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# Comparison of *pepck* gene expression in developing seeds and leaves of chickpea (*Cicer arietinum* L.) plant

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## Abstract

Phosphoenolpyruvate Carboxykinase, encoded by the *pepck* gene, plays an important role in gluconeogenesis. It also seems to be important 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. In this research, *pepck* gene expression in mRNA level, in different genotypes of chickpea (*Cicer arietinum* L.), was determined. Two low protein genotypes (MCC291 and MCC373) and two high protein genotypes (MCC458 and MCC053) out of 20 chickpea genotypes were selected. Total RNA were extracted through different stages of seed development, and the expression of the *pepck* gene was estimated by semi-quantitative RT-PCR. The results of the RT-PCR showed that two isoforms of this gene are expressed in high protein genotypes, whereas in the low protein genotypes, the expression of these isoforms was not obvious. Also this method showed a differential expression of *pepck* gene in different stages of flowering and seed development. *pepck* gene is expressed in higher levels during the sheet formation and developing seeds compared to the flowering and seed formation stages. Probably, the differential expression of *pepck* gene is related to its possible role in metabolism of seed components, particularly in determination of the protein content of chickpea seeds.

**Keywords**: chickpea, nitrogen metabolism, phosphoenolpyruvate Carboxykinase, seed development

## Introduction

Chickpea (*Cicer arietinum* L.) is one of the most important grain legumes in Western Asia, Northern Africa, Southern Europe, Central America and Southern Australia. This crop holds the third place among legumes as of the global cultivation acreage. Its grain is reach in carbohydrates (48.2-67.6%), protein (12.4-31.5%), starch (41-50%), fatty acids (6%), B-group vitamins, and nutritional elements (Icrisat, 2005); and is recommended to be consumed as an amino acids (esp. lysine) supplement to cereals.

Studies on the genes contributing to the seed filling in chickpea and its protein content might lead to engineer plants with seeds of a higher nutritional value. A gene of concern is phosphoenolpyruvate carboxykinase (*pepck*), encoding a protein with a substantial role in the gluconeogenesis pathway. It catalyses the conversion of oxaloacetate to phosphenolpyruvate (PEP) which will be further converted to sugar (Owusu-Apenten et al., 2002; Walker et al., 2003; Malone et al., 2007; McClements, 2007). An increase in PEPCK activity results in reduced organic acids and increased sugar content (Leegood et al., 1999). A probable role of PEPCK thus has also been recently reported in metabolism of nitrogen and nitrogenous compounds and their conversion into proteins in the grain of some legumes (Aivalakis et al., 2004). This gene is involved in growth, seed filling and amino acid content (esp. that of asparagine) of pea (*Pisum sativum*) seeds. The seed coat is recognized as the tissue reach in nitrogen transporter enzymes and invertases which contribute to amino acid and carbohydrate metabolism. 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

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nitrate, ammonium and asparagine in seed coats, but only by asparagine in cotyledons of pea (ICRISAT, 2005).

Increased PEPCK content is combined with elevated levels of the enzymes involved in amino acid metabolism, such as mitochondrial asparagine amino-transferase, cytosolic alanine amino-transferase and acetyl-CoA carboxylase; thus contributing to metabolism, absorption and transmission of the nitrogenous compounds (Ruffner et al., 1975).

No studies on the expression and role of this gene and its possible relation to protein content has been reported in chickpea yet. In the present paper, the protein content (percentage) is 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. This study aims at revealing the relation between PEPCK transcript level with protein content of chickpea seeds which might, in the longer term, end at protein quality improvement of the crop through the gene manipulation procedures.

## Materials and Methods

### *Plant material and raw protein measurement*

The plant material included 20 genotypes of cultivated chickpea (table1), kindly provided by the seed bank of the plant science institute. The protein content was measured by Kjeldahl method (Roylet et al., 2003).

### *Cultivation of plant material and RNA extraction*

A number of intact seeds of four chickpea genotypes, having the farthest high/low protein content (MCC373 and MCC291 as lowest and MCC458 and MCC053 as highest) were selected and sterilized with 75% ethanol for 30 seconds. The seeds were then placed between two layers of the sterilized moist cloth to germinate in the laboratory. The germinated seeds were cultured in 12×12×12 cm pots containing equal amounts of clay, leaf compost and sand, at a depth of 3-4 cm, and maintained in the glasshouse for 3 months under photoperiod of 14 hrs light at 25±2 ºC and 10 hrs darkness at 15±2 ºC, until the genesis seed stage.

The *pepck* gene expression level was assessed using semiquantitative RT-PCR method. Total RNA was extracted from the mature seeds of the low and high-protein genotypes (referred to as L and H, respectively) by the guanidinum thiosianate protocol (Chomaczynski et al., 1987). The quality and quantity of the extracted RNA were assessed by agarose gel electrophoresis and spectrophotometry, and the loading volumes of the samples were adjusted accordingly.

### *Gene expression assessment at the mRNA level by RT-PCR*

The control primer pair, including a forward 19-mer oligonucleotide of 5'-TTTGTGAAAACTCTCACCG-3' and a reverse 18-mer of 5' GTCTGAGCACCAAATGGA-3', were designed based on the mRNA sequence available for ubiquitin protein as registered in the NCBI database. PEPCK primers were a 18-mer forward of 5'-GAAATCGGCACCTTCTAC-3' and a 19-mer reverse of 5'-CCTCATCCCTAACAACACG-3', designed based on the conserved regions identified in the cDNA sequences of PEPCK enzyme in alfalfa (*Medicago truncatula*)[1], tomato (*Solanum lycopersicum*)[2] and flaveria (*Flaveria trinervia*)[3].

The primers were intended to amplify a 208 bp fragment of the ubiquitin cDNA sequence, and fragments of 398 and 700 bp of the *pepck* cDNA and gDNA, respectively. All primers were designed using the Primer Premier (version 5) software.

cDNAs were constructed according to the standard recommendations (Fermentase) including; 0.4 µg of the template RNA together with 1 µl of the reverse primer (10 pmol/ml) and 8 µl of double distilled water incubating at 70ºC (in the thermocycler) for 5 minutes for denaturation; and immediate cooling on ice. The rest of the reaction was performed according to the standard protocols, 2µl of the first standard cDNA of each samples was subjected to ordinary PCR. The resulting cDNAs were stored at -20ºC.

The thermal scheme for PCR was an initial denaturation at 94ºC for 3 minutes, followed by 35 cycles of 94ºC, 52ºC and 72ºC for 1 minute each, and a final 6-minutes extension at 72ºC. The PCR products were loaded on 1.2% agarose gels. The sizes of the bands were estimated according to 100 bp DNA size marker. For equal loading of the first standard cDNA in RT-PCR reaction, the concentration must be adjusted according to an internal control gene, such as ubiquitin.continusely for equal loading of the samples, several PCR reactions were performed on serially diluted aliquots of the cDNA by using ubiquitin primers. In order to make a quantification of the results, gel photographs were analyzed by Lab Works software package which calculated a value for each amplified band (given in table 2) in form of a ratio

1 NCBI code AF212109.1
2 NCBI code AF327432.1
3 NCBI code AB050473.1

to the 400 bp band of the loaded commercial size marker (which was in turn given a value of 100 as the reference). The samples were then scaled by diluting the more concentrated cDNA.

## Results

### *Protein measurement*

The statistical results of protein measurements are presented in table 1 A remarkable variation in protein percentage is evident among the genotypes, with MCC458 and MCC053 having the highest (mean = 30.5) and MCC291 and MCC373 having the lowest (mean = 21.1) amount of protein. These were chosen as the extreme genotypes for further molecular analysis.

**Table 1.** The statistical results of protein content of different chickpea cultivars.

| Genotype | Seed Protein (percent)[1] | weight of hundred seeds (gr) | type |
|---|---|---|---|
| **MCC 067** | 23.50 | 33.2 | Kabuli |
| **MCC 099** | 25.85 | 9.8 | Desi |
| **MCC 165** | 25.24 | 16.8 | Kabuli |
| **MCC 291** | 21.13 | 19.0 | Dsei |
| **MCC327** | 25.66 | 21.4 | Kabuli |
| **MCC 333** | 24.72 | 30.8 | Kabuli |
| **MCC 476** | 23.12 | 28.2 | Kabuli |
| **MCC 495** | 26.12 | 27.2 | Kabuli |
| **MCC 510** | 24.68 | 32.0 | Kabuli |
| **MCC 053** | 30.41 | 33.2 | Kabuli |
| **MCC 202** | 23.60 | 15.8 | Kabuli |
| **MCC 258** | 25.90 | 31.4 | Kabuli |
| **MCC 332** | 28.46 | 15.0 | Desi |
| **MCC 426** | 28.31 | 33.2 | Kabuli |
| **MCC 458** | 30.57 | 25.4 | Kabuli |
| **MCC 477** | 25.42 | 27.4 | Kabuli |
| **MCC 496** | 24.80 | 25.6 | Kabuli |
| **MCC 498** | 26.30 | 23.6 | Kabuli |
| **MCC 207** | 27.40 | 17.6 | Desi |
| **MCC 373** | 20.92 | 17.4 | Desi |

### *RT-PCR results*

The total RNA extraction was successful, judged by two distinct bands of 26S and 18S rRNAs on agarose gel. The two selected high-protein genotypes showed two bands with sizes of 400 bp and 500 bp; whereas the two low-protein ones showed no detectable bands (figure 1). In order to exclude the possibility of DNA contamination of the extracted RNA samples, the total RNA was also incorporated as the negative control in each RT-PCR reaction (figure 1). PCR amplification of the extracted DNA resulted in a 700 bp band which is missing in the RT-PCR results. It could therefore be suggested that there must be intron/introns causing this deviation from the expected size of 400 bp. On the other hand, this is a confirmation of relatively gDNA free RNA extraction. RNA from alfalfa was used as the positive control to ensure the results throughout the experiments, for which a 400 bp band in the RT-PCR and a 2000 bp band in the gDNA amplification were detected (figure 2 and 3).


**Figure 1.** mRNA level of *pepck* gene in chickpea seeds in four different genotypes. Lanes 1-4 correspond to MCC 458, MCC053, MCC373, MCC291 RT-PCR results, respectively. Lanes 5 and 6 are negative controls of RNA and water, respectively. Lane 7 is size marker.

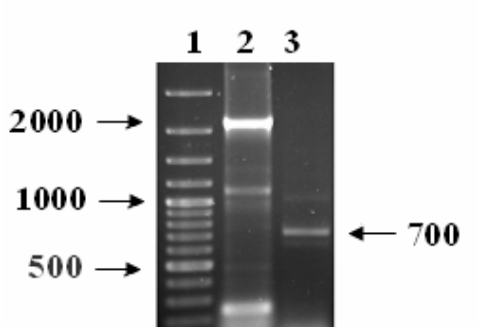


**Figure 2.** Optimization of polymerase chain reaction with pepck specific primers. Lanes 1, 2 and 3 are DNA 100bp Plus size marker, alfalfa gDNA amplified band, and chickpea gDNA amplified band, respectively.

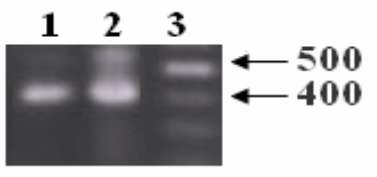


**Figure 3.** Optimization of polymerase chain reaction with pepck specific primers. Lanes 1, 2 and 3 are alfalfa cDNA amplified band (as positive control), chickpea cDNA amplified band and DNA 100bp Plus size marker respectively.

***Comparative analysis of pepck expression throughDifferent stages of chickpea growth and development***

The accumulation of *pepck* (as well as ubiquitin) encoding mRNAs were compared at 6 growth stages of 4 chickpea genotypes. As shown in fig 4, all samples were adjusted to reach equal ubiquitin bands upon PCR. This was also supported by comparison of the ribosomal RNA bands and equal volumes of each RNA were introduced into the RT-PCR reaction using the specific *pepck* primers. The RNA calibration based on the ubiquitin control is shown in figure 4.

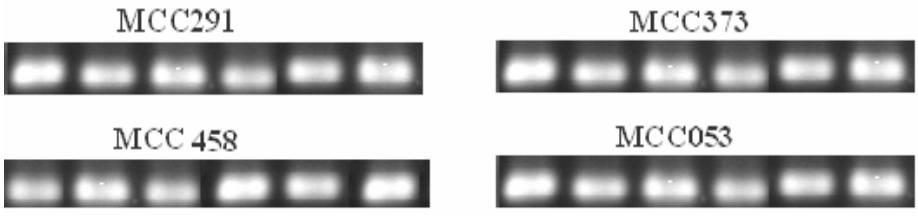


**Figure 4.** Scaling the total RNA concentration in four genotypes (MCC458,MCC053, MCC373, MCC291) of chickpea based on the ubiquitin amplified bands. Lanes 1-6 are loaded correspond to bearing few leaves, flower bud, flowering, sheet formation, seed formation, and full seed maturation, respectively.

The expected band was not amplified in any growth stage of MCC291 and MCC373 genotypes. For MCC458 and MCC053, however the differences in the intensity of the amplified bands are visually evident among different stages of growth and development, and especially in seed genesis stage (figure 5).

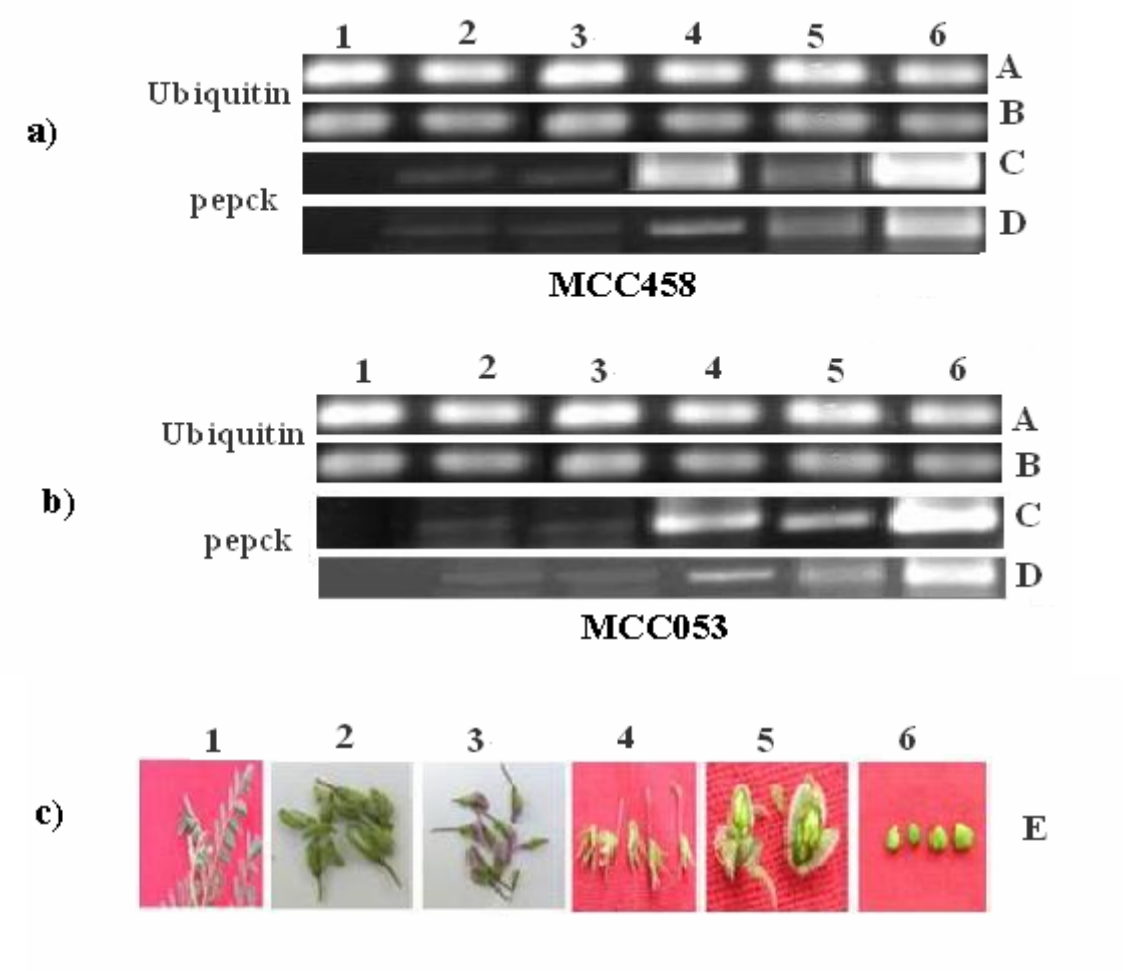


**Figure 5.** Comparative measurement of *pepck* mRNA levels at 6 growth stages of MCC458 (a) and MCC053 (b) chickpea genotypes, based on RT-PCR experiments: (A) calibration of ubiquitin amplified band after 25 cycles of PCR, (B) calibration of ubiquitin amplified band after 20 cycles of PCR, (C) amplified cDNA band related to *pepck* gene from each sample at the same conditions after 35 cycles of PCR, (D) amplified cDNA band related to *pepck* gene from each sample at the same conditions after 30 cycles of PCR, (E) developing stages of the examined samples including: 1) a few leaved 2) flower bud, 3) flowering, 4) sheet formation, 5) seed formation, 6) fully mature seeds (c).

**Table 2.** Quantification of the RT-PCR gel images for *pepck* (amplified at 35 PCR cycles) in MCC458 and MCC053 chickpea genotypes, using Lab Works software. Values are represented as percentage of the reference sample (size marker) which is given a value of 100.

| Genotype | Size marker | Flower bud | flower | Sheath formation | Seed formation | Seed development |
|---|---|---|---|---|---|---|
| **MCC 053** | 100 | 65.30 | 78.08 | 531.52 | 332.76 | 1288.40 |
| **MCC 458** | 100 | 59.07 | 65.13 | 498.57 | 284.36 | 985.50 |

## Discussion

Studies on mature seeds of pea and alfalfa has revealed involvement of the *pepck* gene in nitrogen storage, grain filling and amino acid enrichment, and thus metabolism of storage proteins during seed development (Aivalakis et al., 2004; Delgado et al., 2007). Similarly, our results suggest that in mature seeds of chickpea, the expression of this gene is related to the metabolism of nitrogenous compounds and increasement of seed protein content. It has also been shown that the Arabidopsis genome contains two related genes named *pck1* and *pck2* with the former being expressed at a higher level (Malone et al., 2007). It therefore appears that the two 400 and 500 bp bands, amplified in our studies might be counterparts of the *pck1* and *pck2*; and the higher amount of the 400 bp RT-PCR product could suggest that, in chickpea the same isoform could be of more involvement in chickpea development.

As shown here, *pepck* is not expressed at younger stage (a few – leaved), and experiences the lowest levels of expression at flowering and seed formation and the highest levels at bundle formation and full seed maturation stages. Table 2 also shows a higher expression level of *pepck* at seed filling stage. These results together with the fact that high protein plants are also high *pepck* ones, might add more weight on the notion that *pepck* is a key determinant of protein content in seeds.

The *pepck* gene has been proven to be controlled spatially and temporally (in different tissues and at different growth stages) in other plants such as tomato, pea, alfalfa, cucumber, grape and arabidopsis (Ruffner et al.,1975; Ruffner,1982; Bahrami et al., 2001; Walker et al., 2001; Roylet et al., 2003; Aivalakis et al., 2004; Delgado et al., 2007; Malone et al., 2007). Western blots, mRNA-level analyses, and PEPCK-activity measurement have revealed the highest expression level of the gene to happen in the pericarp of the ripe fruits and the lowest in stems, roots, and germinating seeds (Ruffner et al.,1975; Bahrami et al., 2001). Several studies have also proven that elevated levels of the PEPCK enzyme through pea development coincides with higher absorption and transmition of the nitrogenous compounds, and consequently deposition of the storage proteins in the cotyledon (Delgado et al., 2007).

In addition, studies on alfalfa have revealed a differential expression pattern of this gene through seed development; with the highest level of expression at the torpedo embryo stage and the lowest level at bending cotyldons and mature embryo (Aivalakis et al., 2004). Here we similarly observed very obvious differences in the expression level among the stages of seed development in both genotypes (MCC458 and MCC053). This data, together with observation of the highest expression at the full seed maturation stage, empowers the conclusion that elevated expression of this gene contributes to increments in seed protein content.

As mentioned earlier, arabidopsis genome contains two genes namely *pck1* and *pck2* with different expression patterns. *pck1* expression is more abundant at pre-budding stage, roots, flowers and mature leaves; whereas *pck2* is expressed only in roots and flowers (Roylet et al., 2003; Malone et al., 2007). As results shown, the 500 bp PCR product seems more intense at the flowering and seed development stages, while at the same time the 400 bp product shows a much less intensity. It appears, in orchestra with the previously published reports, of existing two isoforms for PEPCK with different pattern of gene expression, as we also suggest for them to happen in chickpea. Meanwhile the results should be verified by immunohistochemical assays, Real Time-PCR and Western blot techniques in different tissues of the plant.

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