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Research Note

Effects of sucrose and ammonium nitrate on phosphoenolpyruvate carboxylase and ribulose-1, 5-bisphosphate carboxylase activities in wheat ears

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Abstract

The effects of sucrose and ammonium nitrate (NH_4NO_3) levels on the activities of phosphoenolpyruvate carboxylase (PEPC) and ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) in ear organs (glume and grain), and on grain mass and nitrogen concentration were investigated using a culture of detached ears of wheat (*Triticum aestivum* L.). Results showed that PEPC and Rubisco activities in the glume and grain, as well as grain mass and grain nitrogen accumulation differentially increased as sucrose and NH_4NO_3 levels increased. Grain N concentration progressively increased as the NH_4NO_3 level increased, but decreased as sucrose level increased. Correlation analysis showed that PEPC activity in the glume and grain was positively correlated with grain N concentration. These results suggest that PEPC and Rubisco can be manipulated by external C and N supplies, and PEPC may play an important role in grain protein accumulation.

Keywords: Phosphoenolpyruvate carboxylase, Ribulose-1, 5-bisphosphate carboxylase, Detached ears culture, Sucrose, NH_4NO_3, Wheat.
Abbreviations: C – Carbon; DAC – Days after culture; N – Nitrogen; NH_4NO_3 –Ammonium nitrate; PEP – Phosphoenolpyruvate; PEPC – Phosphoenolpyruvate carboxylase; Rubisco – Ribulose-1, 5-bisphosphate carboxylase/oxygenase; WSC –Water-soluble carbohydrate.

Introduction

Ear organs make a substantial contribution to grain yield in wheat (Araus et al., 1993), especially during drought (Abbad et al., 2004). The importance of the ear as a source of assimilates seems to be related to its better photosynthetic performance compared to the flag leaf under stress, including a higher water use efficiency, delayed senescence, higher drought or heat tolerance, the refixation of the CO_2 respired by developing grains and some degree of C_4 metabolism (either constitutive or drought/heat -induced) (Araus et al., 1993; Abbad et al., 2004). Among these characters, at least the last three are involved in phosphoenolpyruvate carboxylase (PEPC) (Tambussi et al., 2007). PEPC catalyzes irreversible β-carboxylation of phosphoenolpyruvate (PEP) in the presence of HCO_3^- and Mg^{2+} to yield oxaloacetate and inorganic phosphate. PEPC is widely distributed in all photosynthetic organisms including vascular plants, algae, cyanobacteria, and photosynthetic bacteria, and also in nonphotosynthetic bacteria and protozoa (Izui et al., 2004). An abundance of PEPC has been reported in the chaff and grain of wheat (Singal et al., 1986; Araus et al., 1993). In vascular plants, the reaction catalyzed by PEPC is the primary fixation step of photosynthetic CO_2 assimilation in C_4 photosynthesis and crassulacean acid metabolism (Izui et al., 2004). In most nonphotosynthetic tissues and in the leaves of C_3 plants, the primary function of PEPC is anaplerotic, replenishing the tricarboxylic acid cycle with intermediates that are withdrawn for a variety of biosynthetic pathways and nitrogen (N) assimilation (Izui et al., 2004; Chollet et al., 1996). PEPC also plays a role in the provision of malate in guard cells and legume root nodules (Vidal and Chollet, 1997). PEPC in leaves and roots is regulated allosterically by various metabolites such as PEP, glucose-6-phosphate (an activator), malate (an inhibitor) (Wedding et al., 1989), sucrose (Hdider and Desjardins, 1994; Sima et al., 2001), and N (Van Quy et al., 1991). Sucrose (Hdider and Desjardins, 1994; Sima et al., 2001) and N (Sharma and Sirohi, 1988; Van Quy et al., 1991, Pasqualini et al., 2001) are reported to enhance PEPC activity of leaves and roots in wheat and other C_3 plants. However, little is known about their effects on PEPC activity in ear organs of wheat. Our previous study showed that there was high PEPC activity in ear organs of wheat, and PEPC activity in the glume and grain were positively correlated with grain mass and protein concentration (Zhang et al., 2008). We hypothesized that PEPC may participate in manipulating grain carbon (C) and N metabolism and protein synthesis; to support this point, a detailed study was needed. Culturing of detached ears is a very effective method to clarify the mechanisms of modulation and accumulation of carbohydrate and protein in cereal grains because the environmental

conditions and source supply level can be easily controlled, and it has been used in wheat (Singh and Jenner, 1983; Barlaw et al., 1983; Cervantes et al., 1989), barley (Corke and Atsmon, 1988) and rice (Sasaki et al., 2005). This study applied the method to investigate the effects of exogenous sucrose and NH_4NO_3 concentrations on the activities of PEPC and ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) in ear organs (glume and grain) and the accumulation of C and N in grain. We also analyzed their possible relationships to provide some reference for simultaneous improvement of grain weight and protein concentration in wheat.

Results and discussion

Effect of sucrose supply on PEPC and Rubisco activities in wheat ears

It is known that there is competition for C assimilates during synthesis of starch and protein (Banziger et al., 1994), therefore, sufficient sucrose was added to the culture solution. Table 1 showed that water-soluble carbohydrate (WSC) concentration in the peduncle and chaff increased as sucrose level increased. However, WSC concentration in the grain only increased up to 60 g l^{-1}sucrose, suggesting that excess sucrose was stored in the peduncle and chaff and not transported to the grain when the supply of sucrose was too high. The dry mass of the peduncle, chaff and grain all increased as sucrose level increased up to 60 g l^{-1}, but above that, grain dry mass significantly decreased while dry mass of the peduncle and chaff were not significantly increased. Other studies also showed that beyond an optimal level of sucrose, there is no further increase in starch synthesis or dry mass in the grain of wheat (Barlow et al., 1983; Cervantes et al., 1989), or rice (Sasaki et al., 2005), indicating that dry matter accumulation in the grain was determined partially by assimilate supply and partially by the storage capacity of the grain. Grain N concentration decreased with the increase in sucrose level, which may be simply the result of the dilution effect caused by dry mass increase in the grain (Austin et al., 1980; Calderini et al., 1995). Grain N accumulation increased when sucrose increased from 0 to 40 g l^{-1}. However, N accumulation significantly decreased when sucrose was above 40 g l^{-1} (Table 1), which may be mainly due to the decrease in grain protein concentration. PEPC activity in the glume and grain increased as sucrose concentration increased, however it began to decrease when sucrose concentration was over 40 g l^{-1} (Fig. 1). In the glume and grain, cytosolic sucrose degradation may provide phosphoglycerate that increases pH, thus inducing PEPC activation (Vidal and Chollet, 1997). The inhibition of PEPC at a high sucrose concentration suggested that sucrose led to an enhanced phosphorylation level, thus protecting the enzyme and desensitising it to malate (Krömer et al., 1996). Rubisco is the key enzyme for C_3 photosynthesis. This enzyme in the glume and grain increased as sucrose level increased and peaked at 80 g l^{-1} and 60 g l^{-1} sucrose, respectively (Fig. 1). This may be the reason that sucrose degradation provided phosphoglycerate for C_3 photosynthesis thus activating Rubisco. In addition, the CO_2 from anaplerotic supply by PEPC may also induce Rubisco activation. That the peak of enzyme activity occurred at a different sucrose concentration for the glume than for the grain may be due to different sucrose concentrations between these two sites. The WSC concentration in the grain peaked at 60 g l^{-1}sucrose, while in the glume it peaked at 80 g l^{-1}sucrose (Table 1).

Effect of NH_4NO_3 supply on PEPC and Rubisco activities in wheat ears

Post-anthesis N uptake by crops has a significant influence on grain N accumulation (Oscarson et al., 1995). When increased concentrations of NH_4NO_3 were added to the culture solution, grain dry mass did not significantly increase. However, N concentrations in the peduncle, chaff, and grain did all significantly increased with the increase in NH_4NO_3 level, and grain N accumulation also significantly increased (Table 1). Studies with other plant species have also shown that, under conditions of high N availability, amino acids or proteins in plant tissue increase substantially (Corke and Atsmon, 1988; Warren and Adams, 2000; Ruan et al., 2010). The grain was the strongest N sink within the ear, and the flow of N entering grain was not restricted (Barlow et al., 1983). Decreasing the C/N ratio in grain by increasing N supply may promote more C being partitioned to compounds containing N and result in higher N concentration. This interpretation is supported by the change of PEPC activity. The activities of PEPC and Rubisco in the glume and grain increased as NH_4NO_3 level increased, and the enzyme activities in the glume peaked at 2.28 g l^{-1} NH_4NO_3, while those in the grain peaked at 1.14 g l^{-1} NH_4NO_3 (Fig. 2). PEPC consumes a-ketoglutarate, a product of the Krebs cycle, to provide C skeletons for the synthesis of amino acids and protein. Addition of N may both lead to an activation of PEPC by phosphorylation (Van Quy et al., 1991; Champigny and Foyer, 1992) and induce gene expression of this enzyme (Sugiharto and Sugiyama. 1992; Pasqualini et al., 2001). An increased PEPC activity at high N-nutrition should therefore indicate a higher demand for such C skeletons. The increase in N supply and the increased PEPC activity promoted grain protein accumulation. In addition, the increase in PEPC activity may further increase Rubisco activity by anaplerotic C flow (i.e., malate), and may promote starch synthesis. But there was no significant difference in grain weight among NH_4NO_3 treatments, which showed that the C flow through the anaplerotic pathway catalysed by PEPC may mostly enter into protein synthesis. The correlation analysis showed that the PEPC activity in the glume (r=0.82) and grain (r=0.54) was positively correlated with grain nitrogen (or protein) concentration (data not shown). A strong correlation between PEPC and protein content in seeds was also reported in soybean (Smith et al., 1989; Sugimoto et al., 1989), *V. faba* (Golombek et al., 2001), *V. narbonensis* (Rolletschek et al., 2004) and our previous study in wheat (Zhang et al., 2008). Ectopic expression of phosphoenolpyruvate carboxylase in *V. narbonensis* seeds also resulted in an increased seed protein content (Rolletschek et al., 2004; Radchuk et al., 2007). Using an electron-microscopic immunolabeling technique, Araus et al. (1993) detected a substantial amount of PEPC in protein bodies of immature durum wheat (*Triticum durum*) grains, where it might contribute to amino acid and protein biosynthesis during grain development. In our study, the positive correlations of PEPC activities of the glume and grain with final protein concentration of grain suggested that PEPC in the ear parts may be involved in N metabolism and contribute to grain protein synthesis in wheat. In this study, NH_4NO_3 was used for the N source. In another study, Pasqualini et al. (2001) used different N sources. They showed that the PEPC activity in Alfalfa (Medicago *sativa* L.) roots was highest with NO_3^- nutrition, lowest with NH_4^+, and intermediate in plants that were fed mixed N sources. The effects of different N sources on PEPC activity in ear organs of wheat need to be studied in the future. In conclusion, our results showed that grain C and N accumulation responded to

Table 1. Effect of sucrose and NH_4NO_3 concentrations on final dry mass, water-soluble carbohydrate (WSC) and N concentration in tissues of detached wheat ears.

Treatment	Concentration ($g\ l^{-1}$)	Dry mass ($mg\ culm^{-1}$)			WSC concentration ($mg\ g^{-1}$)			N concentration ($mg\ g^{-1}$)			Grain N accumulation ($mg\ culm^{-1}$)
		Peduncle	Chaff	Grain	Peduncle	Chaff	Grain	Peduncle	Chaff	Grain	
Sucrose	0	160.8 ± 4.7 b	286.9 ± 8.3 b	395.9 ± 5.0 e	33.9 ± 1.0 e	51.1 ± 0.7 e	175.5 ± 1.6 c	12.7 ± 0.1 a	27.7 ± 0.1 a	36.4 ± 0.2 a	14.4 ± 0.2 d
	20	165.6 ± 5.3 b	299.2 ± 10.0 b	913.8 ± 6.3 d	69.1 ± 1.3 d	75.3 ± 1.0 d	210.6 ± 2.3 b	8.2 ± 0.1 b	10.1 ± 0.1 b	29.0 ± 0.3 b	26.5 ± 0.4 a
	40	167.0 ± 5.3 b	300.5 ± 6.3 b	1064.4 ± 8.0 b	77.2 ± 1.2 c	140.6 ± 1.6 c	226.5 ± 1.7 a	6.2 ± 0.1 c	6.4 ± 0.2 c	24.7 ± 0.1 c	26.3 ± 0.3 a
	60	193.4 ± 6.3 ab	543.1 ± 11.0 a	1167.4 ± 8.7 a	136.1 ± 1.5 b	365.3 ± 2.1 b	234.1 ± 1.0 a	5.5 ± 0.2 c	4.8 ± 0.1 d	19.6 ± 0.1 d	22.9 ± 0.3 b
	80	240.1 ± 8.0 a	586.0 ± 8.7 a	1022.1 ± 7.3 c	232.7 ± 1.6 a	426.1 ± 1.9 a	223.8 ± 2.6 a	7.3 ± 0.3 b	5.1 ± 0.2 d	18.9 ± 0.2 d	19.3 ± 0.3 c
NH_4NO_3	0	179.9 ± 5.0 a	441.3 ± 4.7 a	1029.0 ± 13.0 a	98.8 ± 0.4 b	368.6 ± 2.9 a	190.8 ± 2.4 c	4.7 ± 0.0 d	4.9 ± 0.1 c	14.4 ± 0.1 d	14.8 ± 0.3 d
	0.57	171.8 ± 5.7 a	347.8 ± 6.7 b	1048.0 ± 8.0 a	62.3 ± 0.8 d	189.9 ± 0.7 c	158.2 ± 1.8 d	5.1 ± 0.1 c	6.3 ± 0.1 b	21.6 ± 0.2 c	22.6 ± 0.4 c
	1.14	167.0 ± 5.3 a	300.5 ± 6.3 c	1064.4 ± 8.0 a	77.2 ± 1.2 c	140.6 ± 1.6 d	226.5 ± 1.7 b	6.2 ± 0.1 b	6.4 ± 0.2 b	24.7 ± 0.1 b	26.3 ± 0.3 b
	2.28	190.2 ±6.3 a	371.7 ± 9.0 b	1059.5 ± 11.3 a	105.7 ± 1.6 a	269.7 ± 2.9 b	244.2 ± 2.1 a	12.5 ± 0.1 a	8.7 ± 0.0 a	29.9 ± 0.1 a	31.7 ± 0.4 a

Values are means ± SE. The values with different letters indicate significant differences among sucrose or NH_4NO_3 treatments at $p<0.05$.

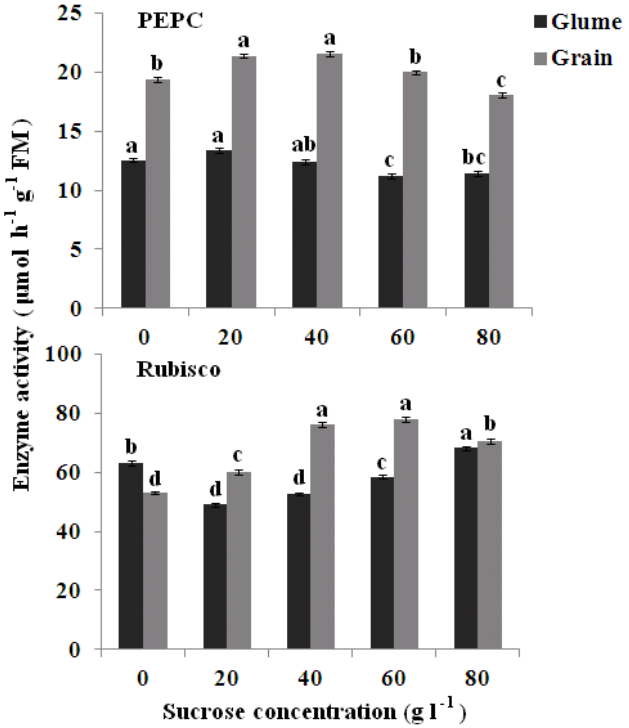

Fig 1. Effect of sucrose concentrations in culture media on the activities of PEPC and Rubisco in wheat glume and grain. Values with different letters indicate significant differences among sucrose treatments for glume and grain at $p<0.05$; FM-Fresh mass.

external sucrose and NH_4NO_3 supply. Therefore, sufficient C and N supplies are required to maintain higher grain mass and N accumulation in wheat grain. The increase in PEPC and Rubisco activities under higher sucrose and NH_4NO_3 levels indicated that PEPC and Rubisco can be manipulated by external C and N supplies, and PEPC may play an important role in the C and N metabolism in ear organs of wheat, especially in protein synthesis. Further study will be conducted by using C^{14} and N^{15} in the culture solution to clarify the contribution of C flow through the anaplerotic pathway catalysed by PEPC of non-leaf organs to protein accumulation, and the detailed physiological and biochemical mechanisms of PEPC participating in protein synthesis.

Materials and methods

Plant materials

The winter wheat cultivar, Shijiazhuang 8, was planted on 15 October 2008 at the Experimental Farm of China Agricultural University, Beijing, China. Irrigation was applied at jointing and flowering stages at the rate of 750 m^3 ha^{-1}. Fertilizer was applied before planting to provide 157.5 kg N ha^{-1} (as urea), 60.7 kg P ha^{-1} (as ammonium monoacid phosphate), and 90.4 kg K ha^{-1} (as potassium sulfate).

Detached ears culture

Detached ears were cultured according to the method of Singh and Jenner (1983) and Lee et al. (1989), with slight modifications. Primary culms flowering on the same day with similar plant height and ear length were tagged. Then, fifteen days after flowering, the tagged culms were detached below the peduncular node, surface sterilized with a sodium hypochlorite solution (0.5% available chlorine), and then recut 2 cm under sterile distilled H_2O. The flag leaf was removed to leave 3 cm of leaf sheath above the peduncular node. Detached ears were inserted through cotton plugs into sterile flasks containing 120 ml of sterile culture media. The basic culture media was prepared according to Lee et al. (1989) except for varying NH_4NO_3 and sucrose concentrations. In the sucrose regulation experiment, the concentrations of sucrose were 0, 20, 40, 60, and 80 g l^{-1}, and that of NH_4NO_3 was 1.14 g l^{-1}. In the NH_4NO_3 regulation experiment, the concentrations of NH_4NO_3 were 0, 0.57, 1.14, and 2.28 g l^{-1}, and that of sucrose was 40 g l^{-1}. The treatments were arranged as a randomized complete block design with three replications. Each replication included 16 flasks, and each flask contained one plant. The flasks containing the detached ears were placed in a circular water bath at 2~4 ℃ in a growth chamber with 16 h of light (high output, cool-white fluorescent tubes providing about 160 μmol m^{-2} s^{-1} light at the level of ears), at a constant air temperature of 24 ℃.

Assays of PEPC and Rubisco activities

At 5 and 10 days after culturing (DAC), 5 ears of each replicate were harvested, immediately frozen in liquid nitrogen and stored at -80 ℃ until the assay of PEPC and Rubisco activities of the glume and grain (whole grain). The extraction and assays of Rubisco and PEPC activities were carried out as described previously in Xu et al. (2003). Magnesium and bicarbonate were included in both the extraction and the assay media to maintain the enzyme in the active form. The enzyme was preincubated in the assay medium at 25 ℃ to measure the maximum activity.

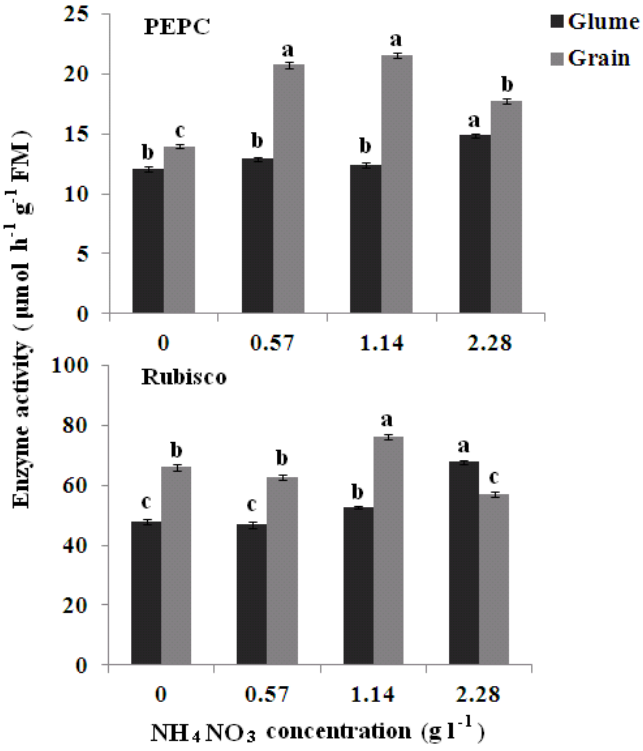

Fig 2. Effect of NH_4NO_3 concentrations in culture media on the activities of PEPC and Rubisco in wheat glume and grain. Values with different letters indicate significant differences among NH_4NO_3 treatments for glume and grain at $p<0.05$; FM-Fresh mass.

Measurement of dry mass and the concentrations of WSC and N

At maturity, six ears of each replicate were harvested and separated into peduncle, chaff (spike axis and kernel husks) and grain; all samples were oven-dried at 80 ℃ to a constant mass and weighed, then the three replicates of each treatment were mixed, ground and preserved. The concentrations of water-soluble carbohydrate (WSC) in the peduncle, chaff and grain were measured on 300 mg of dry powder taken at maturity by extracting three times with 10 ml (v/v) distilled water at 80 ℃ for 30 min, and the supernatants were combined and measured by the phenol-sulphuric acid method (Dubois et al. 1956) using sucrose as the standard. The N concentrations of the peduncle, chaff and grain were measured on 200 mg of dry powder, taken at maturity, using the standard macro-Kjeldahl procedure (Nitrogen Analysis System, Büchi, Switzerland).

Data analysis

For each parameter, three runs were made for each sample and the mean was used for statistical analyses. Data were analyzed using the analysis of variance (SAS, 1997). Duncan's multiple range test was used to compare mean differences among treatments at the 5% probability level. For PEPC and Rubisco activities, the means of 5 DAC and 10 DAC were used.

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