Activities of C₄ Photosynthetic Pathway Enzymes in Different Bread Wheat Genotypes under Field Conditions

(Aktiviti Laluan Fotosintesis Enzim C₄ dalam Genotip Gandum yang Pelbagai pada Keadaan Lapangan)

BACHIR GOUDIA DAOURA, IQBAL SAEED, QUANHAO SONG, YANG YANG, LIANG CHEN & YIN-GANG HU

ABSTRACT

The activities of key C₄ photosynthetic enzymes including phosphoenolpyruvate carboxylase (PEPcase), NADP-malic enzyme (NADP-ME), malate dehydrogenase (MDH) and pyruvate phosphate dikinase (PPDK) were assayed in flag leaves at three major growth stages (heading, anthesis and grain filling) among 59 winter wheat genotypes grown in field conditions. All C₄ enzymes expressed in the flag leaves and their activation showed a wide range of variation in relation to different growth stages in all the genotypes. PEPcase, NADP-ME and MDH displayed the highest mean activities of 1.018, 0.758 and 0.731 µmol. min⁻¹.mg⁻¹ protein at heading stage, respectively; while PPDK showed the highest mean activity (0.888 µmol. min⁻¹.mg⁻¹ protein) at grain filling stage. The activities of PEPcase and PPDK were higher at heading stage, decreased at anthesis and again increased at grain filling stage, while NADP-ME and MDH exhibited a decreasing trend at the three stages. The results of the current study could be valuable and useful for wheat researchers in improving photosynthetic capacity of wheat.

Keywords: C₄ enzymes; flag leaf; photosynthetic efficiency; transgenic plants; wheat

INTRODUCTION

Based on the differences in the mechanism of CO₂ assimilation, green plants can be categorized into C₃, C₄ and Crassulacean acid (CAM). Under unfavorable environmental conditions, C₄ plants have higher efficiency of CO₂ fixation than C₃ by cooperative action of C₄ enzyme system such as phosphoenolpyruvate carboxylase (PEPcase), nicotinamide adenine dinucleotide-phosphate malic enzyme (NADP-ME), malate dehydrogenase (MDH) and pyruvate orthophosphate dikinase (PPDK) (Ku et al. 1999). The C₄ pathway is a complex trait that has evolved from ancestral C₃ plants in response to changes in environmental conditions that caused a decrease in CO₂ availability (Christin et al. 2010; Ludwig et al. 2012). Therefore, many productive crops such as maize and foxtail millet use the C₄ photosynthetic pathway. However, some important major crops such as wheat and rice are C₃ plants exhibiting a lower photosynthetic efficiency (Matsuoka et al. 1998). Hibberd and Quick (2002) reported over-expression of PEPc, NADP-ME and PPDK in cells of stems and petioles in Tobacco, a typical C₃ plant and since then, CO₂-refixation function has been given a great concern.

The transfer of C₄ traits to C₃ plants is thus one strategy being adopted for improving the photosynthetic performance and raising the potential yield of C₃ plants (Surridge 2002). Several previous studies succeeded in introducing the maize C₄-specific PEPC cDNA into wheat and obtained transgenic plants with enhanced photosynthetic capacity (Han et al. 2013; Hu et al. 2012; Wu et al. 2011; Zhang et al. 2012). C₄-specific PPDK, or NADP-ME were introduced into rice (Fukayama et al. 2001;
Jiao et al. 2002; Ku et al. 2000; Taniguchi et al. 2008), *Arabidopsis thaliana* (Wang et al. 2012), oat (Tolley et al. 2012) and potato (Gehlen et al. 1996). Studies on elevated CO₂ concentrations showed a positive correlation between potential leaf photosynthesis and maximal crop growth rate (Murata 1981; Zheng et al. 2011), which indicates that increasing leaf photosynthesis efficiency could provide an attractive approach to improve crop yields. Although some C₄ enzymes have been transferred into C₃ plants, only few were successful in improving the photosynthetic efficiency (Zhang et al. 2009). Additionally, CO₂ metabolism inside the chloroplast of C₃ plants can greatly be disturbed by introduction of a foreign enzyme (Miyao 2003). For example Takeuchi et al. (2000) reported a 20-70-fold increase in maize NADP-malic enzyme in rice leaves which led to aberrant chloroplast structure with agranal thylakloid membranes. Over-expression of maize NADP-malic enzyme in rice was reported to negatively affect chlorophyll content and growth while enhancing photo-inhibition (Tsuchida et al. 2001). It is therefore, questionable to improve photosynthesis and yield of C₃ plants by transferring C₄ enzymes into C₃ plants to induce over-expression (Zhang et al. 2007). Thus, selecting C₃ plant with relatively high expression of C₄ enzymes is an alternative way to enhance photosynthesis in C₃ plants. Knowledge about variation in activities of C₄ enzymes at different growth stages in wheat could help to screen wheat genotypes having higher activities of these enzymes.

The aim of this work was to determine the activities of key C₄ photosynthetic enzymes including PEPCase, NADP-ME, MDH and PPDK in flag leaves of bread wheat. Therefore, we investigated the variations on the activities of these enzymes among different bread wheat genotypes at three major growth stages under field conditions.

### MATERIALS AND METHODS

#### PLANT MATERIAL AND GROWTH CONDITIONS

The experimental material consisted of 59 bread wheat genotypes (Table 1) from the major winter wheat production regions of China. They were sown under natural field conditions at the experimental farm of Northwest A&F University, Yangling, Shaanxi, China (N 34°10', E 108°10', 526 m elevation) during wheat growing seasons 2014-2015 and 2015-2016. Each genotype was planted into three major growth stages under field conditions.

#### ASSAYS FOR THE ACTIVITIES OF C₄ ENZYMES

Flag leaves of three plants of each genotype were sampled at heading (Z55), anthesis (Z67) and grain filling (Z73) stages and stored at −20°C. Frozen leaves were ground in liquid nitrogen to make fine powder using a chilled mortar and pestle. One milliliter of extraction buffer containing 50 mM Tris–HCl (pH7.5), 10 mM MgCl₂, 5 mM dithiothreitol (DTT), 1 mM EDTA, 2% (w/v) insoluble polyvinylpolypyrrolidone (PVP) and 10% (w/v) glycerol were added to each sample. Crude extracts were centrifuged at 13000 g for 20 min at 4°C and the supernatants were used immediately to measure enzyme activities. A final enzyme concentration of 5 mg/mL was used to assess the activities of specific enzymes. All measurements were performed at 30°C using Tecan Infinite 200 Pro (Tecan, Mannedorf, Switzerland) microplate reader. The molar extinction coefficient of 6.22 mM cm⁻¹ was used for NADH and NADPH, respectively. The following formula (Forrester et al. 1976) was used to calculate enzyme activities:

\[
\text{Enzyme activity} = \frac{(\Delta A_{\text{sample}} - \Delta A_{\text{blank}}) \times Vs \times 10^6}{c \times \Delta t \times V_s \times \text{Protein conc.}},
\]

where: \(\Delta A_{\text{sample}}\): Change in the absorbance from the beginning to the end of measurement period; \(\Delta A_{\text{blank}}\): Sample containing all the reagents except the enzyme; \(\Delta t\): Time interval the absorbance was measured (min); \(V_s\): Total volume (L); \(Vs\): Sample volume (mL); Protein conc: Protein concentration (mg/mL); 10⁶: This converts the moles of \(e\) to mmoles.

#### PEPCase ACTIVITY

Phosphoenolpyruvate carboxylase activity was assayed in a mixture containing 50 mM tricine-KOH (pH8.0), 10 mM MgCl₂, 10 mM NaHCO₃, 0.1 mM EDTA, 0.2 mM NADH, 3 U malate dehydrogenase (MDH), 20 µL of the enzyme extract and distilled water. The reaction was initiated by adding phosphoenolpyruvate to a final concentration of 2 mM and the rate of NADH consumption was determined by the absorbance change at 340 nm (Gonzalez 1984; Ku et al. 1999). One unit of enzyme activity is the capacity of the enzyme to catalyze the formation of 1 µmol of oxalacetate min⁻¹.

#### NADP-ME ACTIVITY

The NADP-ME assay medium contained 50 mM Tris–HCl (pH7.5), 1 mM MgCl₂, 1 mM MnCl₂, 1 mM EDTA, 0.5 mM NADP, 20 µL of the enzyme extract and distilled water. The reaction was started by adding 5 mM malate and the reduction of NADP⁺ was monitored by absorbance change at 340 nm (Tsuchida et al. 2001). 1 U of enzyme activity is defined as the amount of enzyme that results in the production of 1 µmol of NADPH min⁻¹.

#### MDH ACTIVITY

The assay mixture contained 100 mM Tris–HCl (pH7.5), 1 mM EDTA, 0.2 mM NADH, 20 µL of the enzyme extract and distilled water. Oxaloacetic acid with a final concentration of 2 mM was added to start the assay and the change of absorbance at 340 nm was monitored (López-Calcagno et al. 2009).

#### PPDK ACTIVITY

PPDK assay buffer consisted of 25 mM Tricine-KOH, 10 mM MgCl₂, 10 mM NaHCO₃, 10 mM DTT, 2 mM Sodium pyruvate, 5 mM (NH₄)₂SO₄, 2.5 mM K₂HPO₄,
1 mM glucose-6-phosphate, 0.2 mM NADH, 2 U NAD-MDH, 50 mM ATP, 0.5 U PEPC, 20 µL of the enzyme extract and distilled water (Hatch 1975). 1 U of PPDK activity corresponds to 1 µmol of pyruvate converted min⁻¹ at 30°C.

STATISTICAL ANALYSIS

Wheat genotypes were grouped based on the activities of each of the C₄ pathway enzymes using the hierarchical cluster analysis across the three growth stages, with the help of SPSS statistics 20.0 (IBM SPSS Statistics, USA). Variations in the activities of the PEPCase, NADP-ME, MDH and PPDK among the groups were assessed by analysis of variance (ANOVA) using SAS 8.1 (SAS Institute Inc., Cary, NC, USA). The multiple comparisons among groups were conducted by the least significant difference (LSD) test at the 0.05 level.

RESULTS

ENZYME ACTIVITIES

C₄ pathway key enzymes PEPCase, NADP-ME, MDH and PPDK existed in different activities in the flag leaves of bread wheat genotypes at the three growth stages (Table 2). The activities of PEPCase and PPDK were high at heading, started decreasing at anthesis and again increased at grain filling stage. At heading, PEPCase showed the highest mean activity (1.018 µmol. min⁻¹ mg⁻¹ protein) with a range of 0.0−2.414 µmol. min⁻¹ mg⁻¹ protein, while PPDK displayed the lowest mean activity (0.521 µmol. min⁻¹ mg⁻¹ protein) with a range of 0.005−2.117 µmol. min⁻¹ mg⁻¹ protein. At anthesis NADP-ME presented the highest mean activity (0.672 µmol. min⁻¹ mg⁻¹ protein) with a range of 0.0−2.117 µmol. min⁻¹ mg⁻¹ protein. At grain filling stage, MDH exhibited a decreasing trend at the three growth stages.
CLUSTER ANALYSIS BASED ON THE ENZYME ACTIVITIES

The 59 wheat genotypes were classified into three groups (high activity, intermediate activity and low activity) based on the activities of each of the C4 pathway enzymes across the three growth stages. Combined cluster analysis, based on the activities of the four C4 pathway enzymes, showed representative genotypes in the three groups with significant differences among wheat genotypes. The activities of the C4 pathway enzymes displayed significant differences among the three groups ($p<0.05$) at heading, anthesis and grain filling stages, with variations among genotypes within the groups (Table 3; Figures 1 to 4). The group I genotypes exhibited significantly higher mean activities than those with intermediate and low activities in group II and group III. Across the three stages, genotypes No 58, 37, 58 and 39 presented the highest PEPCase, NADP-ME, MDH and PPDK activities, respectively. The lowest activities of PEPCase, NADP-ME, MDH and PPDK were displayed by genotypes No 47, 7, 50 and 49, respectively. Based on the combined cluster analysis of the mean activities of the PEPCase, NADP-ME, MDH and PPDK, genotypes No 58, 10 and 34 showed the highest activities of the four C4 enzymes.

DISCUSSION

Activities of four key C4 pathway enzymes were investigated in the flag leaves of 59 diverse wheat genotypes at three major growth stages. The significant variations among the 59 wheat genotypes for PEPCase, NADP-ME, MDH and PPDK in flag leaves are encouraging to transform C4 enzyme genes into C3 plants to improve

<table>
<thead>
<tr>
<th>C4 enzyme</th>
<th>Growth stage</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
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<tr>
<td>PEPCase</td>
<td>Heading</td>
<td>1.74±0.57a</td>
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<td>0.19-2.15</td>
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<td>Grain filling</td>
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<td>1.16-2.76</td>
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<tr>
<td>NADP-ME</td>
<td>Heading</td>
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<td>1.37-2.67</td>
<td>1.58±0.33b</td>
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<td>0.77-1.85</td>
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<td>MDH</td>
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<td>PPDK</td>
<td>Heading</td>
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<td>2.19±0.45a</td>
<td>1.61-2.92</td>
<td>0.82±0.45b</td>
</tr>
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</table>
FIGURE 1. PEPCase activities in flag leaves of three groups of 59 wheat genotypes at heading, anthesis and grain filling stages. Group I: high activity; Group II: intermediate activity; Group III: low activity.

FIGURE 2. NADP-ME activities in flag leaves of three groups of 59 wheat genotypes at heading, anthesis and grain filling stages. Group I: high activity; Group II: intermediate activity; Group III: low activity.

their photosynthetic efficiency and ultimately the yield. Furthermore, activities of these enzymes were different with the age of flag leaf. As the key enzyme of the C₄ pathway, PEPCase displayed the highest mean activities (1.018 and 0.998 µmol min⁻¹ mg⁻¹ protein) at heading and grain filling stages, respectively. These are in agreement with the findings of Huang et al. (2013), where enzyme activities of PEPcase, NADP-MDH, NADP-ME and PPDK showed considerable variations in different organs of C₃ soybean cultivars at different growth stages. NADP-ME has
FIGURE 3. MDH activities in flag leaves of three groups of 59 wheat genotypes at heading, anthesis and grain filling stages. Group I: high activity; Group II: intermediate activity; Group III: low activity

FIGURE 4. PPDK activities in flag leaves of three groups of 59 wheat genotypes at heading, anthesis and grain filling stages. Group I: high activity; Group II: intermediate activity; Group III: low activity

been found in varied tissues of C3 plants, where it plays non-photosynthetic roles (Drincovich et al. 2001). Babayev et al. (2013) reported different activity levels of NAD-MDH, NADP-MDH and PEPCase in leaves and grains of durum wheat and bread wheat under continuous soil drought conditions. The activity of PEPcase, NADP-MDH and PPDK were also reported to increase with the ages of flag leaves of super high-yield hybrid rice and maize (Ana-Luz et al. 1994; Yang et al. 2003; Zhang et al. 2007). The variation in the activities of the C4 enzymes at the three stages could be due to their photosynthetic performance under field conditions. It has been reported that the activities of...
the enzymes of the main metabolic pathways (glycolysis, Krebs cycle and oxidative pentose phosphate pathway) have increased under the influence of the unfavorable environmental conditions (Riccardi et al. 1998; Umeda et al. 1994).

Although we found low level of these enzymes in the flag leaves of the studied wheat genotypes as compared to other transgenic C3 plant, but it is confirmed that these enzymes are existing which is a positive sign. For example, Zhang et al. (2014) reported 4.3- and 2.1-fold higher activities of PEPC and PPDF in transgenic wheat lines than in the untransformed control lines, respectively. Maize C4-specific PEPC activity of 1.40-fold greater than that of untransformed plants was also reported in the flag leaves of transgenic wheat plants (Lin et al. 2012). In a study by Wang et al. (2002), higher activities of C4 pathway enzymes in both flag leaves and lemmas of super high-yield hybrid rice (Liangyoupeijiu) were reported. The activity of the three C4 enzymes increased at early stages and gradually decreased at grain filling stage. The photosynthetic activity of flag leaves is especially important during grain filling when the older leaves begin senescing (Reynolds et al. 2000).

CONCLUSION

The tested wheat genotypes exhibited significant differences in the activities of the C4 pathway enzymes. Therefore, it is possible that genotypes containing high enzyme activities could be an indicator for breeding wheat with high photosynthetic efficiency. This study can also be helpful for food security in future.

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REFERENCES


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