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## REGULATION OF PHOSPHOENOLPYRUVATE CARBOXYLASE GENE EXPRESSION AND PROTEIN CONTENT IN DEVELOPING AND GERMINATING GRAINS OF *BRACHYPODIUM*

**Sh.M.Bayramov**

(Presented by Academician of ANAS I.M. Huseynova)

*Gene expression and protein content levels of different Phosphoenolpyruvate carboxylase (PEPCase) isoforms were investigated in developing and germinating Brachypodium distachyon grains. Western blot experiments detected two main PEPCase polypeptides with apparent molecular masses of 110 and 102 kD. According to the obtained data there was an increase in the amount of 102 kD polypeptide of PEPCase beginning from the embryo formation till the wax ripeness stage of grain filling and it began to decrease gradually at the final stages of grain filling. The higher intensity of the expression of the studied PEPCase genes at the final grain filling stages is probably related to the intensifying biosynthesis of storage proteins and lipids.*

**Keywords:** *B.distachyon, phosphoenolpyruvate carboxylase, gene expression, polypeptide content, grain*

### Introduction

PEPCase catalyzes the conversion of phosphoenolpyruvate (PEP) to oxaloacetate using bicarbonate. The critical role of PEPC in assimilating atmospheric CO<sub>2</sub> during C<sub>4</sub> photosynthesis and *Crassulacean* acid metabolism has been studied extensively. Plant types PEPCs also fulfill a wide range of crucial non-photosynthetic functions, particularly the anaplerotic replenishment of tricarboxylic acid cycle (TCA) intermediates consumed during biosynthesis (O'Leary et al., 2011). The produced oxaloacetate is consumed to provide carbon skeletons of the TCA cycle members, which are used in many biosynthetic reactions such as amino acid and fatty acid synthesis. Vascular plants have two types of PEPCs of different evolutionary lineages, namely plant and bacterial types (Sanchez and Cejudo, 2003). High activity of PEPCase has been observed in the seeds of many crops, indicating that this enzyme plays some crucial roles in seeds. In C<sub>3</sub> plants, several studies emphasized the potential contribution of PEPCase to nitrogen metabolism in developing seeds. PEPCase is thought to be involved in the carbon partitioning in developing seeds (Plaxton and Podest, 2006; Andre et al., 2007; Junker et al.,

2007). Several studies of seeds support the contribution of PEPCase to the accumulation of seed storage compounds such as storage proteins and lipids (González et al., 1998; Sebei et al., 2006; Gennidakis et al., 2007; Radchuk et al., 2007; Fan et al., 2013; Yamamoto et al., 2014). For monocot plant seeds, the accumulation of PEPCase proteins in developing wheat seeds also supports their contribution to seed storage compound accumulation (Gonzalez et al., 1998). Studies of bacterial-type PEPCase also provide evidences for roles of bacterial-type PEPCase (BTPECase) in fatty acid synthesis, for instance, RcPpc4 is expressed abundantly in developing castor oil seeds and involved in seed oil accumulation. A study of the castor oil plant revealed that PEPCase proteins were accumulated in its developing seeds, especially during grain-filling stages (Gennidakis et al., 2007). PEP metabolism plays an important role also in partitioning developing seed carbohydrates toward plastidic fatty acid biosynthesis, and mitochondrial production of ATP and the organic acids required for amino acid synthesis through PEPCase and cytosolic pyruvate kinase (Uhrig et al., 2008).

*B. distachyon* has been proposed as a new model species for grasses (Draper et al., 2001;

Bevan et al., 2010). With a fully sequenced genome, a short life-cycle *Brachypodium* appears to be a good model for dissecting numerous aspects of grass biology and development. In particular, it could be used to get a better understanding of the metabolic switches responsible for carbon partitioning during grain filling. *Brachypodium* is much more closely related to wheat than to rice or maize. It was shown recently that seeds of *B. distachyon* were developed in approximately 36 days and dry seeds were obtained on the 50th day after flowering. The endosperm contains storage compounds and is the largest compartment of the grain (Opanowicz et al., 2011). *B. distachyon* seed development was also found to be significantly different from that of other cereals. These properties were found to be unique to small grain plants studied until now. The protein content of *B. distachyon* grain approaches the highest level measured in cereal grains, in fact it is close to that of oats. In this work we first characterized the *B. distachyon* PEPCase gene expression and protein content during seed development and imbibition.

## Materials and methods

### *Plant material and growth conditions.*

Seeds of *B. distachyon* variety Bd21 were sterilized in 15% bleach for 15 min. Sterilized seeds were sown on damp filter paper, cold treated at 4°C for 2 d in order to synchronize germination. For growing mature plants experiments were conducted in a glasshouse under natural light with day/night temperatures of 25/18 °C and 16 h photoperiod with an additional illumination of 400  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and 60–70% relative humidity during the day. The collected developing seed samples were immediately frozen in liquid nitrogen and stored at –80°C until use.

**Immunocharacterization and electrophoresis.** Grains were frozen in liquid nitrogen and ground with a mortar and pestle. Crude extracts were prepared from ground tissue with extraction buffer (50 mM Tris–HCl pH 7.9, 1.0 mM EDTA, 0.5 mM PMSF). The homogenized mixture was centrifuged for 20 min at 15 000  $\times$  g. Extracted total soluble proteins were mixed with SDS-PAGE sample buffer (100 mM Tris–

HCl, 4% (w/v) SDS, 20% glycerol, 0.1% (w/v) bromophenol blue and 0.2 M DDT) and heated at 95 °C for 5 min. Denaturing (SDS-PAGE) polyacrylamide gel electrophoresis was conducted as described by Laemmli (Laemmli, 1970). 10% (w/v) resolving gel coupled to a 4.5% (w/v) stacking gel were utilized. After electrophoresis, the gel was electroblotted onto a nitrocellulose membrane (N-8017; Sigma). Membranes were then soaked at room temperature for at least 1 h in 15 mM Tris–HCl pH 7.4 containing 0.2 M NaCl (buffer A) and 5% w/v dry powdered milk. After overnight incubation at 4°C with polyclonal sorghum anti-PEPC antibodies (diluted 1:3000 in phosphate-buffered saline plus 1% nonfat milk), sheets were washed four times each for 15 min in buffer A containing 0.1% v/v Tween 20. Subsequent detection was with a peroxidase assay using affinity-purified goat anti-rabbit IgG horseradish peroxidase (diluted 1:10,000 in phosphate-buffered saline plus 5% nonfat milk conjugate) (Bio-Rad). The enhanced chemiluminescence western-blotting detection kit (Amersham) was used to develop the reaction. Immunoreacting bands were detected with Quantity One software. Protein was measured by the method of Bradford.

**Total RNA extraction and RT-PCR analyses.** Total RNA was extracted from developed and imbibed *B. distachyon* grains using modified sodium dodecyl sulphate (SDS)/TRIzol method. The quantity and quality of total RNA were measured by spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, MA) and 1.2% agarose/Ethidium bromide gel electrophoresis. One microgram of the isolated total RNA was used to synthesize first-strand cDNA using quantiscript reverse transcriptase. PCR amplification was carried out in a 20  $\mu\text{l}$  reaction mixture containing a 200  $\mu\text{M}$  concentration of each dNTP, a 20  $\mu\text{M}$  concentration of each primer was as follows: 0.5–1  $\mu\text{l}$  cDNA, 4  $\mu\text{l}$  5xMyTaq reaction buffer, and 0.1  $\mu\text{l}$  of MyTaq™ DNA polymerase (Promega). PCR was carried out 30 cycles for 1 min of initial denaturation at 95°C, 15 sec at 95°C, 15 s annealing temperature (For *Brppc1* annealing temperature was 60 °C, and 55°C for *Brppc2* and *Brppc4* fragments) and 10 s at 72 °C, followed by a final

extension step for 4 min at 72°C. Primers designed on the base of all three studied PEPC genes (*Brppc1*, *Brppc2*, *Brppc4*) were used. The *Brppc1* forward and reverse primer sequences were 5'-GGTTGCTG-GTCACAAGGATG-3' and 5'-GACAGG-GGTGGCTGCTG-3', *Brppc2* forward and reverse primer sequences were 5'-GAATTTCCACATGCTCCAGG-3', and 5'-CTGTCA-ATGCTGGAAGTATGG-3', *Brppc4* forward and reverse primer sequences were 5'-GCCCTTC-TTCCAGTCCACC-3' and 5'-CCGAGA-GCTTGCTGTGCC-3', respectively. The PCR products were loaded onto a 1.5% agarose gel containing ethidium bromide, and electrophoresis was performed. The gel was examined and photographed using a UV imager.

**Statistical analysis.** ImageJ 1.38 program was used to analyze the intensity of the Western blot and RT-PCR products. PCR reactions were run with three replicates.

### Results and discussion

Expression of the three PEPC genes has been examined in germinating and developing seeds of *B. distachyon*. This analysis was performed using the relative RT-PCR analysis with gene-specific primers. The expression of the three PEPC genes was found to change differently in the germinating seeds, which had been imbibed and kept in a dark place, at room temperature for 6 and 24 hours. In spite of the absence of the expression after 6 hours the *Brppc1* gene was detected to be induced in the samples taken after 24 hours. The *Brppc2* gene, contrary to *Brppc1* was expressed after 6 hours and the expression was not observed in the samples taken after 24 hours. Whereas the expression of the *Brppc4* gene was at the same level for both examined variants (fig. 1A). By placing 3-day-old early seedling on the filter paper, time dependence of the expression changes of the three PEPCase genes were also examined in germinating seeds of the plant exposed to severe drought stress. The obtained results showed a decrease in the expression of the three genes subjected to a short-term severe dehydration (fig. 1A). However, this decrease was more pronounced in the *Brppc1* gene ex-

pression. PEPCase polypeptide content was higher in imbibed seeds of early seedlings compared with the seeds of 3-day-old seedlings. The polypeptide contents in the seeds of 3-day-old seedlings decreased gradually.

There were no significant differences between the dynamics of the PEPCase protein changes in samples of the germinating seeds exposed to gradual drought and salt stress and in samples of normal watered plants (fig. 1B). The development of seeds was divided into five stages according to size and color of developing seeds. The expression of the PEPCase genes changed differently in developing *B. distachyon* seeds depending on the phases of development (fig. 2A).

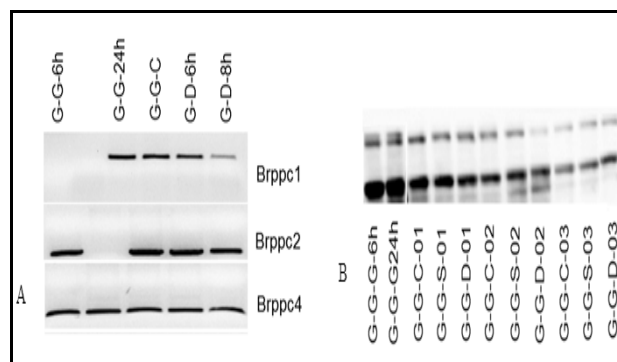


**Fig.1.** A) The changes of the *Brppc1*, *Brppc2*, *Brppc4* genes in *B. Distachyon* seeds during imbibitions and in early seedlings subjected to a short-term severe drought; B) Western-blot analysis of changes in PEPCase polypeptide contents in 6 h and 24 h imbibed *B. distachyon* seeds and during the seed germination (G-G)

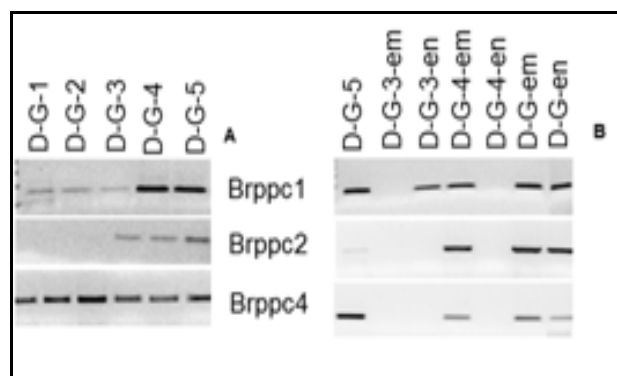


**Fig.2.** A) Changes in the expression of the *Brppc1*, *Brppc2* and *Brppc4* genes in different developmental phases, B) in embryo (-em) and endosperm (-en) parts of the developing *B. distachyon* grains (D-G-)

Thus, the low expression level of the *Brppc1* gene at the initial stages of seed formation increased to the end of this stage. *Brppc2* was not expressed at the initial stages of the development of seed, whereas beginning from the wax ripeness stage of seed development till the complete maturation its expression changed similarly. The expression of the *Brppc4* gene was observed at all stages of seed development, beginning from the initial seed formation. But the stable high levels of its expression in initial phases decreased evenly during the final stages of the grain germination. The expressions of the three genes were also examined separately in embryo and endosperm parts of the developing seeds. Thus, beginning from the third stage of grain filling the *Brppc1* gene was expressed in embryo as well as in endosperm of the developing grains. But the level of the expression was higher in endosperm than in embryo. The *Brppc2* gene was expressed at the beginning of the wax ripeness stage in embryo and at the next stages of grain filling in both embryo and endosperm. Similar to the *Brppc1* gene the expression level of the *Brppc2* gene was higher in embryo compared with endosperm. The expression of the *Brppc4* gene in embryo and endosperm of developing grains matches the expression of the *Brppc2* gene (fig. 2B). Using the immunoblotting method dynamics of the changes of the protein content of PEPCase isoforms was determined in *B. distachyon* plants during different stages of the grain development, beginning from the flowering period (fig. 3).

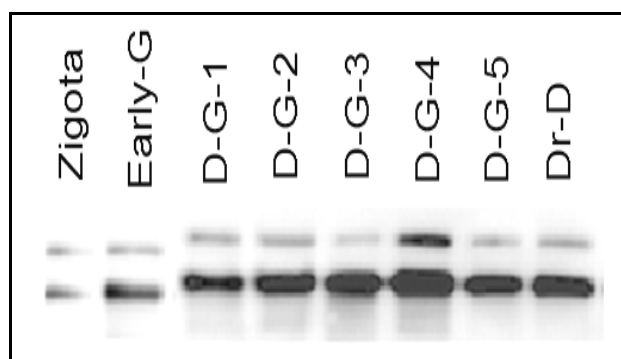


**Fig. 3.** Immunoblot analysis of the dynamics of changes in PEPC protein beginning from the formation of zygote till the full ripeness of grains

According to the obtained data the amount of the 102 kD polypeptide increased gradually beginning from the zygote formation till the wax ripeness stage of grain filling. Its amount began to decrease at the final stage of grain filling. The 110 kD polypeptide quantity was less than the quantity of 102 kD polypeptide. The influence of dehydration on the PEPCase polypeptide content was determined at different stages of the plant development in grain samples taken from ears, detached and kept at room temperature for a day. The obtained results showed that the 102 kD PEPCase polypeptide content in grains at different stages of grain filling increased under dehydration compared with the normal watered plants (fig. 4A). High levels of the PEPCase polypeptide content were also found in green glumes at initial stages of grain ripening. The PEPCase polypeptide content was higher in glumes compared with awns of the ripening *B. distachyon* plants and the polypeptide content in both ear elements decreased gradually under water stress conditions. In contrast, the PEPCase polypeptide content in the ripening grains from plants exposed to gradual drought stress increased compared with normal watered plants (fig. 4B).

Previously PEPCase activity and appropriate mRNA levels were studied in developing and germinating wheat (*Triticum aestivum*) grains (Gonzalez et al., 1998).

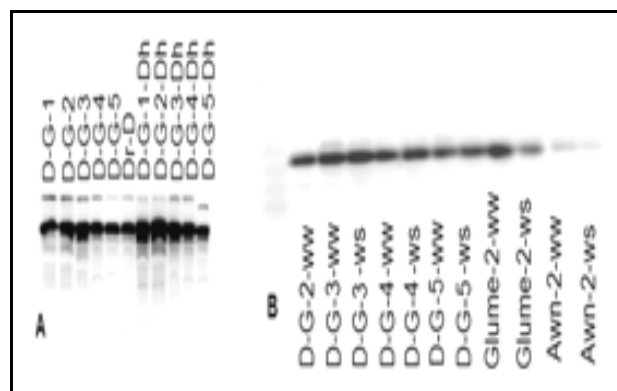


**Fig. 4.** A) Dynamics of the changes of the PEPCase polypeptide content related to the growth phases in ripening and exposed to dehydration *B. distachyon* grains (–Dh); B) changes of the plant type PEPCase polypeptide content in the developing grains and ear elements under well watered (–WW) and water stress (–WS) conditions

Housekeeping (103 kD) and inducible (108-kDa) subunits were detected in germinating barley (*Hordeum vulgare*) grains (Feria et al., 2008). Phosphorylation levels of PEPCase was shown to be at high levels at initial stages of grain filling and also in imbibed barley grains during germination. The expression of the PEPCase genes – *Osppc1* and *Osppc3* in developing rice grains has been studied recently. The obtained results showed that in spite of the expression of both genes throughout the grain developmental stages, the dynamics of the changes of their expression are different. The authors noted also a positive correlation between the expression of *Osppc1* and genes of aspartate aminotransferase and alanine-aminotransferase participating in nitrogen metabolism. They suggested that both aminotransferases are correlated functionally with *Osppc1* participate in the synthesis of amino acids (Yamamoto et al., 2014). The less accumulation of the general reserve proteins in ripening barley and wheat seeds compared with *B. distachyon* seeds and more active expression of the three studied PEPCase genes during the final phases of the ripening of *B. distachyon* seeds suggest the active participation of PEPCase in the biosynthesis of reserve proteins and lipids.

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*Institute of Molecular Biology and Biotechnology  
of ANAS  
sbayramov@hotmail.com*

**BRACHYPODIUM DISTACHYON** BİTKİSİNİN CÜCƏRƏN VƏ YETİŞMƏKDƏ OLAN DƏNLƏRİNDƏ  
FOSFOENOLPİRUVAT KARBOKSİLAZA FERMENTİNİN GEN EKSPRESSİYASININ VƏ ZÜLAL  
MİQDARININ TƏNZİMLƏNMƏSİ

Ş.M.Bayramov

*Brachypodium distachyon* bitkisinin cücərən və inkişaf edən dənələrində FEPK-aza fermentinin bəzi izoformalarının gen ekspressiyasının və zülal miqdarının dəyişməsi müqayisəli tədqiq olunmuşdur. İmmunoblotinq analizlərin nəticələrinə əsasən FEPK-azanın molekul cəkiləri təxminən 102 və 110 kDa olan iki polipeptidi aşkarlanmışdır. İlk inkişafın əmələ gəlməsindən başlayaraq dəndolmanın müvafiq yetişmə mərhələsinə qədər FEPK-azanın molekul kütləsi 102 kDa olan polipeptidinin miqdarı tədricən artsa da, dəndolmanın son mərhələlərində isə onun miqdarı tədricən azalaraq stabil qalır. Yetişməkdə olan dənələrin inkişafının son mərhələlərində yoxlanılan hər üç FEPK-aza geninin ekspressiyasının güclənməsi görünür ehtiyat zülalların və lipidlərin biosintezinin artması ilə bağlıdır.

*Açar sözlər:* *B. distachyon*, fosfoenolpiruvat karboksilaza, genin ekspressiyası, polipeptidlərin miqdarı, dən

**РЕГУЛЯЦИЯ ЭКСПРЕССИИ ГЕНА И КОЛИЧЕСТВА БЕЛКА  
ФОСФОЭНОЛПИРУВАТКАРБОКСИЛАЗЫ В РАЗВИВАЮЩИХСЯ И ПРОРАСТАЮЩИХ  
ЗЕРНАХ BRACHYPODIUM DISTACHYON**

Ш.М.Байрамов

Изучались экспрессия гена и количество белка различных изоформ фосфоэнолпируваткарбоксилазы (ФЭПК) в развивающихся и прорастающих зернах *Brachypodium distachyon*. Методом вестерн-блоттинга обнаружены два основных полипептида ФЭПК с приблизительной молекулярной массой 110 и 102 kDa. Согласно полученным данным, содержание полипептидов ФЭПК массой 102 kD возрастает, начиная с формирования эмбриона вплоть до стадии восковой спелости и налива зерна, и постепенно уменьшается на конечной стадии налива зерна. Наиболее высокая интенсивность экспрессии генов ФЭПК на конечной стадии налива зерна связана, вероятно, с интенсификацией биосинтеза запасных белков и липидов.

*Ключевые слова:* *B. distachyon*, фосфоэнолпируваткарбоксилаза, экспрессия гена, количество полипептидов, зерно