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 azides 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 MCC357) 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 RTPCR 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 shoot 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 rich in carbohydrates (48.2-67.6%), protein (1.4-31.5%), starch (41-50%), fatty acids (6%), B-group vitamins, and nutritional elements (Kerst, 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 phosphoenolpyruvate (PEP) which will be further converted to sugar (Owasu-Abatang 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 (Alvalakits 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...
interacts with aspartate and asparagine in seed coats, but only by asparagine in catalysis of pea (ICRISAT, 2005).

Inosine PEPCK content is combined with elevated levels of the enzymes involved in amino acid metabolism, such as mitochondrial asparagine amino- transferase, cytosolic asparagine aminotransferase and aspartate aminotransferase. It can be induced by asparagine itself, and by asparagine in catalysis of pea. The present paper shows that the presence or absence of these enzymes is measured in a number of the investigated 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 26 genotypes of cultivated chickpea (table 1) kindly provided by the seed bank of the plant science institute. The protein content was measured by Kjeldahl method (Kosjet 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 (MC2373 and MC2291 as lowest and MC452 and MC053 as highest) were selected and sterilized with 75% ethanol for 30 seconds. The seeds were then placed between two layers of the sterilized muslin cloth to germinate in the laboratory. The germinated seeds were cultured in 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°C and 16 hrs darkness at 15 ± 2°C, until the seedling 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 guanidinium thiocyanate protocol (Chomczynsky 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 primers pair, including a forward 19-mer oligonucleotide of 5'-TTTGTUAACACTCTACC-3' and a reverse 19-mer of 5'-GGACAAACGTACCTTCA-3', were designed based on the mRNA sequence available for ubiquitin protein as registered in the NCBI database. PEPCK primers were a 15-mer forward of 5'-GAATGCGACATTTCTTC-3' and a 19-mer reverse of 5'-CUCATTCCCTAACAACAGC-3', based on the conserved regions identified in the cDNA sequence of PEPCK enzyme in alfalfa (Medicago truncatula), tomato (Solanum lycopersicum) and flax (Linum usitatissimum).

The primers were used to amplify a 208 bp fragment of the ubiquitin cDNA sequence, and fragments of 579 and 706 bp of the peapc cDNA and gDNA, respectively. All primers were designed using the Primer Premier (version 5) software.

cDNAs were constructed according to the standard recommendations (Femenia et al.) including: 0.4 μl of the template RNA together with 1 μl of the reverse primer (16 pmol/ml) and 8 μl of double distilled water, heating at 70°C (in the thermocycler) for 5 minutes for denaturation, and immediate cooling on ice. The reaction was performed according to the standard protocols, 2 μl of the first standard cDNA of each sample was subjected to ordinary PCR. The resulting cDNAs were stored at 20°C.

The thermal scheme for PCR was an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of 34°C, 52°C and 72°C for 1 minute each, and a final 6-minutes extension at 72°C. 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 reactions, the concentration must be adjusted according to an internal control gene, such as ubiquitin, continuously for equal loading of the templates. 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, which calculated a value for each amplified band (given in table 2) in form of a ratio.
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>
<thead>
<tr>
<th>Genotype</th>
<th>Seed Protein (percent)</th>
<th>weight of hundred seeds (gr)</th>
<th>type</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCC 067</td>
<td>25.50</td>
<td>33.2</td>
<td>Kabuli</td>
</tr>
<tr>
<td>MCC 099</td>
<td>25.85</td>
<td>9.8</td>
<td>Deui</td>
</tr>
<tr>
<td>MCC 165</td>
<td>25.24</td>
<td>16.8</td>
<td>Kabuli</td>
</tr>
<tr>
<td>MCC 201</td>
<td>21.13</td>
<td>19.0</td>
<td>Deui</td>
</tr>
<tr>
<td>MCC 227</td>
<td>25.66</td>
<td>21.4</td>
<td>Kabuli</td>
</tr>
<tr>
<td>MCC 333</td>
<td>24.72</td>
<td>30.8</td>
<td>Kabuli</td>
</tr>
<tr>
<td>MCC 476</td>
<td>23.12</td>
<td>28.2</td>
<td>Kabuli</td>
</tr>
<tr>
<td>MCC 490</td>
<td>26.12</td>
<td>27.2</td>
<td>Kabuli</td>
</tr>
<tr>
<td>MCC 510</td>
<td>24.68</td>
<td>32.0</td>
<td>Kabuli</td>
</tr>
<tr>
<td>MCC 053</td>
<td>30.41</td>
<td>33.2</td>
<td>Kabuli</td>
</tr>
<tr>
<td>MCC 202</td>
<td>23.60</td>
<td>15.8</td>
<td>Kabuli</td>
</tr>
<tr>
<td>MCC 258</td>
<td>25.90</td>
<td>31.4</td>
<td>Kabuli</td>
</tr>
<tr>
<td>MCC 332</td>
<td>28.46</td>
<td>15.0</td>
<td>Deui</td>
</tr>
<tr>
<td>MCC 426</td>
<td>28.31</td>
<td>33.2</td>
<td>Kabuli</td>
</tr>
<tr>
<td>MCC 458</td>
<td>30.57</td>
<td>25.4</td>
<td>Kabuli</td>
</tr>
<tr>
<td>MCC 477</td>
<td>25.42</td>
<td>27.4</td>
<td>Kabuli</td>
</tr>
<tr>
<td>MCC 496</td>
<td>24.80</td>
<td>25.6</td>
<td>Kabuli</td>
</tr>
<tr>
<td>MCC 498</td>
<td>26.30</td>
<td>23.6</td>
<td>Kabuli</td>
</tr>
<tr>
<td>MCC 207</td>
<td>27.40</td>
<td>17.6</td>
<td>Deui</td>
</tr>
<tr>
<td>MCC 373</td>
<td>20.92</td>
<td>17.4</td>
<td>Deui</td>
</tr>
</tbody>
</table>

**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/intron 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 peck 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.](image-url)
**Figure 2.** Optimization of polymerase chain reaction with peck 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 peck specific primers. Lanes 1, 2 and 3 are alfalfa cDNA amplified band (as positive control), chickpea cDNA amplified band and DNA 500bp Plus size marker respectively.

**Comparative analysis of peck expression through different stages of chickpea growth and development**

The accumulation of *peck* (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 *peck* primers. The RNA calibration based on the ubiquitin control is shown in figure 4.

**Figure 4.** Scaling the total RNA concentration in four genotypes (MCC291, MCC293, MCC458, MCC053) of chickpea based on the ubiquitin amplified bands. Lanes 1-6 size loaded correspond to bearing few leaves, flower bud, flowering, first pod, second pod, and full seed maturation, respectively.

The expected band was not amplified in any growth stage of MCC291 and MCC293 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).
The alfalfa cDNA are expressed in the RT-PCR products. The specificity control is supported by bands and equal intensity of the RT-PCR products.

Figure 5. Comparative measurement of pepk 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 pepk gene from each sample at the same conditions after 35 cycles of PCR, (D) amplified cDNA band related to pepk gene from each sample at the same conditions after 30 cycles of PCR, (E) developing stages of the examined samples including: 1) a few leaves, 2) flower bud, 3) flowering, 4) sheath formation, 5) seed formation, 6) fully matured seeds.

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>size marker</th>
<th>flower bud</th>
<th>flower</th>
<th>sheath formation</th>
<th>seed formation</th>
<th>seed development</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCC 05</td>
<td>100</td>
<td>65.30</td>
<td>78.08</td>
<td>531.52</td>
<td>152.16</td>
<td>174.40</td>
</tr>
<tr>
<td>MCC 45</td>
<td>100</td>
<td>59.10</td>
<td>65.13</td>
<td>498.57</td>
<td>174.16</td>
<td>90.50</td>
</tr>
</tbody>
</table>

Discussion

Studies on mature seeds of pea and alfalfa have revealed involvement of the pepk gene in nitrogen storage, grain filling and amino acid enrichment, and thus metabolism of storage proteins during seed development (Avivala 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 increase of seed protein content. It has also been shown that the Arabidopsis genome contains two related genes named pekl and pekl2, 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 pekl and pekl2, 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 transmigration 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 cotyledon 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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carboxykinase in Arabidopsis thaliana is essential for seedling establishment. Plant Physiology 131: 1834-1842.


