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Research article

C4 photosynthetic enzymes play a key role in wheat spike bracts primary carbon metabolism response under water deficit

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ABSTRACT

C4 photosynthetic enzymes are present in C3 plants and participate in non-photosynthetic metabolism. Wheat spike bracts had a higher drought tolerance, photosynthesis and senesced later compared to the flag leaves under water deficit. This research was conducted to investigate the different response of primary carbon metabolism induced by C4 photosynthetic enzymes in wheat flag leaves and spike bracts including glumes and lemmas under water deficit. The activities of C4 photosynthetic enzymes and Ribulose bisphosphate carboxylase oxygenase (Rubisco), the expression of related genes and primary carbon metabolism contents were demonstrated in wheat flag leaves and spike bracts exposed to water deficit. Results showed that drought stress strongly inhibited wheat photosynthetic metabolism by decreasing Rubisco activity in flag leaves. The activities of phosphoenolpyruvate carboxylase (PEPC), NADP-malic enzyme (NADP-ME), phosphate dikinase (PPDK) and NADP- malic dehydrogenase (NADP-MDH) increased in wheat spike bracts under water deficit. Transcript levels of C4 photosynthetic genes in wheat spike bracts were higher under water deficit than that of control. Furthermore, the results indicated that drought stress induced changes in the contents of primary carbon metabolism including malate, oxaloacetic acid (OAA), citric, fumaric acid were organ-specific. In conclusion, the functions of C4 photosynthetic enzymes appear to be important for wheat spike bracts primary carbon metabolism and defence response under drought stress.

1. Introduction

Northern China is one of the major regions for wheat production, accounting for roughly 85% of summer production, and, future wheat yield gains is immense important to the food security of China (Wang et al., 2016a). However, water resources depletion exists worldwide but is a special problem in arid and semiarid regions (Lesk et al., 2016; Boyer and Westgate, 2004). Water deficit induces a series of physiological and biochemical responses in plants, in which include repression of cell growth, stomatal closure, activation of respiration and photosynthesis (Yang et al., 2016; Mastalerczuk et al., 2017; Aziz et al., 2018).

The flag leaves of wheat are foremost photosynthetic assimilation organs, and photosynthetic rate is dropped significantly under water deficit. But nonleaf organs (spikes) still display a certain degree of photosynthetic at the grain filling (Yousfi et al., 2013; Araus et al., 1993a; Araus et al., 1993b). Wheat spike bracts have multiple advantage compared to flag leaves, which include higher photosynthetic and strong osmotic adjustment ability under water deficit. Previous study indicated that spike bracts photosynthetic contributes largely to grain yield under water deficit (Jia et al., 2015; Lou et al., 2018).

C4 photosynthesis plants have advantages in extreme growth conditions such as high temperature and drought stress (María Valeria et al., 2006). Wheat is a C3 plant, it was less-efficient photosynthetic than that of maize under water deficit. The enzymes of C4 photosynthetic primary fixation of CO_2 assimilation and process acid metabolism. Whereas, the C4 photosynthetic enzymes have different non-photosynthetic roles in C3 plant, which participated in tricarboxylic acid cycle (TCA), play an important role in the replenishment of Calvin cycle, hence process the biosynthetic of amino acids and provide NADPH for the antioxidant system (Chojak-Kozniewska et al., 2018; Doubnerova and Ryslava, 2011; Hýsková et al., 2016; Gao et al., 2018). Rangan et al., (2016) indicated that the presence of a C4 photosynthetic in the developing wheat grain that is absent in the leaves. But previous study showed that it was poor evidence for C4 photosynthetic in the wheat grain (Busch and Farquhar, 2016). C4 photosynthetic enzymes including NADP-malic enzyme (NADP-ME), Phosphoenolpyruvate carboxylase (PEPC), pyruvate, phosphate dikinase (PPDK) and NADP-malic dehydrogenase (NADP-MDH) participate in the assimilation process of CO_2 in C4 photosynthesis (Sage, 2004). In *Arabidopsis*

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Abbreviation

2-OG	Ketoglutaric acid
AsA	Ascorbic Acid
CK	Control with normal water supply
Critic	Citric Acid
DAA	Day after anthesis
DTT	Dithiothreitol
Fumaric	Fumaric acid
FW	Fresh weight
GSH	glutathione
Malate	Malate acid
MD	Moderate water deficit
NADP-ME	NADP- Malate dehydrogenase
NADPH	Nicotinamide adenine dinucleotide phosphate
NADP-MDH	NADP- Malate Dehydrogenase
OAA	Oxaloacetic acid
PEP	Phosphoenolpyruvic acid
PEPC	Phosphoenolpyruvate carboxylase
PGA	3-phosphoglycerate
PPDK	Phosphoenolpyruvatekinase
RuBP	Ribulose bisphosphate
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
ROS	Reactive oxygen species
RWC	relative water content

thaliana PEPC genes has been identified (Sanchez and Cejudo, 2003). Moreover, ten PEPC genes were identified in soybean, the expression of *GmPEPC6*, *GmPEPC8* and *GmPEPC9* were significantly increased under salt, drought and cold stress (Wang et al., 2016b). In the monocotyledon, the characteristic of C4 photosynthetic were also identified in rice (Shen et al., 2016). The C4 photosynthetic enzymes activity (PEPC, NADP-ME, NADP-MDH, and PPDK) were obviously enhanced in wheat under water deficit (Jia et al., 2015). However, little reports on the identified of C4 photosynthesis gene and the specific expression of these genes in wheat. OAA is the production of the reaction catalyzed by PEPC, which reduced by MDH to relatively acidic malate (Brendan et al., 2011). The malate content was enhanced in the winter rye by increased PEPC activity under cold stress (Crecelius et al., 2003a). NADP-ME catalysis the oxidative decarboxylation of malate and $NADP^+$ as a coenzyme to synthesis pyruvate, CO_2 and NADPH (Saher et al., 2005). NADP-ME enzyme has been reported in many C3 plants, such as *A. thaliana* (Wheeler et al., 2005) and rice (Wei et al., 2004), which the gene expression was specified in different period and tissue. *At-NADP-ME* is participated in the metabolic response of plants to drought stress. The increased activity of At-NADP-ME provide enough NADPH for biosynthetic metabolic pathway. Antioxidative defense systems, for instance the ascorbate-glutathione cycle (ASA-GSH) also requires NADPH to protect plants against drought stress. PPDK catalysis a reaction converting pyruvate to PEP in C4 plants, and part of the TCA cycle, which is also play a vital role in most tissue in C3 plants (Doubnerova and Ryslava, 2011). Previous research has been suggested that *AtPPDK* controls starch syntheses and amino acid metabolism. Moons et al., 2010 described that the rice PPDK activity was enhanced in the roots under water deficit. Although the change of C4 photosynthetic enzyme activities were reported in wheat under water deficit, however the carbon biosynthetic metabolic change in spike bracts response to water deficit by regulation of C4 photosynthetic enzyme activity are still unknown.

In our previous studies, drought stress was found to increase the activity of NADP-ME, PEPC, PPDK and NADP-MDH in wheat spike bracts. However, the study of C4 photosynthetic enzymes at the transcription and metabolic level is not completely understood. So, this research focused on investigating the different enzymes activity, genes expression and carbon metabolism between wheat flag leaves and spike bracts under water deficit. To fully understand the mechanism of drought tolerance between wheat spike bracts (glumes and lemmas) and flag leaves. This study could be reasonable explanation for wheat spike bracts senesced later than flag leaves and the more stable photosynthetic capacity under water deficit.

2. Materials and methods

Wheat cultivar Zhengyin1 (St1472/506) was carried out in our pot experiment, which was executed from October 2017 to June 2018. Seeds were sown in each plastic pot containing 7 kg soil. The field water capacity was 29.2%. The soil was fully watered and then equilibrated for approximately 20 days. In total, 20 seeds were seeded into the soil in each pot and retained 12 main stems per pot after removing tillers at the jointing stage. Water control was carried out from anthesis (April 12, 2018) by weighing method. The soil water content was implemented with 70–75% (control) and 45–50% (water deficit) of the largest filed capacity respectively. The pots were weight every day. The flag leaves, glumes and lemmas of wheat were collected at 0, 6, 12, 18, and 24 days after anthesis (DAA). The sample plants were stored at −80 °C for subsequent analysis.

2.1. Identification and sequence analysis of PEPC genes

We searched the C4 photosynthetic genes and downloaded from Phytozome database (http://phytozome.jgi.doe.gov/pz/portal.html). Multiple alignments of the sequences were used MEGA 7.0. Phylogenetic Tree was constructed using MEGA v7.0 based on the neighbor-joining (NJ) method. The stability analysis of the internal nodes was assessed with 1000 replicates bootstrap. The chromosomal location of the C4 photosynthetic genes were acquire from Ensembl Plants website (http://archive.plants.ensembl.org/Triticum_aestivum/).

2.2. Enzymes assay

Enzyme extraction was assayed according to the method of Sayre and Kennedy, 1979. The wheat tissues were ground with 4 mL grinding media, in which containing 1.0 mM EDTA, 0.1 M Tris-HCl (pH 7.8), 20 mM mercaptoethanol, 10 mM $MgCl_2$, and 2% (w/v) polyvinylpyrrolidone-10. The extract solution was centrifuged at 15,000 × *g* for 10 min at 4 °C, and the supernatant was used for enzymes assay.

PEPC (EC 4.1.1.31) activity was carried out using the method of Blanke and Ebert (1992). The extract solution was added to the mixture consisting of 0.25 mM EDTA, 50 mM Tris-HCl (pH 7.8), 5.0 mM $NaHCO_3$, 10 mM $MgCl_2$, 2.0 mM DTT, 0.1 mM NADH, 4U MDH and 2.0 mM phosphoenolpyruvate. Enzyme was assayed by following the change in absorbance at 340 nm.

PPDK (EC 2.7.9.1) activity were determined with method of Camp et al. (1982). The reaction solution consisting of 0.1 mM EDTA, 0.1 M Tris-HCl (pH 8.0), 5.0 mM DTT, 10 mM $MgCl_2$, 1.25 mM pyruvate, 2.5 mM K_2KPO_4, 0.16 mM NADH, 50 mM $NaHCO_3$, 2 U PEPC, 3 U MDH, and the enzyme extract. Reactions were start with 1.25 mM ATP, and the change absorbance was calculated at 340 nm.

NADP-ME (EC 1.1.1.40) activity was assayed by method of Sayre and Kennedy, 1979. Enzyme supernatant was added to a mixture consisting of 2.5 mM Tris-HCl (pH 8.3), 0.5 mM EDTA, 0.75 μM CoA, 0.25 mM NADP and 2.5 mM malate. Reactions begin with the addition of 5.0 mM $MnCl_2$. The absorbance of the supernatant was assayed at 340 nm.

NADP-MDH (EC 1.1.1.37) activity was determined by the method of

Sayre and Kennedy, 1979. The extract solution consisting of enzyme extract, 0.2 mM EDTA, 100 mM Tricine (pH 7.5) and 0.1 mM NADH. Reactions were start with Oxaloacetic acid.

Rubisco (EC 4.1.1.39) activity was spectrophotometrically carried out by the method of Camp et al. (1982). The reactions contained 1 mM EDTA, 50 mM Tricine-NaOH (pH 7.9), 2 mM DTT, 15 mM $MgCl_2$, 10 mM KCl, 10 mM $NaHCO_3$, 5 mM phosphocreatine, 2 U mL^{-1} creatine phosphokinase, 4 U mL–1 glyceraldehyde-3P-dehydrogenase and 0.2 mM NADH. Reaction were start with 0.5 mM RuBP.

2.3. Quantitative real -time RT-PCR

The RNA was extract from wheat by TRIzol Reagent and reverse-transcribed using PrimeScriptTM RT Reagent Kit (TaKara, China). The gene-specific primers (Table S1) were designed by Primer Premier 6.0. Real-time PCR reaction using SYBR green with an CFX96TM (Bio-Rad). Each reaction in a total volume of Real-time PCR was set as the follows. At 95 °C denaturation 30 s, and then followed by 39 cycles of 5 s at 95 °C and 60 °C for 30 s, and at last a melting curve (65°C–95 °C, at increments of 0.5 °C) generated to check the amplification. Relative gene expression was calculated using $2^{-\Delta\Delta CT}$ method.

2.4. Determination of metabolite

The wheat tissues were ground with 4 mL distilled water. Extract in 75 °C water baths for 15 min. The extracts solution was centrifuged a t 20,000 × *g* for 15 min at 4 °C. The supernatant was passed through 0.22 μm filter and determinate by HPLC (Waters, USA).

3. Results

3.1. Identification, chromosomal localization of C4 photosynthetic gene in wheat

The wheat C4 photosynthetic genes blast search was performed by using the maize sequence as baits, and the corresponding sequences were identified on NCBI database. The homologues sequence of *TaPEPC* (Y15897, AJ007705), *TaPPDK* (AF475130, AK333343), *TaNADP-ME* (EU082065, EU170134), *TaNADP-MDH* (AK333412) were obtain. Both of *TaPEPC* (Y15897, AJ007705) sequence were contain conserved domain of PEPCase. Using the obtained *TaPEPC* cDNA sequence in the URGI databank for blast search, it was revealed the homologous sequence of *TaPEPC* (AJ007705) were on the long arms of chromosome 5 and designated as *TaPEPC-5*, while that of *TaPEPC* (Y15897) located on

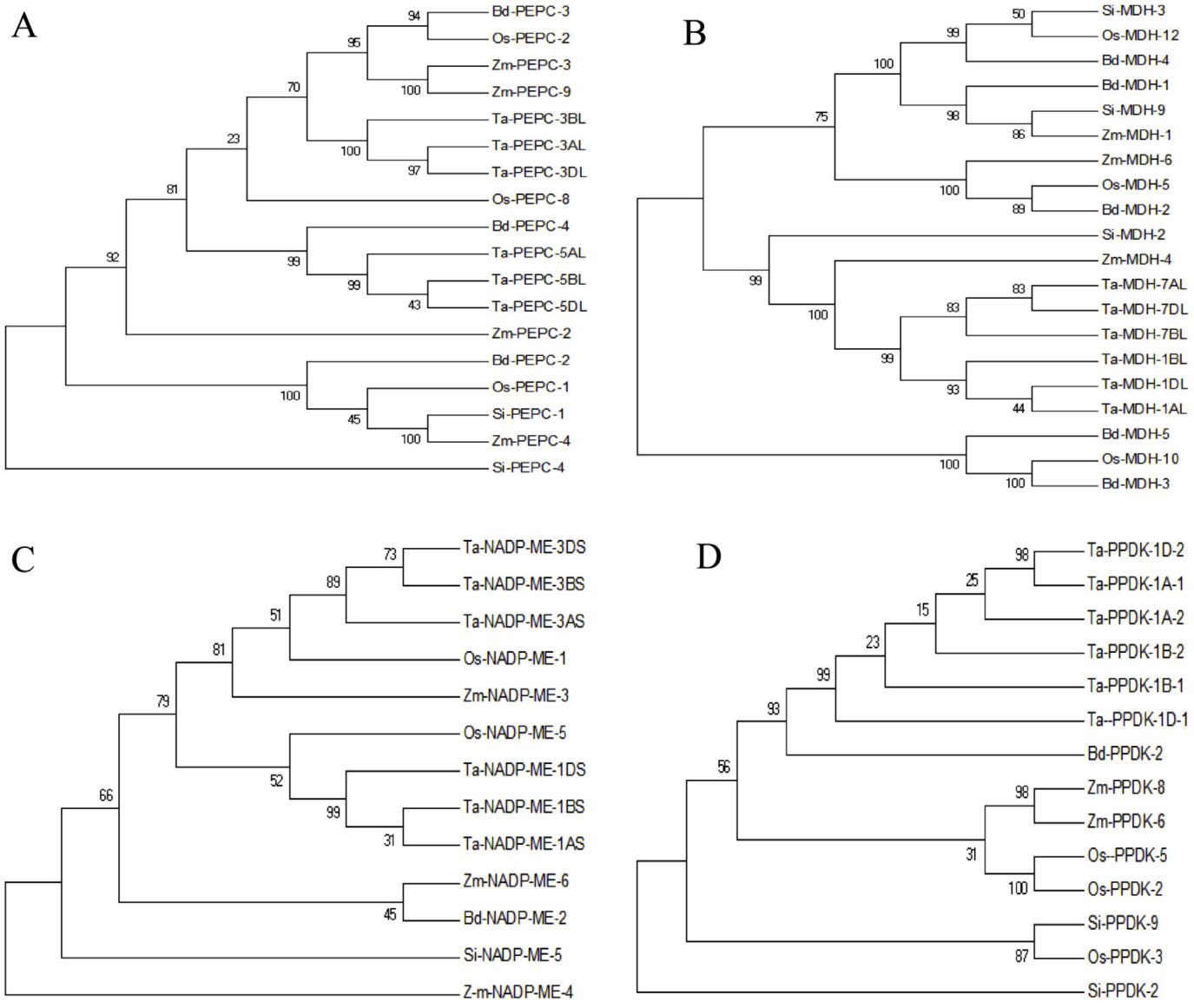

Fig. 1. Phylogenetic analysis of C4 photosynthesis pathway genes (A: *PEPC*, B: *NADP-MDH*, C: *NADP-ME*, D: *PPDK*) in wheat and other plant species.

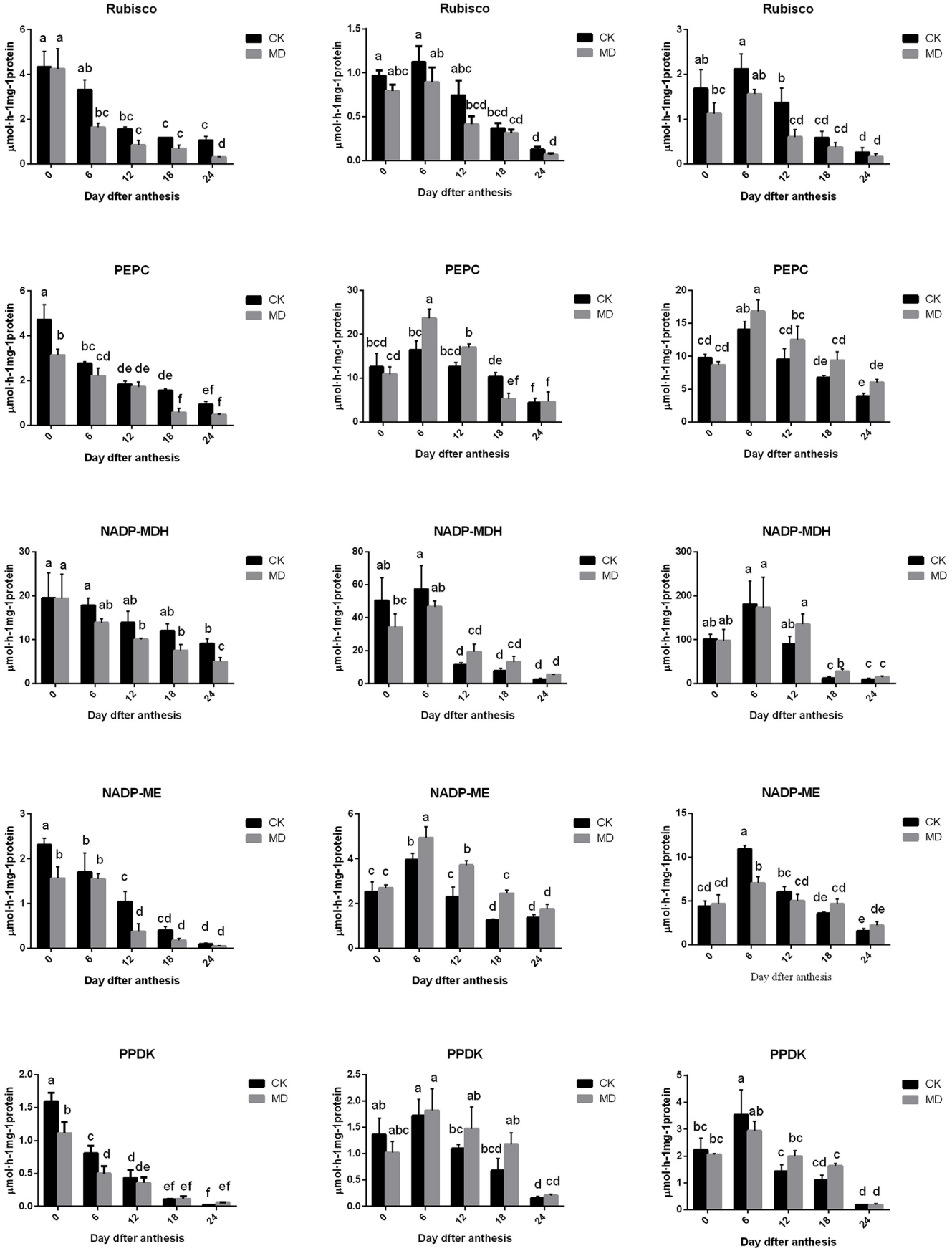

Fig. 2. Activities of C4 photosynthetic pathway enzymes and Rubisco in wheat flag leaves (A, D, G, J, M), glumes (B, E, H, K, N) and lemmas (C, F, I, L, O) under control and water deficit. MD -moderate water deficit; CK- control with normal water supply. Differences at $P < 0.05$ were considered significant. Data were expressed as mean ± standard deviation (SD).

the long arms of chromosome 3 and named as TaPEPC-3. Identification of Copies of each gene corresponding to the A, B and D chromosomes of wheat. Similarly, the cDNA sequence of *TaPPDK* (AF475130, AK333343) were on the chromosome 1 long arm and named as *TaPPDK-1-1* and *TaPPDK-1-2*. *TaNADP-ME* (EU082065, EU170134) were on the chromosome of 1 and 3 short arms and named as *TaNADP-ME-1* and *TaNADP-ME-3*. *TaNADP-MDH* (AK333412) were on the chromosome 1 and 7 long arms (*TaNADP-MDH-1* and *TaNADP-MDH-7*). The full-length cDNA sequence was obtained. Both of the three sub-genomes cDNA sequence were high similarity.

The polygenetic trees (Fig. 1) of PEPC, NADP-ME, NADP-MDH and PPDK showed the relationships between the amino acid sequence of C4 photosynthetic genes from wheat, maize, rice, *Brachypodium distachyon* and *Setaria italica*. The results indicated that wheat, rice and *Brachypodium distachyon* PEPC gene are high degree of homology. Both of *TaPPDK* and *TaNADP-MDH* are more related to *Brachypodium*

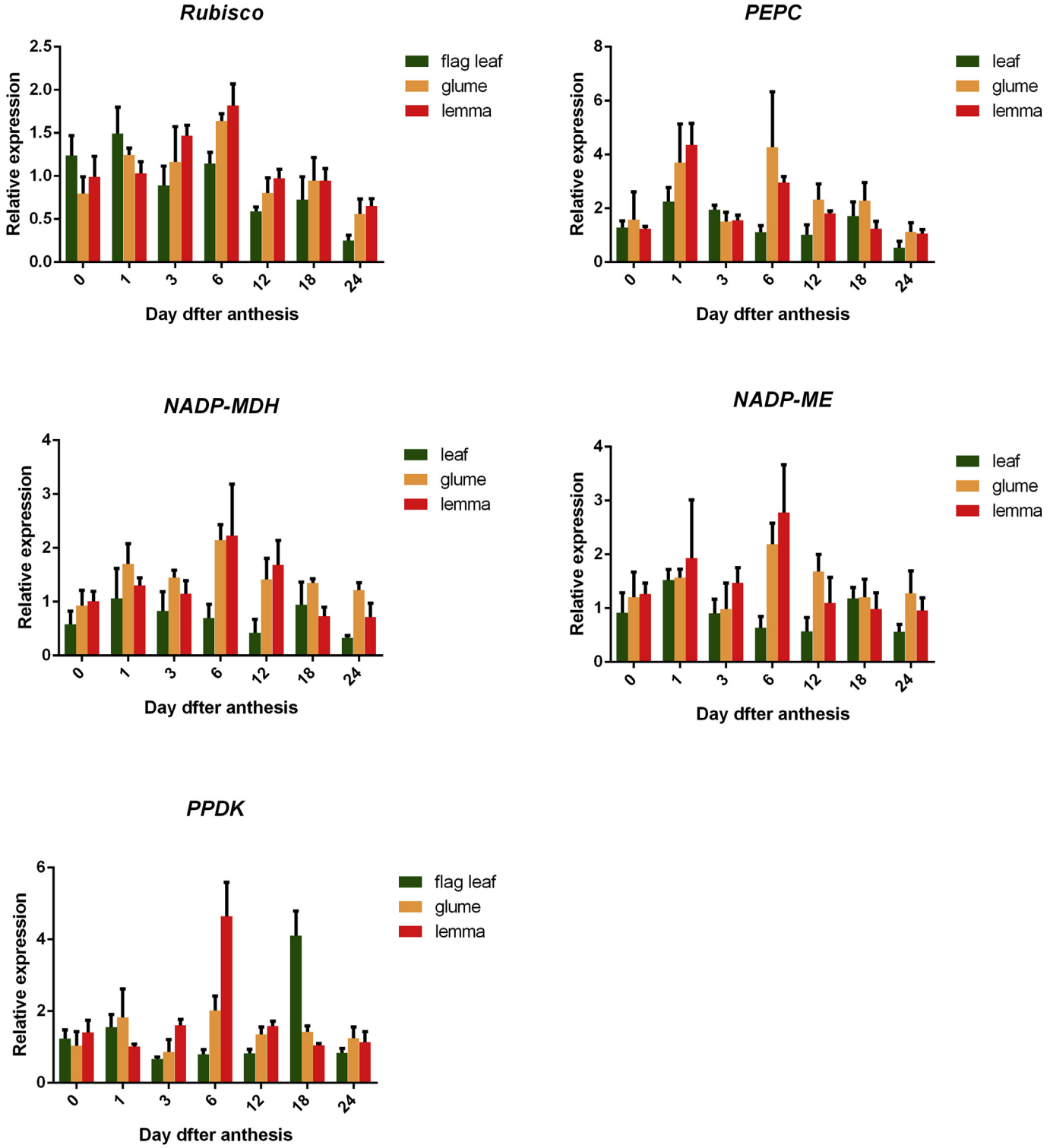

Fig. 3. Relative expression of C4 photosynthetic pathway genes in wheat flag leaves, glumes and lemmas under control and water deficit. Differences at $P < 0.05$ were considered significant. Data were expressed as mean + standard deviation (SD).

distachyon PPDK and *NADP-MDH*. The *TaNADP-ME* genes are more similar with maize and rice.

3.2. Activities of Rubisco and C4 photosynthetic enzymes

Activities of Rubisco and the C4 photosynthetic enzymes PEPC, PPDK, NADP-MDH, NADP-ME were assayed in flag leaves and spike bracts (glumes and lemmas) at anthesis and fully grain-filling stages. As shown in Fig. 2A, Rubisco activity in flag leaves decreased by 46.6% and 25.3% at 6 DAA, 12 DAA respectively under water deficit, while the decline in the rest of periods was not obvious. Moreover, Rubisco activity gradually decreased from 0 DAA to 24 DAA in both of control and water deficit. Glumes (Fig. 2B) and lemmas (Fig. 2C) Rubisco activity were the highest one at 6 DAA and keep decreasing until 24 DAA. Drought stress significantly decreased the Rubisco activity at the middle-late grain-filling stage, and especially at 12 DAA that declined

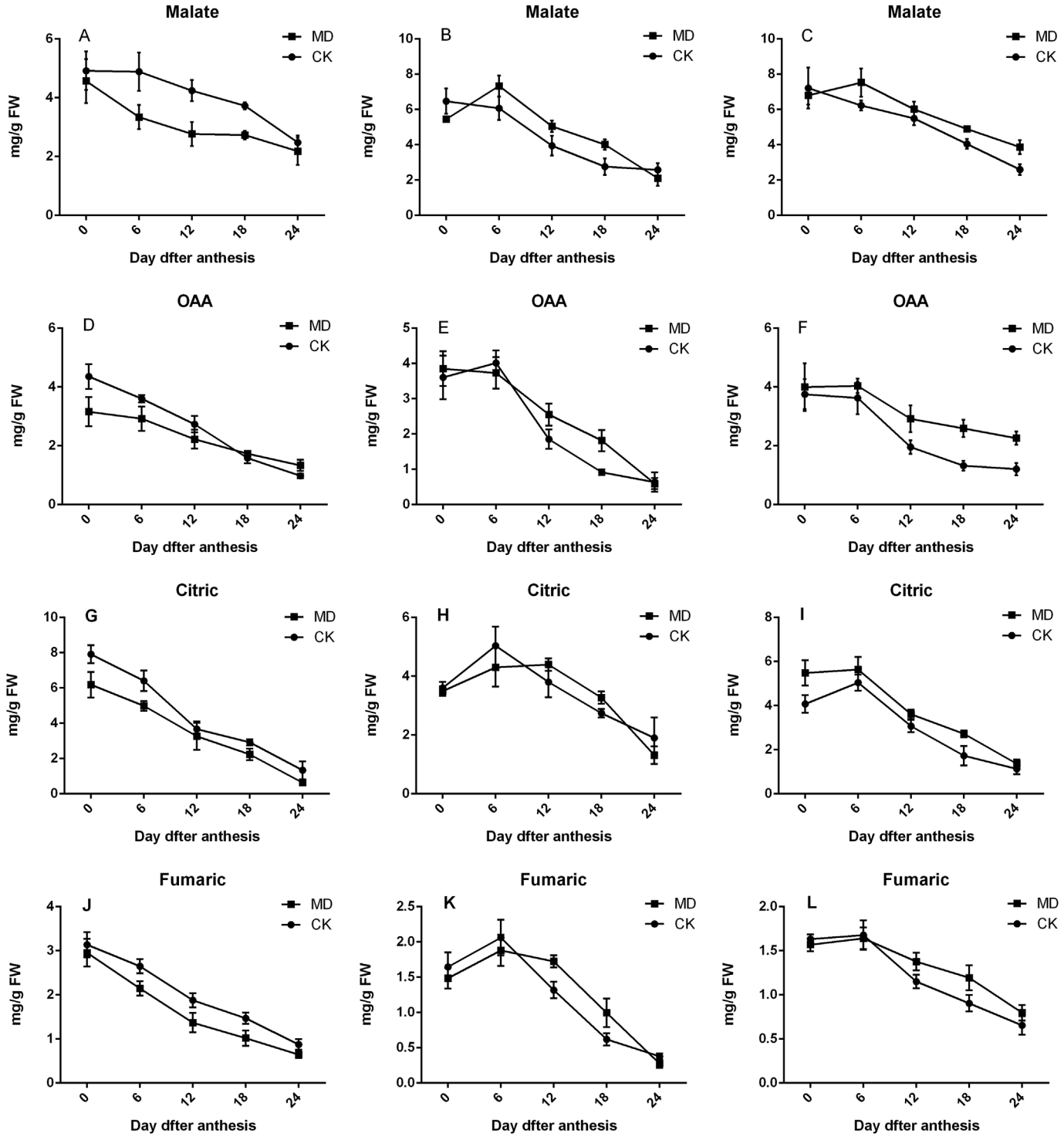

Fig. 4. The contents of malate, OAA, citric, fumaric acid in wheat flag leaves (A, D, G, J), glumes (B, E, H, K) and lemmas (C, F, I, L) under control and water deficit. MD -moderate water deficit; CK- control with normal water supply. Differences at $P < 0.05$ were considered significant. Data were expressed as mean + standard deviation (SD).

significantly by 43.9% in glumes and by 57.1% in lemmas. The higher activity was found in glumes and lemmas compared to flag leaves.

A parallel change in PEPC and Rubisco activities at flag leaves from 0 DAA to 24 DAA was observed. The activity of PEPC in spike bracts were the highest at 6 DAA and keep decreasing until 24 DAA. Under water deficit, glumes PEPC activity (Fig. 2E) were significantly higher (92.3%) at 6 DAA than 0 DAA, and slightly increased under control conditional. Lemmas PEPC (Fig. 2F) activity increased by 73.19% and 40.95% at 6 DAA compared to 0 DAA in both of water deficit and control. Moreover, glumes PEPC activity increased by 40.90% and 25.6% at 6 DAA and 12 DAA, respectively, and no obvious change in other stages under water deficit than control. Lemmas PEPC activity at 6 DAA (18.8%), 12 DAA (34.6%), 18 DAA (39.8%), and 24 DAA (25.4%) was higher under water deficit than that of control.

NADP-MDH activity significantly decreased in flag leaves (Fig. 2G) during grain-filling stage from 0 DAA to 24 DAA and decreased by 11.92%, 25.8%, 34.7%, 51.6% at 6 DAA, 12 DAA 18 DAA and 24 DAA. Under water deficit, glumes NADP-MDH activity (Fig. 2H) obviously increased from 12 DAA to 24 DAA, with the highest increase of 60.65% at 12 DAA. Lemmas NADP-MDH activity (Fig. 2I) significantly rose by 40.6% and 27.8% at 12 DAA and 18 DAA. Furthermore, both of glumes and lemmas NADP-MDH activity were the highest at 6 DAA than other stage. The higher activity was found in glumes and lemmas compared to flag leaves.

Glume NADP-ME activity (Fig. 2K) obviously rose during 6 DAA to 24 DAA under water deficit, with the highest increase (87.6%) at 18 DAA. However, lemmas NADP-ME activity (Fig. 2L) just rose by 49.8%, 37.6% at 18 DAA and 24 DAA under water deficit. Same as PEPC activity, flag leaves NADP-ME activity (Fig. 2J) decreased from 0 DAA to 24 DAA, and under water deficit it significantly decreased compared to control. In spike bracts, NADP-ME activity was also higher than flag leaves.

The activity of PPDK in flag leaves (Fig. 2M) significantly decreased by 47.3% at 6 DAA under water deficit. The highest glumes PPDK activity (Fig. 2N) was observed at 6 DAA, lemmas PPDK activity (Fig. 2O) was also found at grain-filling stage. Glumes PPDK activity increased by 37.5%, 45,7%, 21.4% at 12 DAA, 18 DAA and 24 DAA under water deficit. Lemmas PPDK activity increased by 48.3%, 36.2% at 12 DAA and 18 DAA, but no obviously change at 24 DAA. Compared with flag leaves the glumes and lemmas activity increased significantly under water deficit.

3.3. The expression of C4 photosynthetic enzyme genes and rubisco

Comparative transcriptional levels were performed in glumes, lemmas and flag leaves to analyses change of C4 photosynthetic enzyme genes and *Rubisco* under water deficit (Fig. 3). The *Rubisco* expression in all organs slightly up-regulated at early grain-filling stage under water deficit. In glumes and lemmas, the *Rubisco* expression were the highest at 6 DAA. However, in flag leaves *Rubisco* expression was down-regulated under water deficit at late grain-filling stage. *PEPC* expression at 1 DAA and 6 DAA significantly up-regulated in glumes (4.1 fold), (4.3 fold) and slightly increased at other grain-filling stages under water deficit compared to control. Drought stress promotes the expression of *PEPC* in lemmas at 6 DAA (3.7 fold), it was slowly up-regulated at other grain-filling stage. *NADP-ME* and *NADP-MDH* genes exhibited similar expression patterns in glumes and lemmas under water deficit. The expression level of *NADP-ME* was the highest at 6 DAA in glumes (2.3 fold) and in lemmas (2.8 fold), respectively. *NADP-MDH* expression was also the highest in glumes (2.1 fold) and in lemmas (2.4 fold) at 6 DAA. The drought stress treatment up-regulated the *PPDK* expression in glumes (2.1 fold) and in lemmas (4.8 fold) compared to the control at 6 DAA. Transcript levels of these five gene were up-regulated at early grain-filling stages under water deficit and slowly downregulated thereafter. Furthermore, in glumes and lemmas related genes showed generally higher expression levels than flag leaves.

3.4. The organic acid contents

The organic acid contents in flag leaves gradually decreased during grain-filling stages, and it decreased under water deficit. In glumes and lemmas, the organic acid contents increased at early grain-filling stage and then decreased during late grain-filling stage. Malate content significantly decreased by 31.7%, 35.5%, 26.8% at 6 DAA, 12 DAA, 18 DAA in flag leaves (Fig. 4A) under water deficit compared to control, it rose by 27.9%, 48.1% at 12 DAA, 18 DAA in glumes (Fig. 4B). It also increased by 21.1%, 49.6% at 18 DAA and 24 DAA respectively in lemmas (Fig. 4C). Meanwhile, drought stress increased the OAA content in glumes (Fig. 4E) and lemmas (Fig. 4F) at middle grain-filling stage, while there is no obvious change in flag leaves. OAA content (Fig. 4E) in glumes increased by 37.2% and 87.9% at 12 DAA and 18 DAA under water deficit compared to the control. A similar change of fumaric acid decrease was observed in glumes and lemmas under water deficit, fumaric acid contents were obviously increased by 31.2%,51.2%, 20,1%, 32.2% in glumes (Fig. 4K) and lemmas (Fig. 4L) at 12 DAA and 18 DAA, and it remained unchanged at other grain-filling stages. Furthermore, it gradually decreased until the 24 DAA in flag leaves. The citric acid content in flag leaves (Fig. 4G) decreased by 23.4% at 6 DAA under water deficit compared to the control, it continues to decrease throughout the grain-filling stage. The citric acid content of glumes and lemmas increased to a maximum at 6 DAA and decreased thereafter, which was increased by 15.5%, 19.7% at 12 DAA and 18 DAA in glumes (Fig. 4H), and was increased by 17.8%, 58.4% at 12 DAA and 18 DAA in lemmas (Fig. 4I) under water deficit compared to the control.

4. Discussion

C4 photosynthetic enzymes plays an important role in CO_2 assimilation during C4 and CAM photosynthesis, and also participates in C3 plants non-photosynthetic processes (Doubnerova and Ryslava, 2011; Chojak-Kozniewska et al., 2018). Wheat is a typical C3 plant with relatively low photosynthetic efficiency. Previous study has indicated all the C4 photosynthetic genes are encoded by the wheat genome (Rangan et al., 2016; Bachir et al., 2017), but little reported focused on their function in wheat spikes. The sequences of the C4 photosynthetic genes (*NADP-MDH*, *PEPC*, *PPDK*, *NADP-ME*) have been identified in rice and soybean (Wang et al., 2016b; Muthusamy et al., 2018). C4 photosynthetic genes were identified and expression in the wheat photosynthetic tissue (Rangan et al., 2016). In this research, we identified the C4 photosynthetic genes in the wheat and comparison with C4 photosynthetic genes in C4 plants, including rice, maize, *Brachypodium distachyon* and *Setaria italica*. Phylogenetic analysis indicated that wheat C4 photosynthetic genes were more correlative to *Brachypodium distachyon* and rice than to maize.

It has been well known that C4 photosynthetic enzymes play an vital role in C4 plants photosynthetic and also important for C3 plant drought tolerance (Wei and Wang, 2003; Hýsková et al., 2016; Liu et al., 2007). In additional to, the part in supplying metabolites to the TCA cycle, C4 photosynthetic enzymes are participated in other reactions which could be beneficial for C3 plants under water deficit including providing CO_2 for Calvin cycle and NADPH for metabolic processes (Doubnerova and Ryslava, 2011; Chojak-Kozniewska et al., 2018).

Non-photosynthetic PEPC enzyme produced OAA that supply intermediates of the TCA cycle in C3 plants. OAA is an important intermediate metabolite in the TCA cycle (Brendan et al., 2011; Gao et al., 2018). When the stomata are closed PEPC can provide carbon skeletons for biosynthesis by prefix the internally released CO_2 and nitrogen assimilation, it helps wheat to avoid drought stress (Seki et al., 2007; Kuźniak et al., 2016). In this research PEPC activity and transcript level were significantly increased in glumes and lemmas but decreased in flag leaves under water deficit. In additional, OAA content increased in glumes and lemmas under water deficit, no significantly in flag leaves.

The contents of malate, citric and fumaric acid have same pattern under water deficit. This result suggested that drought stress positively effect on PEPC activity and transcript level in wheat spike bracts. Similar results were observed in wheat roots under NaCl and drought stress (Gonzalez et al., 2003). In *Arabidopsis thaliana*, salts stress up-regulates the expression of genes *Atpepc1* and *Atpepc3* (Sanchez et al., 2006). This mechanism by allocation the limited carbon resources are beneficial to defence under water deficit.

NADP-MDH enzyme catalyzes a reaction converting OAA to malate acid (Doubnerova and Ryslava, 2011). Malate can act as a vacuolar osmolyte and also sever as an additional sink for carbon assimilation and reducing equivalents (Guo et al., 2018). In addition, when the cell structures damage, malate function as a transport metabolite at drought stress (Crecelius et al., 2003b). Present study indicated that the activity and expression level of NADP-MDH increased resulting in malate content enhanced in glumes and lemmas under water deficit. However, lowly malate content due to gene expression down-regulated and reduced activity of NADP-MDH in flag leaves under water deficit. These characteristics could be related to the spikes senesced later than flag leaves under water deficit. Qi et al. (2017) study indicated that *ZmPEPC* gene can enhance antioxidant enzyme activity, up-regulate the expression of photosynthesis-related genes, increased malate content and other metabolites in wheat, and increased it drought tolerance, which is also verified our results.

NADP-ME enzyme catalyze malate and $NADP^+$ to synthesis pyruvate, NADPH and CO_2 (Ryšlavá et al., 2007). NADP-ME expression provides reduced NADPH for biosynthetic metabolic pathway to response to abiotic stress (Doubnerová et al., 2009; Valderrama et al., 2010; Doubnerova and Ryslava, 2011). Antioxidative defence systems such as the ASA-GHS cycle require NADPH to protect plants detoxifying ROS under abiotic stress (Chojak-Kozniewska et al., 2018; Liu et al., 2007). Liu et al. (2007) study indicated that *OscytME2* responded to NaCl and mannitol, the activity and expression of *NADP-ME* were enhanced. In our study, spike bracts NADP-ME activity and gene expression were increased, which indicated that spike bracts could be synthesize more NADPH than that of flag leaves under water deficit. This results confirm our previous conclusions, the spike bracts exhibited higher activity of antioxidant enzymes involved in ASA-GSH metabolic under water deficit, it could be the explanation the spike bracts senesced later than flag leaves (Lou et al., 2018). Muller et al. (2008) suggested that NtNADP-ME1 in leaves responded to drought stress by play a key role in providing NADPH and pyruvate, the release of CO_2 could be useful for Calvin cycle and increase the fixation of CO_2, therefore in this research the activity and expression of Rubisco was increased under water deficit at early grain-filling stage. Furthermore, the role of NADP-ME is control stomatal closure by decomposed the malate under drought stress. It has been indicated that the increased expression of NADP-ME resulted in enhanced organic acid content, which may be an important mechanism for drought tolerance (Laporte et al., 2002; Guo et al., 2009).

PPDK enzyme catalyzes a reaction converting pyruvate to PEP and PP_i, moreover, reaction catalyzed by PPDK and PEPC to PEP and OAA was participated in TCA cycle to synthesis 2-oxoglutarate, which is the precursor compound of glutamine and glutamate. (Parsley and Hibberd, 2006). The previous research indicated that PPDK activity was assayed in C3 plants (Chojak-Kozniewska et al., 2018; Jia et al., 2015). Lucy et al. (2010) study showed that PPDK play a pivotal part in amino acids transport, the enhanced activity of PPDK could be significantly accelerate nitrogen mobilization and increase protein content. We found that PPDK were induced at the activity and transcript level in wheat spike bracts, however in flag leaves it was decreased under water deficit. These results indicated that the enzyme reaction capable of regenerating substrate for PEPC is also induced under water deficit. Abscisic acid and other drought stress markedly induced PPDK expression in rice roots, in this condition, PEPC activity is also increased, which hypothesized that the recovery of respired CO_2 is occurring (Moons et al., 2010; Kuźniak et al., 2016).

Drought stress response in wheat spike bracts is always more obviously than that of flag leaves and accompanied by the rearrangement of primary metabolism. Our results found that all C4 photosynthetic enzymes were induced at the activity and transcript level. In addition, the higher activity, genes expression of C4 photosynthetic enzyme and the enhanced content of organic acid were found in glumes and lemmas compared to flag leaves under water deficit. This study indicated C4 photosynthetic enzymes positively contributed to wheat spike bracts drought tolerance. When the stomata are closed, and photosynthesis is decreased under water deficit, the recovery of respired CO_2 by PEPC will be important. Malate, OAA, citric and fumaric acid are most important metabolic under drought stress. Our findings shown that all acid contents increased in spike bracts compared to flag leaves under water deficit. The increased acid contents in spike bracts under water deficit could indicated that malate, OAA, citric and fumaric acid were used to support the increased activity of spike bracts C4-photosynthetic enzymes supplying NADPH and pyruvate to the TCA cycle for biosynthesis and the antioxidative system. Moreover, the cycle reaction catalyzed by NADP-ME provide CO_2 (Kuźniak et al., 2016; Bort et al.,

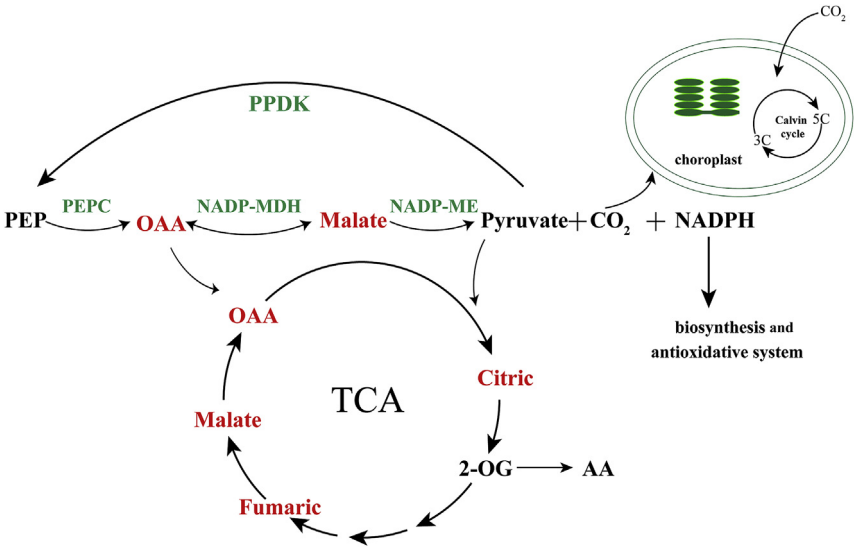

Fig. 5. Schematic representation of changes in wheat carbon metabolic pathways.

1996; Gao et al., 2018), which can participate in Calvin cycle, increased the photosynthesis product (Fig. 5). Similar results were also observed in tobacco plants, C4 photosynthetic enzymes activity increased under biotic stress by viral infection (Ryšlavá et al., 2003). In other research, abscisic acid and cold of *Egeria densa*, enhanced activities of C4 photosynthetic enzymes also has been observed (Crecelius et al., 2003a). The results of this research further confirm our previous conclusions at the transcript and primary carbon metabolism level, which was C4 photosynthesis enzymes appear to be important for wheat spike bracts defence response and senesced later compared to the flag leaves under water deficit. (Lou et al., 2018; Jia et al., 2015).

5. Conclusion

Drought stress stimulated the wheat spike bracts C4 photosynthetic enzymes activity, up-regulated related genes expression, increased the contents of malate, OAA, citric and fumaric acid, led to the accumulation of more metabolic in the TCA cycle, enhanced NADPH for antioxidative system and provide CO_2 for Calvin cycle (Fig. 5). These results could be reasonable explanation for the spike organs more drought tolerance and stable photosynthetic capacity at grain-filling stage compared to the flag leaves under water deficit., which provides a theoretical basis for developing a strategy to improve wheat drought tolerance through genetic engineering and has implication for increase wheat yield in arid areas.

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Contribution

Xu Zhang performed the experiments and analyzed the data of the experiments. Peng Pu helped with the organic acid contents study. Lixin Zhang helped to revise the manuscript. Jinyin Lv helped to analyze the data of the experiments and draft the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.06.013.

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