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Activities of C_4 Photosynthetic Pathway Enzymes in Different Bread Wheat Genotypes under Field Conditions

(Aktiviti Laluan Fotosintesis Enzim C_4 dalam Genotip Gandum yang Pelbagai pada Keadaan Lapangan)

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ABSTRACT

The activities of key C_4 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 C_4 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: C_4 enzymes; flag leaf; photosynthetic efficiency; transgenic plants; wheat

ABSTRAK

Aktiviti enzim fotosintesis C_4 yang utama termasuk fosfoenol piruvat karboksilase (PEPcase), enzim NADP-malik (NADP-ME), malat dehidrogenase (MDH) dan piruvat fosfat dikinase (PPDK) diasai pada daun bendera dalam tiga peringkat pertumbuhan utama (kepala, antesis dan isi bijirin) dalam kalangan 59 genotip gandum musim sejuk yang ditanam dalam keadaan lapangan berbeza. Semua enzim C_4 dinyatakan pada daun bendera dan pengaktifan mereka menunjukkan pelbagai variasi berhubung dengan peringkat pertumbuhan yang berbeza di semua genotip. PEPCase, NADP-ME dan MDH menunjukkan aktiviti min tertinggi sebanyak 1.018, 0.758 dan 0.731 µmol. protein $min^{-1}.mg^{-1}$ di peringkat tajuk, masing-masing; manakala PPDK menunjukkan aktiviti min tertinggi (0.888 µmol. $min^{-1}.mg^{-1}$ protein) pada peringkat pengisian bijian. Kegiatan PEPcase dan PPDK lebih tinggi pada peringkat tajuk, menurun pada antesis dan sekali lagi meningkat pada peringkat pengisian bijirin, sementara NADP-ME dan MDH menunjukkan penurunan pada tiga tahap. Keputusan kajian ini bernilai dan bermanfaat untuk penyelidik gandum dalam meningkatkan kapasiti fotosintesis gandum.

Kata kunci: Daun bendera; enzim C_4; gandum; kecekapan fotosintesis; tumbuhan transgenik

Introduction

Based on the differences in the mechanism of CO_2 assimilation, green plants can be categorized into C_3, C_4 and Crassulacean acid (CAM). Under unfavorable environmental conditions, C_4 plants have higher efficiency of CO_2 fixation than C_3 by cooperative action of C_4 enzyme system such as phosenolpyruvate carboxylase (PEPcase), nicotinamide adenine dinucleotide-phosphate malic enzyme (NADP-ME), malate dehydrogenese (MDH) and pyruvate orthophosphate dikinase (PPDK) (Ku et al. 1999). The C_4 pathway is a complex trait that has evolved from ancestral C_3 plants in response to changes in environmental conditions that caused a decrease in CO_2 availability (Christin et al. 2010; Ludwig et al. 2012). Therefore, many productive crops such as maize and foxtail millet use the C_4 photosynthetic pathway. However, some important major crops such as wheat and rice are C_3 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 C_3 plant and since then, CO_2-refixation function has been given a great concern.

The transfer of C_4 traits to C_3 plants is thus one strategy being adopted for improving the photosynthetic performance and raising the potential yield of C_3 plants (Surridge 2002). Several previous studies succeeded in introducing the maize C_4-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). C_4-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 CO_2 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 C_4 enzymes have been transferred into C_3 plants, only few were successful in improving the photosynthetic efficiency (Zhang et al. 2009). Additionally, CO_2 metabolism inside the chloroplast of C_3 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 thylalkoid membranes. Over-expression of maize NADP-malic enzyme in rice was reported to negatively affect chlorophyll content and growth while enhancing photoinhibition (Tsuchida et al. 2001). It is therefore, questionable to improve photosynthesis and yield of C_3 plants by transferring C_4 enzymes into C_3 plants to induce over-expression (Zhang et al. 2007). Thus, selecting C_3 plant with relatively high expression of C_4 enzymes is an alternative way to enhance photosynthesis in C_3 plants. Knowledge about variation in activities of C_4 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 C_4 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 in 2014-2015 and 2015-2016. Each genotype was planted in 3 rows of 1.67 m length, with 25 cm rows spacing and 6.7 cm plant spacing.

Assays for the activities of C_4 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 $MgCl_2$, 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:

$$\text{Enzyme activity} = \frac{(\Delta A_{\text{sample}} - \Delta A_{\text{blank}}) \times V_t \times 10^6}{\varepsilon \times \Delta\text{time} \times V_s \times \text{Protein conc.}},$$

where: ΔA_{sample}: Change in the absorbance from the beginning to the end of measurement period; ΔA_{blank}: Sample containing all the reagents except the enzyme; ΔTime: Time interval the absorbance was measured (min); V_t: Total volume (L); V_s: Sample volume (mL); Protein conc: Protein concentration (mg/mL); 10^6: This converts the moles of ε to mmoles.

PEPCase activity

Phosphoenolpyruvate carboxylase activity was assayed in a mixture containing 50 mM tricine-KOH (pH8.0), 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 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 $MgCl_2$, 1 mM $MnCl_2$, 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 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 $MgCl_2$, 10 mM $NaHCO_3$, 10 mM DTT, 2 mM Sodium pyruvate, 5 mM $(NH_4)_2SO_4$, 2.5 mM K_2HPO_4,

TABLE 1. Name, origin and production region of the 59 winter wheat genotypes

Code	Genotype	Origin	Production Region	Code	Genotype	Origin	Production Region
1	Linhan 51329	Shanxi	NWWR	31	Zhongyu 8	Henan	HHWWR
2	Linhan 536	Shanxi	NWWR	32	Bainong 160	Henan	HHWWR
3	Luohan 2	Henan	HHWWR	33	Luomai 21	Henan	HHWWR
4	Luohan 6	Henan	HHWWR	34	Lunxuan 061	Beijing	NWWR
5	Shijiazhuang 8	Hebei	NWWR	35	Luo 9908	Henan	HHWWR
6	Zhonghan 110	Beijing	NWWR	36	Xinyuan 958	Henan	HHWWR
7	Youmai 2	Shandong	NWWR	37	Shaanken 81	Shaanxi	HHWWR
8	Jinmai 47	Shanxi	NWWR	38	Jinmai 33	Shanxi	NWWR
9	Changwu 135	Shaanxi	HHWWR	39	Han 6172	Hebei	NWWR
10	Shaan 229	Shaanxi	HHWWR	40	Huaimai 21	Jiangsu	HHWWR
11	Xiaoyan 6	Shaanxi	HHWWR	41	Yunong 982	Henan	HHWWR
12	Shaanhan 187	Shaanxi	HHWWR	42	Xifeng 20	Gansu	HHWWR
13	Pubing 143	Shaanxi	HHWWR	43	Lunxuan 715	Beijing	NWWR
14	Liken 2	Shaanxi	HHWWR	44	Shijiazhuang 54	Hebei	NWWR
15	Luohan 3	Henan	HHWWR	45	Nongda 198	Beijing	NWWR
16	Jing 411	Beijing	NWWR	46	Kedong 81	Beijing	NWWR
17	Jinan 18	Shandong	NWWR	47	Linfen 10	Shanxi	NWWR
18	Heng 95 Guan 26	Hebei	NWWR	48	Fengkang 5	Beijing	NWWR
19	Tongmai 3	Shaanxi	HHWWR	49	Changfeng 1	Beijing	NWWR
20	Mianyang 11	Sichuan	SWWR	50	Jingwang 9	Beijing	NWWR
21	Taishan 5	Shandong	NWWR	51	Jingdong 1	Beijing	NWWR
22	Jining 18	Shandong	NWWR	52	Jinmai 21	Shanxi	NWWR
23	Xinmai 13	Henan	HHWWR	53	Jimai 23	Hebei	NWWR
24	Xinmai 18	Henan	HHWWR	54	Hanxuan 10	Shanxi	NWWR
25	Zhoumai 16	Henan	HHWWR	55	Hanxuan 1	Shanxi	NWWR
26	Xinong 2000-7	Shaanxi	HHWWR	56	Lumai 1	Shandong	NWWR
27	Shaanmai 150	Shaanxi	HHWWR	57	Wenmai 6	Henan	HHWWR
28	Yuanfeng 139	Shaanxi	HHWWR	58	Aifeng 3	Shaanxi	HHWWR
29	Fengchan 3	Shaanxi	HHWWR	59	Yunhan 618	Shanxi	NWWR
30	Xinong 979	Shaanxi	HHWWR				

NWWR: Northern Winter Wheat Region; HHWWR: Huang-Huai Winter Wheat Region; SWWR: Southwestern Winter Wheat Region; Origin: Name of province or state

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 C_4 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

C_4 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, while NADP-ME and MDH exhibited a decreasing trend at the three growth stages. 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.

TABLE 2. Mean C_4 enzyme activities in the flag leaves of 59 wheat genotypes at three growth stages

Growth stages	Mean	PEPCase	NADP-ME	MDH	PPDK
Heading	Mean±SD	1.018±0.81a	0.758±0.80a	0.731±0.67a	0.521±0.49b
	Range	0.02-2.414	0.002-2.666	0.016-2.238	0.005-2.117
Anthesis	Mean±SD	0.589±0.71b	0.672±0.55a	0.616±0.71a	0.410±0.42b
	Range	0.006-2.153	0.032-1.846	0.026-2.490	0.024-2.353
Grain filling	Mean±SD	0.988±0.79a	0.652±0.48a	0.552±0.61a	0.888±0.74a
	Range	0.077-2.764	0.03-2.250	0.041-2.473	0.014-2.916

Data are presented as mean±SD (standard error)
Enzyme activity is expressed as μmol. $min^{-1}.mg^{-1}$ protein
Lowercase letters represent significant differences among the three groups ($p<0.05$)

$min^{-1}.mg^{-1}$ protein) with a range of 0.032−1.846 μmol. $min^{-1}.mg^{-1}$ protein, whereas the lowest mean activity was recorded for PPDK (4.10 μmol. $min^{-1}.mg^{-1}$ protein) with a range of 0.024−2.353μmol. $min^{-1}.mg^{-1}$ protein. At grain filling stage, PEPcase exhibited the highest mean activity (0.998 μmol. $min^{-1}.mg^{-1}$ protein) with a range of 0.077−2.764 μmol. $min^{-1}.mg^{-1}$ protein, while the lowest mean activity was displayed by MDH (0.552 μmol. $min^{-1}.mg^{-1}$ protein) with a range 0.041−2.473 μmol. $min^{-1}.mg^{-1}$ protein.

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 C_4 pathway enzymes across the three growth stages. Combined cluster analysis, based on the activities of the four C_4 pathway enzymes, showed representative genotypes in the three groups with significant differences among wheat genotypes. The activities of the C_4 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 C_4 enzymes.

DISCUSSION

Activities of four key C_4 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 C_4 enzyme genes into C_3 plants to improve

TABLE 3. Mean C_4 pathway enzyme activities in the three groups of 59 wheat genotypes at three growth stages

C_4 enzyme	Growth stage	Grouping of 59 wheat genotypes					
		Group I		Group II		Group III	
		Mean ±SD	Range	Mean ±SD	Range	Mean ±SD	Range
PEPCase	Heading	1.740±0.57a	0.31-2.41	1.373±0.38b	0.77-1.95	0.213±0.16c	0.02-0.579
	Anthesis	1.329±0.66a	0.19-2.15	0.219±0.17b	0.02-0.49	0.116±0.12b	0.006-0.39
	Grain filling	1.919±0.45a	1.16-2.76	0.452±0.26b	0.13-0.83	0.416±0.25b	0.08-1.01
NADP-ME	Heading	2.011±0.43a	1.37-2.67	1.548±0.33b	0.98-2.01	0.234±0.22c	0.002-0.78
	Anthesis	1.474±0.33a	0.77-1.85	0.615±0.51b	0.03-1.51	0.481±0.48b	0.08-1.80
	Grain filling	1.383±0.61a	0.42-2.25	0.621±0.16b	0.38-0.93	0.473±0.28b	0.03-1.08
MDH	Heading	1.781±0.35a	1.18-2.24	0.559±0.26b	0.07-1.15	0.075±0.05c	0.02-0.19
	Anthesis	1.805±0.47a	0.93-2.49	0.356±0.28b	0.06-1.37	0.087±0.05c	0.03-0.23
	Grain filling	1.456±0.77a	0.34-2.47	0.326±0.17b	0.05-0.82	0.220±0.11b	0.04-0.40
PPDK	Heading	1.412±0.34a	0.93-2.12	0.523±0.09b	0.41-0.69	0.183±0.13c	0.01-0.40
	Anthesis	1.111±0.56a	0.41-2.35	0.333±0.16b	0.03-0.59	0.225±0.18c	0.02-0.64
	Grain filling	2.191±0.45a	1.61-2.92	0.827±0.45b	0.27-1.52	0.493±0.39c	0.01-1.67

Data are presented as the mean±SD (standard deviation).
Group I: high activity; Group II: intermediate activity; Group III: low activity. Lowercase letters represent significant differences among the three groups ($p<0.05$)

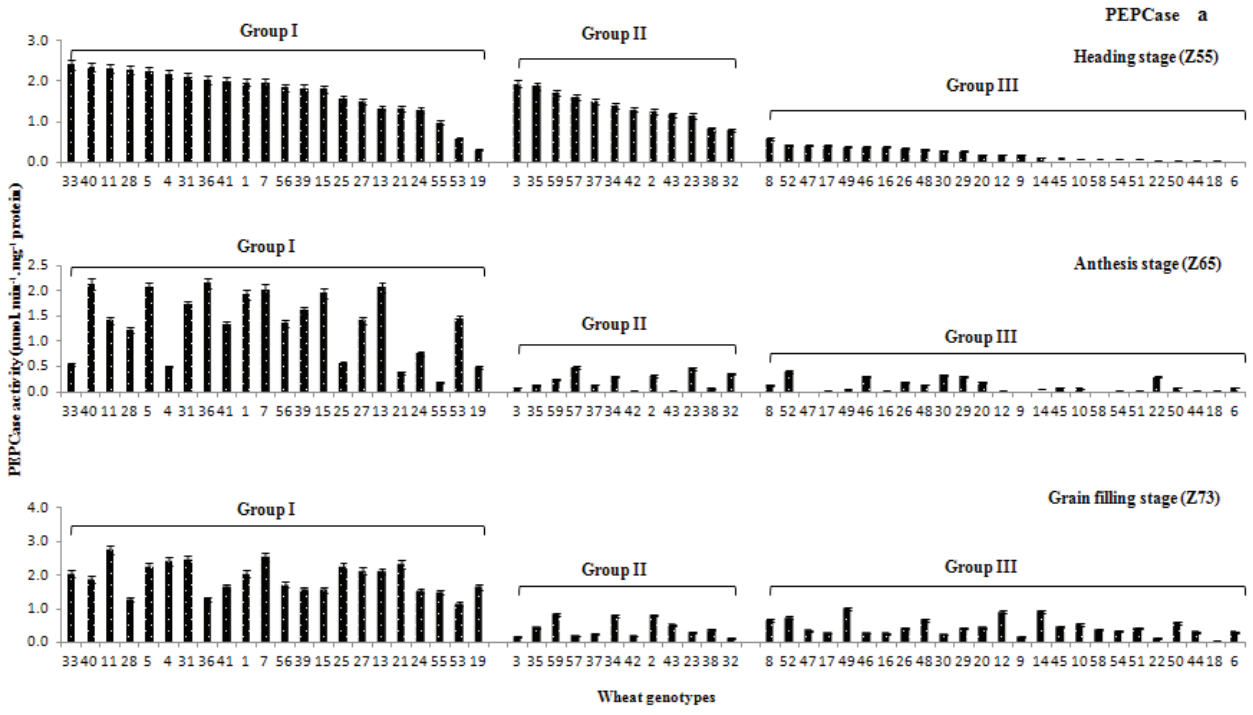

FIGURE 1. PEPCase activities in flag leaves of three groups of 59 wheat genotypes at heading, anthesis and grain filling stages. Group I: high activity; Group II: intermediate activity; Group III: low activity

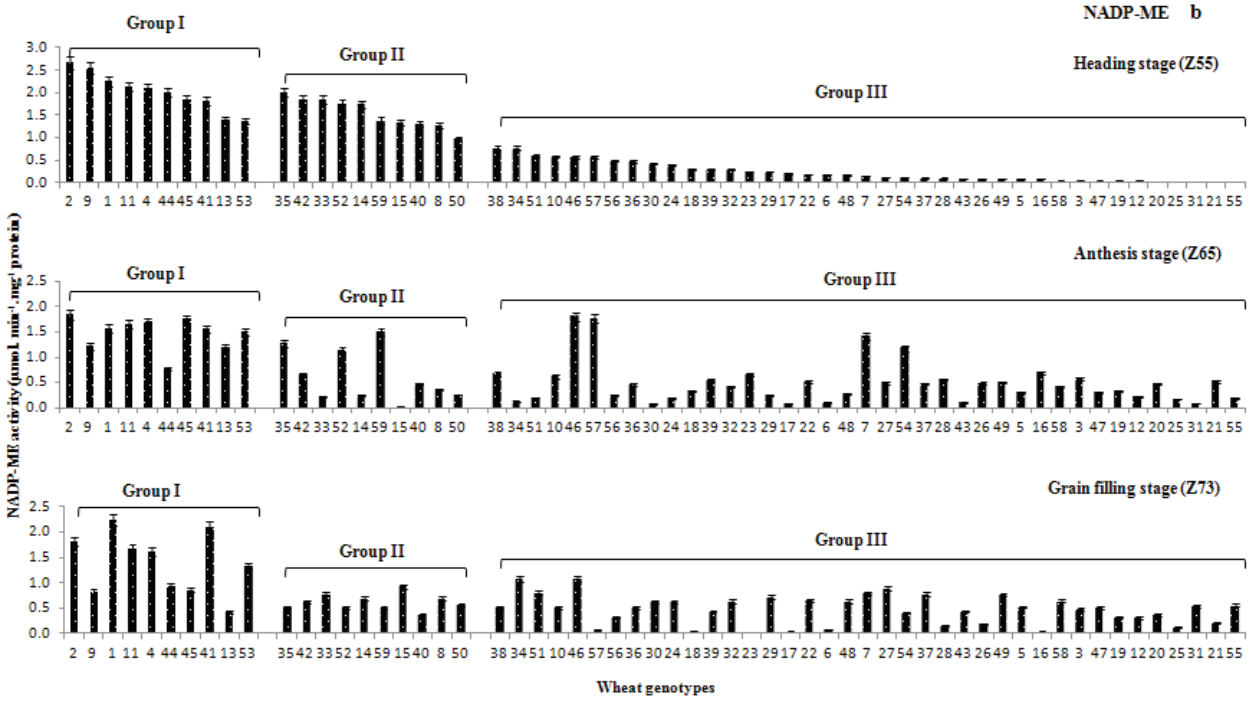

FIGURE 2. NADP-ME activities in flag leaves of three groups of 59 wheat genotypes at heading, anthesis and grain filling stages. Group I: high activity; Group II: intermediate activity; Group III: low activity

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 C_4 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 C_3 soybean cultivars at different growth stages. NADP-ME has

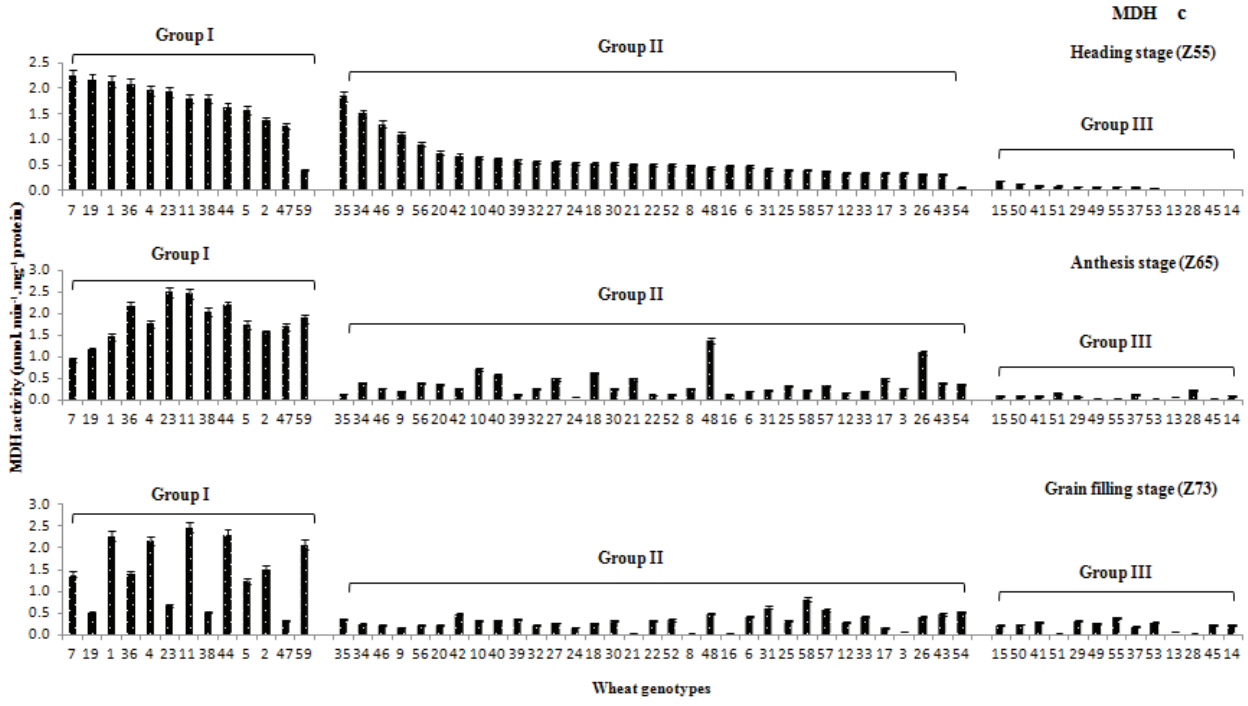

FIGURE 3. MDH activities in flag leaves of three groups of 59 wheat genotypes at heading, anthesis and grain filling stages. Group I: high activity; Group II: intermediate activity; Group III: low activity

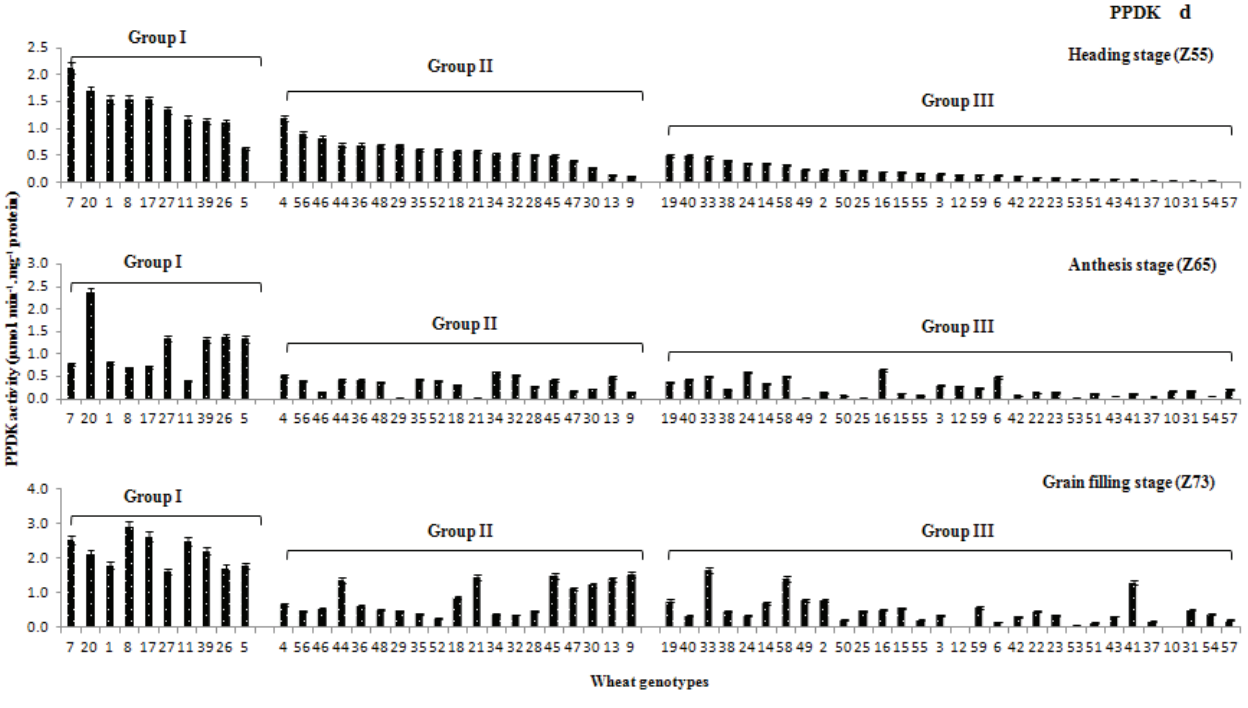

FIGURE 4. PPDK activities in flag leaves of three groups of 59 wheat genotypes at heading, anthesis and grain filling stages. Group I: high activity; Group II: intermediate activity; Group III: low activity

been found in varied tissues of C_3 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 C_4 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 C_3 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 PPDK in transgenic wheat lines than in the untransformed control lines, respectively. Maize C_4-specific PEPCase 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 C_4 pathway enzymes in both flag leaves and lemmas of super high-yield hybrid rice (Liangyoupeijiu) were reported. The activity of the three C_4 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 C_4 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.

Acknowledgements

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