are carried out in the thylakoid membrane in PSII and PSI (Ferreira *et al.* 2004). In this study, we found that the expression of *psbB*, *psbC* (encoding the light-harvesting proteins CP47 and CP43), and *cab* (encoding the light-harvesting Chl *a/b* binding protein) genes were down-regulated under WD stress, consistent with previous studies (Seki *et al.* 2002, Yuan *et al.* 2005, Živčák *et al.* 2013, Muhammad Salman *et al.* 2016). The results suggested that the light-harvesting process might be harmed after moderate drought stress, and reduced expression of these genes may also explain the changes in φ_{Po}. Decreased electron transfer activities may be the main factor leading to PSII instability (Li *et al.* 2016). The expression of *psbA* (encoding D1) was down-regulated, indicating that electron transport and PSII stability were affected (Yuan *et al.* 2005, Liu *et al.* 2006). φ_{Ro} was an important indicator that could reflect the status of the reduction end acceptors of PSI (Strasser *et al.* 1995). Decreased φ_{Ro} or damage to PSII function under WD stress might be an indirect result of decreased expression of psa*A* and *psaB* (which encode the PSI reaction center proteins (Tang *et al.* 2002). In addition, the significantly decreased *psbP* [encoding extrinsic nuclear-encoded subunits of the PSII oxygen-evolving complex (OEC)] expression at the late drought stage indicated that the OEC suffered serious injuries. Simultaneously, K band is an indicator of OEC damage (Oukarroum *et al.* 2009, Zhang *et al.* 2015), and in this study, a positive K band (200–300 μs) of the Chl *a* fluorescence curve was also found after prolonged drought stress. Decreased *psbP* as well as increased K peak and L peak, suggested that the balance of electron transport between the donor and acceptor side of PSII was damaged.

Plants have developed several mechanisms to protect the photosynthetic apparatus from injury. For example, it has been reported that increased levels of *rbcL* and *rbcS* compensate for the loss of oxidative stress damage due to WD, maintaining photosynthesis and preventing stress-related damage in rice plants (Xiong *et al.* 2010). In this study, significantly increased expression of *rbcL* and *rbcS* were observed at T13 and T15 in WD plants, which was consistent with results from Xu *et al.* (2013) in Kentucky bluegrass plants and Fu *et al.* (2007) in rice under drought stress. Maintaining higher Rubisco activity is necessary to sustain higher P_N (Zhang *et al.* 2015). Up-regulation of *rbcL* and *rbcS* may help to maintain photosynthesis and prevent damage due to drought. Interestingly, in the present study, P_N still decreased rapidly despite these adaptations, indicating that long-term water stress resulted in a serious damage to photosynthesis. However, some studies have reported that these two genes are down-regulated under drought stress (Bartholomew *et al.* 1991, Pelloux *et al.* 2001, Hayano-Kanashiro *et al.* 2009), which is not consistent with our results. This discrepancy may be due to differences in drought severity and duration. The gene *rca* regulates Rubisco (Portis 1995). Increased expression of *rca* at T15 in WD plants might serve to alleviate damage of Rubisco by drought stress (Ji *et al.* 2012). However, *rca* expression was lower in WD plants at T5, T7, and T11, similar to results of other plant species under drought stress (Pelloux *et al.* 2001). These differing effects of drought on *rca* expression may occur due to species differences and variation in relative stress intensity (Xu *et al.* 2013).

Conclusion: In summary, under long-term WD stress, nonstomatal limitations may be the primary cause of photosynthetic disturbances. Fluorescence parameters combined with transcript levels of photosynthesis-related genes indicated that down-regulation of light-harvesting and electron transport during the photosynthesis process might be caused by the low expression levels of *psbA, psaA, psaB, psbB, psbC,* and *cab*, which in turn impact the photosynthesis. However, higher transcript levels *of rbcL* and *rbcS* in the later stages of WD stress might be associated with maintenance of photosynthetic capacity under long-term WD stress. Despite these adaptations, the drop-offs in P_N and biomass were not prevented, indicating that the damage caused by WD in this study was beyond the repair ability of plants.

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