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Research Article

Supplementary Analysis of Phosphoenolpyruvate Carboxykinase Gene Expression in Developing Seeds of Chickpea

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Abstract

A gene of interest in this study is phosphoenolpyruvate carboxykinase (*pepck*), encoding a protein with a substantial role in the gluconeogenesis pathway and in metabolism of nitrogenous compounds in developing seeds of legumes, including amides and ureides which are then transformed into amino acids necessary for the synthesis of storage proteins. Whereas studies on genes contributing to the seed filling in chickpea and its protein content might be valuable in engineering plants with seeds of a higher nutritional value. In order to investigate *pepck* gene expression in different genotypes of chickpea (*Cicer arietinum* L.), four genotypes of chickpea were studied by Real-time PCR and western blot techniques. So results show that *pepck* expresses in high protein genotypes more than low protein genotypes at different growth stages and there was a differential expression of *pepck* gene at different stages of flowering and seed development. The PEPCK was expressed at higher levels during the shoot formation and in developing seeds compared to the flowering and seed formation stages.

***Keywords*:** Chickpea, Protein, Phosphoenolpyruvate Carboxykinase, Real-time PCR, Western blot.

Introduction

Phosphenolpyruvatecarboxykinase (PEPCK), catalyzes the conversion of oxaloacetate to phosphenolpyruvate (PEP) which will be further converted to sugar (Bahrami et al., 2001), and encoding a protein with a important role in the gluconeogenesis pathway in plants. PEPCK is an inducible enzyme, that is only present in certain plant cells, and in many plant and animal tissues and in microorganisms (Walker and Chen, 2002). In plants, PEPCK has been found in the phloem companion roots, cells, stomatal guard cells, the flesh of fruits, simple and glandular trichomes, latex-producing ducts, germinating seeds, developing seeds, and in the leaves of many C4 and CAM plants (Kim and Smith, 1994; Walker and Chen, 2002; Leegood and Walker, 2003). PEPCK is therefore present in many tissues and cell types than was once thought but, in many of these tissues and cells, it is only present under certain conditions, or at certain stages of development (Leegood and Walker, 2003). Also it is well established that PEPCK functions in gluconeogenesis from lipids and proteins in seeds post germination (Leegood and Walker, 1999). Only in some of the plant cell types the function of PEPCK has been clearly established. For some other cells and tissues, there is evidence that PEPCK plays a role in the metabolism of nitrogenous compounds (Chen et al., 2000; Leegood and Walker, 2003; Delgado-Alvarado et al., 2007) .

Metabolism of nitrogenous compounds in legumes developing seeds, including amides such as asparagines which are then transformed into amino acids necessary for the synthesis of seed storage proteins in the grain of some legumes (Aivalakis et al., 2004; Malone et al., 2007; Delgado-Alvarado et al., 2007).

So it is involved in growth, seed filling and amino acid content (especially asparagines metabolism) of pea (*Pisum sativum*) seeds. The seed coat is recognized as a tissue rich in nitrogen transporter

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enzymes and invertases which contribute to amino acid and carbohydrate metabolism (Delgado-Alvarado et al., 2007). So the relationship between PEPCK and metabolism of amino acids and amides shows that it is a relatively sensitive enzyme to the presence of nitrogenous compounds in seed coat and cotyledons, with its content being affected by nitrate, ammonium and asparagine in seed coats, but only by asparagine in cotyledons of pea (Delgado-Alvarado et al., 2007).

Chickpea (*Cicer arietinum* L.) is an economically important legume cultivated throughout the world (Chickpea 2005). The aim of this work was to investigate the occurrence of phosphoenolpyruvate carboxykinase (PEPCK) in different stage of reproductive growth of chickpea.

In our previous study (Beihaghi et al., 2009) the protein content percentage was measured in a number of cultivated chickpea genotypes, followed by comparison of the expression levels of PEPCK gene at different stages of seed filling in some of the genotypes by semi quantitative RT-PCR. Also PEPCK mRNA level in different genotypes was determined. two high protein genotypes (MCC053 and MCC458) and Two low protein genotypes (MCC291 and MCC373) out of 25 chickpea genotypes were selected and total RNA was extracted at different seed development stages. Results of semi-quantitative RT-PCR showd that; there was a differential expression of *pepck* gene at different stages of flowering and seed development. The PEPCK was expressed at higher levels during the shoot formation and in developing seeds compared to the flowering and seed formation stages.

In this study, *pepck* expression and the occurrence of PEPCK protein and its activity in different growth stages of chickpea was determined (during both vegetative and reproductive growth) by Real-time PCR and Western blot techniques in different tissues of the plant.

Materials and Methods

conditions of Plant growth

Four chickpea (*Cicer arietinum* L.) genotypes, high protein genotypes MCC458 and MCC053 and low protein genotypes MCC 291 and MCC373 were used in this study. Chickpea seeds were obtained from the Ferdowsi University Genebank (Mashhad, Iran). The germinated seeds were cultured in pots containing equal amounts of clay, leaf compost and sand, and maintained in the glasshouse for 3 months under photoperiod of 14 h light at 25±2ºC and 10 h darkness at 15±2ºC, until the genesis seed stage.

RNA extraction

Total RNA was extracted from different growth stage of such as flowering, sheet formation, seed formation and seed development by RNeasy Plant Mini Kit (Qiagene, ZistBaran, Iran). Fresh tissues were homogenized in liquid nitrogen and other steps were performed according to the manufacturer's instructions.

cDNA Synthesis

Easy™ cDNA Synthesis Kit was used for cDNA synthesis. Reverse transcription was performed in a reaction mixture containing 5 µg of total RNA, 1 µl oligo dT primer and DEPC-treated water Up to 10 µL, then the mixture incubated at 65°C for 5 min and chilled on ice, added 10 µL of RT Premix (2X) and mixed by pipetting gently up and down (total reaction volume 20 µL), incubated 10 min at 25°C and 60 min at 50°C.

Primer design

One specific primer pair (Table1) were designed with Primer5 software using the *pepck* (XM_004509665.2) cDNA sequences for Real-time PCR*;* and *hsp90* (NM_001019786.2). The primers were obtained from Gene Fanavaran (Tehran, Iran).

Table1: Primer sequences used in this study

No	Gene	Primer sequence		Amplicon length, (bp)
1	*pepck*	5' -ATGGTTATCCTCGGCACGC 3'	F	127 bp for Real-time PCR product
		5'-CACCATCTTTGCCCATATTGC 3'	R	
2	Hsp90	5' CAGGAACGACAAATCCGTCA 3'	F	142 bp for Real-time PCR product as control
		5' CTCGTCAATGCTCAGTCCAAG 3'	R	

Real-time PCR

The relative expression levels of *hsp90* and *pepck* genes were determined at various post-inoculation times by Real-time PCR. By establishing a gradient of concentration that covered 150–400 nM for primers and 50–200 ng for cDNA, the dynamic range of cDNA and optimum concentration of primers for Real-time PCR were determined. For all selected genes, the best florescence signal was obtained from 150 ng cDNA. Also, a unique peak was acquired at melting step with 1 µM primers. Each cDNA template was run three times. Gene expression variations were monitored by Rotor Gene 3000 (Corbett research) in 20µl final volume reaction of Real-time PCR containing 3 of cDNAs, 1 µl each primer, and 10 µl 2X SYBR GREEN PCR Master Mix (pars tous).

The Real-time PCR conditions included initial activation step at 95°C for 10 min, denaturation at 95°C for 15s, and combined annealing/extension at 55°C for 30s. Then melting curve analysis of PCR product was carried out at 72°C for 30s foe 35 cycle. Extension step of each cycle and melting step data were collected on the FAM/SYBR channel. The analysis of these raw data was done by *LinReg PCR* program, after exporting them to an Excel worksheet. The PCR efficiency and CT value of each reaction were obtained from Lin Reg PCR software. There after, the relative expression value was calculated using ΔΔCT formula. A quantification of relative expression of *pepck* was obtained by calibrating the expression of this gene with the *hsp90* gene used as a reference gene. The relative expression levels of *pepck* gene in different stages of flowering and seed development of high protein genotype were separately compared with low protein genotype that of control. As shown in Fig. 1, the expression levels in flowering stage of *pepck* gene in MCC291(low protein plants) were set equal to 1 and the cDNA levels of this gene in MCC373, MCC458 and MCC053, in different stage of growth, were calculated relative to this control.

Protein extraction and quantification

Equal amounts (5µg) of each protein sample were electrophoresed on 10% SDS-polyacrylamide gel and electrophoretically transferred onto the PVDF membrane. The transfer buffer contained 12 mM Tris base, 95 mM glycin, and 20% glycerol, and transfer was carried out for 4 h using 90 V constant voltage at 4°C. The membrane was blocked in PBS-Tween (0.1% tween 20) for 20 min and incubated with PEPCK polyclonal primary antibody (kindly donated by prof. R. Leegood, University of Sheffield), diluted 1:5000 in blocking solution 2 h at 30°C. The membrane was washed three times in PBS-Tween (0.1 % tween 20) for 25 min and incubated for 1 h in diluted (1:5000 in the blocking solution). Secondary antibody was horseradish peroxidase- conjugated anti goat IgG (Sigma). Then the membrane was transferred to a shallow tray and 10 µl H_2O_2 (30%) was added to 10 ml of 0.05% DAB (diaminobenzidine) in PBS, and mixed well immediately. DAB was poured onto the membrane and was incubated at room temperature with shaking in dark position(5 min), filter was washed in water, and in PBS. Then the membrane was dried and the photograph of it was taken to provide a permanent record of the experiment.

Results

Real-time PCR

Since *pepck* probably plays an effective role in protein content of plant, the presence of mRNA arising from the *pepck* genes, was determined at different stages of development in two high protein genotypes (MCC053, MCC458) and two low protein genotypes (MCC291,MCC373). The results showed that amount of *pepck* expression was significantly higher at the stage of seed filling than other stages of all genotypes. The lowest levels of expression had been at flowering and seed formation (Fig. 1). Under conditions used in this study, the transition from flowering stage to seed developing stage shows that the activity of PEPCK was increased, PEPCK activity in sheet formation is higher than seed formation in all of the genotypes, but in high protein genotypes these differences were more significant than low protein genotypes (Fig.1). As expected, the expression of this gene was detected in both of two low protein genotypes, but comparatives showed that the measure of *pepck*, was fairly lower in these genotypes. Accumulation of these RNAs in high protein genotypes was significantly higher than that of the control ($P \leq 0.001$) and in low protein genotypes were not so significant (Fig.1). As shown in Fig.1, the expression of *pepck* in growth stages of high protein genotypes; MCC458 was induced at seed development, sheet formation and seed formation (6674 fold , 4473 fold and 3450 fold, respectively) and also MCC053 was induced at seed development 7428 fold, sheet formation 3732 fold and 2413 fold in seed formation stages. So as it shows; it has a few differentiation in expression of this gene between these two genotypes. However, the expression of this gene lower at the stage of flowering (265 fold in MCC458 and 206 fold in MCC053) compared to seed development stages. Also, comparison between high protein and low protein genotypes showed that the high protein genotypes had higher *pepck* expression than low protein genotypes in all flowering and seed development stages (Fig. 1). Thus, in high protein genotypes *pepck* expression was induced, than in low protein genotypes. The result of semi quantitative PCR in the last study (Beihaghi et al., 2009) also showed that The expected band was not amplified in any growth stage of MCC291 and MCC373 genotypes. However by Real-time PCR the differences in expression of *pepck*, visually evident among different growth stages (Fig.1). Also, in order to confirm the amplified bands from RT- PCR, these bands were sequenced and the result of sequencing was blast in NCBI database (Fig.2).

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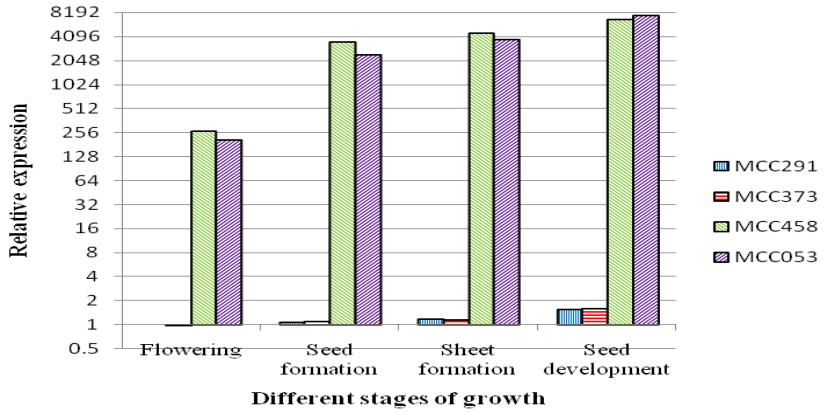

Figure 1. *Pepck* expression at different growth stage of high protein genotypes (MCC053 and MCC458) and low protein genotypes (MCC291 and MCC373). The expression levels of *pepck* gene in flowering stage of MCC291 (low protein genotype) were set equal to 1 as control, and the cDNA levels of this gene in different stage of all of these genotypes have been comparised to this control. Accumulation of these RNAs in high protein genotypes was significantly higher than control ($P \leq 0.001$) and in low protein genotypes were not so significant.

PREDICTED: Cicer arietinum phosphoenolpyruvate carboxykinase [ATP]-like (LOC101503695), mRNA

Sequence ID: refjXM_004512483.1| Length: 2544 Number of Matches: 1

Range 1: 1230 to 1405 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
270 bits(146)	3e-69	166/176(94%)	0/176(0%)	Plus/Plus

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Query  3     CACAAGCATTGATCTTTATCTTGCTAGAAGGGAAATGGTTATCCTCGGCACGCAGTATGC  62
             ||| |||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  1230  CACTAGCATTGATCTTAATCTTGCTAGAAGGGAAATGGTTATCCTCGGCACGCAGTATGC  1289

Query  63    CGGGGAAATGAAGAAAGGTCTCTTCAGTGTCATGCAATAGCTCATGCCTAAGCGCCAAAT  122
             ||||||||||||||||||| ||||||||||||| || ||||||||||||||||||||||
Sbjct  1290  CGGGGAAATGAAGAAAGGTCTTTTCAGTGTCATGCATTATCTCATGCCTAAGCGCCAAAT  1349

Query  123   TCTCTCCTTACACTGAGGTTGCATTATGCGGAAAGATGGTGATGTTGCACTCTTCT  178
             ||||||||||||||  ||||||| |||| | ||||||||||||||||||||||||||
Sbjct  1350  TCTCTCCTTACACTCTGGTTGCAATATGGGCAAAGATGGTGATGTTGCACTCTTCT  1405
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Figure 2. Alignment of amplified bands from Real-time PCR reaction with specific primers.

Western blot analysis

To confidence that if the PEPCK protein express in high protein genotypes more than low protein genotypes; the level of PEPCK protein were just compared in flowering stage of both genotypes. As expected, the expression of this protein was detected in both of them but comparative western blots showed that the level of PEPCK protein was fairly higher in high protein genotype (MCC 053) compared to low protein genotype (MCC 373) (Fig.3).

Figure 3. PEPCK expression at flowering stages of, MCC053 and MCC373 chickpea genotypes, based on western blot experiments.

Discussion

results of this study show that, *pepck* gene is involved in the content of chickpea protein. During development of pea seeds, high amounts of storage protein are rapidly synthesized in the developing cotyledons (Beevers and Poulson, 1972), so these contain the largest seed storage protein in pea. Developing seeds import large amounts of nitrogenous compounds, usually as amino acids, amides or uridines, for the synthesis of seed storage proteins (Murray, 1987; Peoples et al., 1985). In legumes, like other seeds, these high activities of enzymes of nitrogen metabolism found in the seed coat of peas (Sodek et al., 1980). PEPCK was present throughout development, and accumulated in the cotyledons (Delgado-Alvarado et al., 2007). There has been no reported about the role of this gene in protein content of chickpea yet. In plants, phosphenolpyruvatecarboxykinase (PEPCK), encodes a protein with a substantial role in the

gluconeogenesis pathway. It catalyzes the conversion of oxaloacetate to phosphenolpyruvate (PEP) which will be further converted to sugar (Bahrami et al., 2001). Necessary amino acids for protein synthesis in seed, are transported form other organs during the seed development. Glutamine and asparagine are important nitrogen-transport compounds in many plants (Temple et al., 1998). Many amino acids are produced from these amino acids through the transamination and intermediates of the Glycolysis and Citric Acid Cycle (Berg et al., 2002). Glutamine and asparagines, by deamination reaction, produce α-Ketoglutaric acid and oxaloacetate, the two components of the TCA cycle. Accumulation of these components through the large amount of imported amino acids in high protein genotypes, may be needed for PEPCK enzyme to equalize components between TCA cycle and Glycolysis. Also accumulation of storage compounds requires energy and could be limited due to reduced penetration of light particularly and oxygen into the inner parts of the seeds (Galili et al., 2014). In limited oxygen, FAD^+ and NAD^+ components are reduced through the accumulation of $FADH_2$ and $NADH_2$ in TCA cycle and Glycolysis (Berg et al., 2002), and cells may activate the gluconeogenesis pathway to convert accumulating intermediates of these cycle to sugar. Therefore PEPCK is needed for enhancing and equalizing amino acid production in seeds. But maybe the first principle for high protein seeds is larger amounts of amino component that are transported to seeds.

In the present paper by comparison of the expression levels of *pepck* at different seed filling stages in high protein genotypes, it is suggested that PEPCK is involved in increasing of protein content of developing chickpea seeds. Similar results have been reported by Aivalakis and Delgado (2004) on mature seeds of pea and alfalfa has revealed involvement of the *pepck* in grain filling, nitrogen storage, amino acid enrichment, and thus metabolism of storage proteins during seed development. Similarly, our results suggested that in mature seeds of chickpea, the expression of this gene is related to the metabolism of nitrogenous compounds and increasing of seed protein content.` Results of this study suggested that; this gene were regulated differently in two chickpea genotypes (high protein and low protein genotypes). It appears that the significantly higher expression level of *pepck* in genotypes MCC458 and MCC053, further induction of this gene in the growth stage of seed development can effectively enhance rather than other growth stages. Probably, the differential expression of *pepck* is related to its possible role in metabolism of seed components, particularly in determination of the protein content of chickpea seeds. Thus, *pepck* may be has an important role in increase of seed protein content. Further, the results of this study can be useful for constructing transgenic high protein plants in the future.

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