Full Length Research Article
Advancements in Life Sciences – International Quarterly Journal of Biological Sciences

ARTICLE INFO

Date Received: 18/04/2018;
Date Revised: 10/08/2019;
Date Published Online: 25/08/2019;

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How to Cite:
Shah SRU, Rashid M, Jaffar MA, Aslam M, Shah H, Rasool G, (2019). Evolution of Phosphoenolpyruvate carboxylase encoding transcripts in Chickpea (*Cicer arietinum* L.). Adv. Life Sci. 6(4): 152-158.

Keywords:
Phosphoenolpyruvate carboxylase transcripts; Chickpea; Phylogeny

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Evolution of Phosphoenolpyruvate carboxylase encoding transcripts in Chickpea (*Cicer arietinum* L.)

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Abstract

Background: Phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) is an important enzyme encoded by a gene family of at least 2-8 plant type and 1-2 bacterial type genes depending upon genome size or species complexity. This enzyme functions as catalyst for the β-carboxylation of phosphoenolpyruvate (PEP) to form oxaloacetate in cytoplasm. It is involved in carbon fixation and various other plant metabolic pathways.

Methods: In this study we characterized the evolutionary perspective of *PPC* transcripts and their abundance pattern in different plant tissues of chickpea (*Cicer arietinum* L.).

Results: The current study revealed that PEPC enzyme in chickpea is encoded by a gene family of at least 6 transcripts. All active site residues of C3 PEPCs were found in transcripts. Phylogenetic analysis of the amino acid sequences showed two major groups PTPC and BTPC from different ancestral lineages. Divergence of PTPC in two groups and further convergence within species was found in most of the plants while multiple evolutionary divergences was likely to be specific in legumes including chickpea.

Conclusion: *CaPPC* genes are regulated under various abiotic stress. Furthermore, the expression pattern of the identified genes can be helpful to explore plant metabolism of chickpea under abiotic stresses, which can be the next step to explore more into this gene family in chickpea.

Introduction

The phosphoenolpyruvate carboxylase (EC 4.1.1.31) is an important enzyme involved in primary metabolism in bacteria, archaea, algae and vascular plants. It has different catalytic and replenishing roles in C3 plants. It plays the vital role in plant metabolism, such as carbon fixation of C4 and CAM plants, regulation of pH in the cells, act as source of carbon in plant root metabolites specifically in cluster root and nodules [1, 2, 3]. PEPC catalyzes carboxylation of phosphoenolpyruvate (PEP) through bicarbonate (HCO_3^-) fixation to produce oxaloacetate, followed by reduction to malate or transamination to asparagine via aspartate [4, 5, 6]. PEPC proteins in plants are divided in to two classes. Class I PEPCs are homotetramers of PTPC subunits [3] while class II PEPCs are the hetero-octamers of BTPC and PTPC subunits complex. Class II PEPCs are reported in developing oilseeds of *Ricinus communis*, developing pollen of *Lilium longiflorum* and in some green algae [7, 8]. PEPC enzymes are coded by two distinct lineages, named as plant types (PTPCs) and bacterial types (BTPCs) genes [9]. Several candidate genes in *PPC* families are reported in agricultural plants. The gene family *PPC* consists of at least 3 genes of PTPC and 1 gene of BTPC in plants [9, 10]. Initial research on Arabidopsis and *Oryza sativa* have shown that in Arabidopsis there are three PTPC genes named as *Atppc1*, *Atppc2* and *Atppc3*, containing 10 exons, while the *Atppc4* gene, with 20 exons having sequence identity with bacterial *PPCs* than plant-type *PPC* [9]. Later research on these genes showed that there are five PTPCs, including 4 cytosolic and 1 chloroplastic and a bacterial type PPC gene in *Oryza sativa* [11, 12]. In a legume plant, white lupin (*Lupinus albus*), four PTPCs have been reported [13]. These results reveal that number of PTPCs members vary among the plant species in contrast to previous studies for Arabidopsis and rice [9]. Interaction of these two PEPC subunits PTPC and BTPC were proved at transcriptional and translational levels in *Ricinus communis*, where interaction of BTPC *RcPPC4* and the PTPC gene *RcPPC3* results the Class II PEPC protein in developing castor oil seed [7, 14].

Phosphoenolpyruvate carboxylase may have a great role in plant root metabolism, enhanced transcript levels and PEPC activity in cluster roots and nodules [5, 15] suggest its significance in diverse root tissues. In a study on *Lupinus albus*, it was found that phosphorus supply influenced the transcript levels of *PPC* that altered the carbon flux in glyceraldehyde 3-phosphate dehydrogenase and glycolytic bypass [16]. In a leguminous plant alfalfa root nodule exposed to elevated level of CO_2 resulted improvement in the N_2-fixing activity in nodules, where PEPC might have a contribution to malate biosynthesis for N_2-fixing bacteroides through facilitating oxaloacetate for N metabolism [17]. It also contributes in different other metabolic processes such as ripening of fruits, pollen germination, stomatal opening, nitrogen metabolism seed development and germination and against different abiotic stresses [18]. Recently introgression of *PPC* genes from maize into wheat has significantly improved the tolerance against heat and drought [19, 20].

Keeping in view the importance of *PPC* genes in plant metabolism, in this research we address the number of candidate genes of *PPC* family in chickpea and their evolutionary history and tissue specific role.

Methods

Sequence analysis

Full length sequences of *PPC* gene of chickpea were retrieved through "Basic Local Alignment Search Tool" from chickpea transcriptome database (CTDB) (http://www.nipgr.res.in/ctdb.html). Orthologues of *CaPPC* transcripts from model plant *Arabidopsis thaliana* were downloaded from *TAIR* database (https://www.arabidopsis.org). To retrieve *PPC* gene sequences of legume plant including model plant *Medicago truncatula*, the blast analysis was performed against each *AtPPC* gene of the *PPC* gene family using NCBI database. (http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis and Pairwise comparison

The orthologues of the *PPC* gene family of different plants were translated into protein and the protein sequences were aligned with the chickpea *PPCs* using CLC Main Workbench 7 with default settings. Phylogenetic analysis was performed through the Neighbor-Joining algorithm with bootstrapping of 1000 repeats using MEGA5 [21] and a rooted phylogenetic tree was constructed. Pairwise comparisons of Arabidopsis and chickpea PEPC protein sequences were carried out on aligned sequences using CLC Main Workbench 7.

Transcript abundance and hierarchical clustering:

Transcript abundance for each transcript of *PPC* were retrieved from the chickpea transcriptome database (CTDB)(http://www.nipgr.res.in/ctdb.html) and values as fragments (paired-end reads) per kilobase per million fragments (FPKM) mapped against each contig were calculated.. Transcript level (FPKM) in the different samples were normalized with averages of FPKM values from different tissues and used for heat map generation and hierarchical cluster analysis using CLC Main Workbench 7. Hierarchical cluster analysis was confirmed in SIMCA (Umetrics, Umeå, Sweden).

Results

Phylogenetic relationship of chickpea *PPC* genes with *PPCs* in other plants

Phylogenetic analysis of full-length protein sequences translated from RNA seq data (Fig.1 & 2) reflected the results of previous studies by separating plant type and bacterial type *PPCs* of chickpea in separate clades. Phylogenetic tree (Fig. 2) consists of two well-known major clusters of PTPC and a BTPC in all plants. It has been reported that the major lineages are separated before divergence of legumes [9, 18, 22]. In the PTPC cluster, Arabidopsis and *Lupinus albus* were placed in two groups while the legumes such as *Medicago truncatula*, *Glycine max* and *Cicer aritinum* made two major groups including an additional group and further sub-groups within each group. The additional legume specific cluster might have been diverged as different evolutionary lineage. Similar divergence has also been reported in *Glycine max* [18].

Of the chickpea transcripts, transcript *TC15934* shared a group (G.1) with 4 transcripts of *Glycine max*, 2 of *Lupinus albus* and 1 of *Vigna radiata*. Transcript *TC15977* shared a group (G. 2) with 1 transcript of each *G. max*, *V. radiata* and *M. truncatula*. Transcripts *TC19984* and *TC00570* were placed together and made a group (G. 3) with 1 transcript of each *M. truncatula* and *G. max*. Transcript *TC00569* was placed in a group (G. 4) with 1 transcript of each *M. truncatula* and *G. max*. Major cluster of BTPC contained one transcript of each plant species. Arabidopsis and *Lupinus albus* have the simpler *PPC* families consisting of two main groups while *Medicago truncatula*, *Glycine max* and *Cicer aritinum* showed great diversity within the *PPC* family. In all groups two of the legume species *Medicago truncatula*, *Glycine max* shared groups with chickpea transcripts, this indicates a close evolutionary history of these species within the legume family.

PTPC and BTPC Transcript model and catalytic domains

The length of the PTPC protein sequence is shorter (963-967) than BTPCs, which are 1043 amino acids longer. In plant and cyanobacteria BTPC are the largest PEPCs of 118 kDa reported to date while PTPC is of 107 kDa [14] but the insertion (residues 328–423) in BTPC in Arabidopsis (*AtPPC4*) do not match with the insertions observed in the cyanobacterial enzyme [23] which does not prove any co-evolutionary incident with Arabidopsis and cyanobacteria. All the PTPCs and BTPCs showed higher degree of conservation within each type as compared to conserved region between the PTPCs and BTPCs regardless the species differences (Fig. 2). Each type of *PPC* has also a specific region, missing in either of the other type. These different gene structures of PTPC and BTPC in plants as well as their coding sequences clearly suggest independent origins of these genes [9]. All well-known PEPCs catalytic amino acid residues of the deduced protein sequence were present in PEPC protein sequences of *Cicer aritinum*. Alignment of the deduced amino acid sequences of PTPCs and BTPCs indicated the occurrence of catalytic residues and domains typical for C3 plants. All the *CaPPCs* contain alanine instead of the C4 specific serine [24]. The N-terminal PTPC specific phosphorylation site is present in all PTPCs in *C. aritinum* and Arabidopsis. The HCO_3 and PEP binding sites at domain II (GYSDSGKDA) and loop (FHGRGGxxGRGG) are found conserved among the PTPCs and BTPCs [8, 22,25]. The PTPC and BTPC sequences in plants end with last conserved motif Q/RNTG [8, 22]. Alignment of the full-length protein sequences of PTPCs and BTPCs in Arabidopsis and chickpea is shown in supplementary files.

Pairwise comparison of deduced amino acid sequences

Pairwise comparison of full-length protein sequences deduced from PTPC transcripts of *Cicer aritinum* (Fig. 3) reflected the phylogenetic tree which showed high percentage sequence identity (83-98 %) with difference of 20-159 amino acids when compared within or with

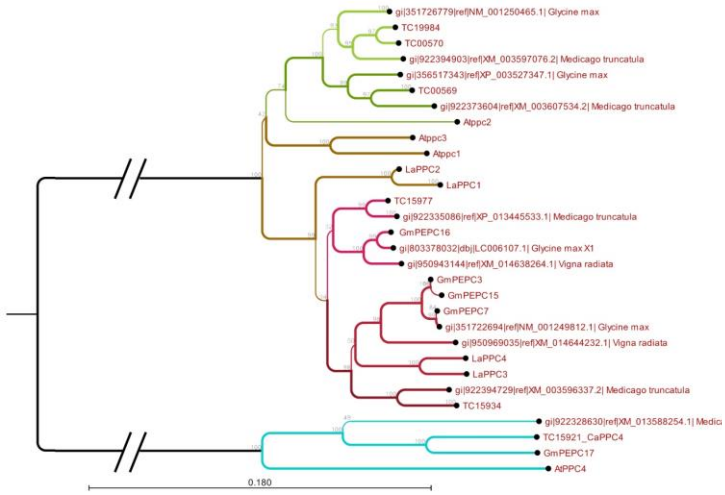

Figure 1: Phylogenetic tree of PEPC and BTPC protein sequences from model plants Arabidopsis thaliana and legume model plant *Medicago truncatula*, other legumes *Lupinus albus* and *Glycine max*. Partial protein sequences were aligned using Clustal W and the phylogenetic tree was constructed using the Neighbor-Joining method and bootstrap analysis was carried out using1000 replicates. Branches with more than 95% bootstrap values are highlighted. Different clades of PTPC and BTPC are shown with different colours. *PPC* transcripts of chickpea present in NCBI data base are shown with their accession numbers.

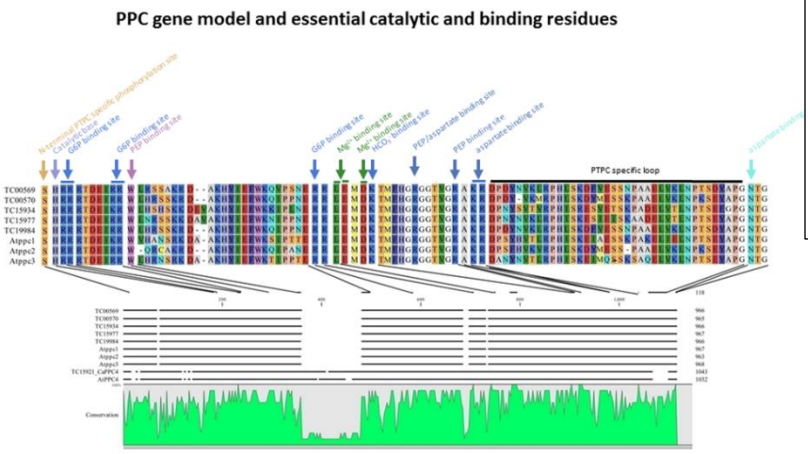

Figure 2: Alignments of selected deduced amino acid sequences of regulatory and catalytic domains of PTPC and BTPC gene models. The gene models based on amino acid sequences from Cicer aritinum and published sequences from Arabidopsis thaliana are shown in bars. Essential residues are indicated with respect to their positions in sequences. Conservation of the sequences is shown with line graph.

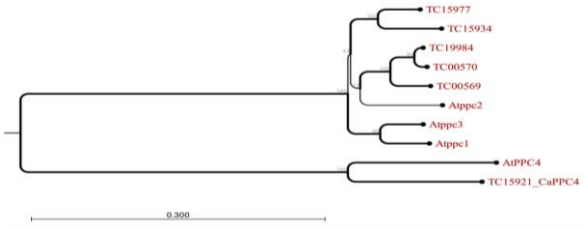

Figure 6: Neighbor-Joining tree using deduced protein sequences. Transcript designation is shown at the ends of the branches and bootstrap values at branch nodes. Branches with more than 95% bootstrap values are highlighted.

	1	2	3	4	5	6	7	8	9
TC00569	1		92.24	85.95	86.67	94.31	85.94	86.65	85.74	37.04
TC00570	2	75		85.74	87.50	97.93	86.14	86.96	86.98	37.04
TC15934	3	136	138		90.59	86.05	84.50	83.57	84.62	36.59
TC15977	4	129	121	91		87.50	86.88	85.12	87.72	37.31
TC19984	5	55	20	135	121		86.25	86.85	87.09	37.40
Atppc1	6	136	134	150	127	133		85.11	91.74	37.04
Atppc2	7	129	126	159	144	127	144		84.50	36.89
Atppc3	8	138	126	149	119	125	80	150		37.73
TC15921_CaPPC4	9	697	697	702	694	693	697	698	690	
AtPPC4	10	692	696	694	687	696	694	695	690	265

Figure 3: Pairwise comparison of PEPC protein sequences. Amino acid sequences deduced from RNAseq contigs of *Cicer aritanum* were compared within the gene family and with PEPC sequences of model plant Arabidopsis. Sequence similarity in percent is shown in upper half while number of different amino acid residues are shown in lower half.

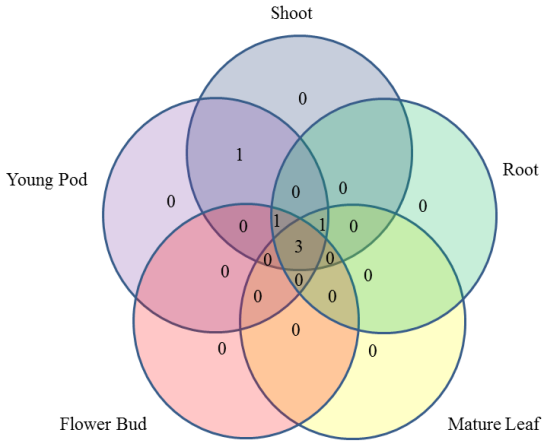

Figure 4: Venn diagram of *CaPPC* transcripts expressed in five different plant tissues with FPKM ≥ 10.

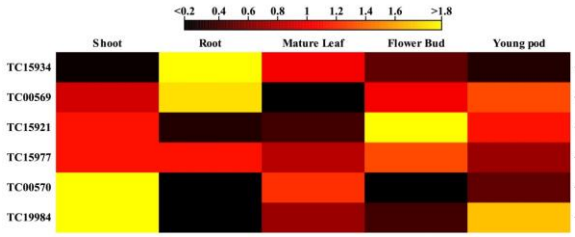

Figure 5: Differential expression of CaPPC transcripts in different tissues. Transcript levels based on fragments per kilobases per million mapped fragments (FPKM) were retrieved from chickpea database and heat maps and hierarchical clustering was generated using CLC workbench 7. Different tissues of chickpea like shoot, root, mature leaf, flower bud and young pod were subjected to experiment. The expression levels indicated from lowest (black) to highest (yellow) as shown by the scale.

Arabidopsis. Very low percent identity was recorded between all PTPCs and BTPC (<38 %) with >687 amino acid difference. Similar results were also reported in Arabidopsis, *Ricinus communis* and *G. max* [9, 18, 22]. Within PTPCs in chickpea, transcript *TC00570* had highest sequence identity (98%) with *TC19984* while lowest identity with transcript *TC15934*. All others PTPCs are ranged between these two transcripts.

Expression pattern of *CaPPCs*

To determine which of the *CaPPC* transcripts are expressed in more than one tissue, a Venn diagram was constructed (Fig. 4). Three of the transcripts were expressed in all tissues, each of the two transcripts were expressed in three tissues while 1 transcript was expressed in two of the studied tissues. To evaluate the *PPC* transcript abundance pattern in various tissues, normalized FPKM values of RNA-seq data revealed differential abundance of the *PPC* transcripts in different tissues of chickpea (Fig. 5). Tissue specific abundance of *PPC* transcripts indicates different functions of this gene family. The PTPCs transcripts showed higher expression in shoot, root and young pod except *TC15977* which had higher expression in flower buds and similarly a BTPC, *TC15921* had also higher expression in flower bud. Previously it has been reported that *PPC* genes were expressed in male reproductive tissues in both lily and *Arabidopsis* and it was mentioned to have a role in pollen maturation [26]. Two of the transcripts, *TC15934* and *TC00569* had higher transcript level in roots which indicates role of this gene in organic acid metabolism in roots. Within root system expression of *PPC* genes may also vary in different developmental stages of roots [5]. In *Arabidopsis*, *AtPPC1* showed higher expression in root [9] while in a recent study *AtPPC3* is reported as root specific gene containing citrate binding sites [27]. Two of the transcripts *TC00570* and *TC19984* showed abundance in shoots which may have different developing organs within shoots. In developing shoots *PPC* have a role as carbon source of building block for organogenesis of the tissues [22, 28] or high expression in shoot may also have a role in photosynthesis. Hierarchical cluster analysis (Fig. 5) revealed co-expression of BTPC, *TC15921* and a PTPC, *TC15977* while the two PTPC transcripts *TC19984* and *TC00570* have co-expression and also close phylogenetic relationship (Fig. 6).

Discussion

Phosphoenolpyruvate carboxylase is an important enzyme involved in CO_2 fixation in photosynthesis in C4 and crassulacean acid metabolism. It plays a role in different metabolic processes in various plant tissues such as fruit, pollen grain, leaf, seed and root in tissue development and against different abiotic stresses [19, 20, 18]. In plants, phosphoenolpyruvate carboxylase comprises a small gene family containing variable number of members in different species such as 4 members in Arabidopsis to 10 members in *Glycine max* [9, 18].

This study was carried out to investigate that how many members of *PPC* genes named as *CaPPC* gene family exist in chickpea and to quantify their transcript levels. We also compared the evolution of *PPCs* in chickpea being edible representatives of legumes with other plant species. Analysis of RNA sequence data from chickpea database revealed that *CaPPC* gene family has at least 5 PTPC transcripts and at least 1 BTPC transcript which is much lower number than *PPC* genes in *Glycine max* [18]. The possible reason for the discrepancy could be no or lower expression of the transcripts in tissues studied for constructing this transcriptomic data. *Arabidopsis* and rice *PPCs* were also consisted of two gene sub-families [9]. Rice has been shown to contain five PTPCs (four cytosolic and one chloroplastic) and one BTPC [11, 12] while four PTPCs have been reported in white lupin [13]. Less number of *PPC* genes in chickpea than soybean could be due to smaller genome size ~738-Mb which is 64-67% of soybean genome [29, 30].

In plants, the plant-type PEPC protein exists as homotetramers (class 1 PEPC) while the bacterial-type PEPCs found in plants exist as heterotetramers of subunits derived from both PTPC and BTPC genes [26]. Co-expression of BTPC, *TC15921* and a PTPC, *TC15977* in chickpea indicates that these two transcripts may encode for the two sub-units of class II PEPC protein. In *R. communis*, the BTPC gene *RcPPC4* and *RcPPC3* had co-expression proposing an interaction of BTPC and PTPC transcripts to participate in a class 2 PEPC protein [7]. Previously in Seabuckthorn PTPC

HrPPC5 and BTPC *HrPPC4* showed similar transcript patterns [5]. Comparing hierarchical cluster (Fig. 5) and phylogenetic tree (Fig. 6) of *CaPPCs* revealed that the transcript *TC19984* and *TC00570* have co-expression and close phylogenetic relationship which specifies that the both regulatory region and coding regions of these transcripts have same evolutionary history.

This study revealed the existence of two diverse types of chickpea *PPC* genes, PTPC and BTPC, which are diverged as separate evolutionary lineage. A legume specific cluster of PPCs was also separated at early divergence of legumes. *CaPPCs* include 5 plant type *PPCs* and at least a bacterial type *PPC*. Differential expression of *CaPPC* gene family members occur in various tissues. There are specific transcripts for root, shoot and flower which indicates its diverse role in chickpea.

Conflict of Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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