

Phosphoenolpyruvate Carboxylase cDNA phylogeny to investigate the C₄ photosynthetic pathway evolution in grasses

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Keywords: C₄ photosynthetic pathway, cDNA, grass, phosphoenolpyruvate carboxylase, phylogeny

Introduction

The grass family is a large group composed of more than 10,000 species of which numerous are cultivated, and, particularly C₄ plants in tropical areas (i.e. maize, sorghum, sugarcane, mil, panicum). All C₄ grasses are classified in the "PACC clade" (Davis and Soreng 1993) which includes the subfamilies Panicoideae, Arundinoideae, Chloridoideae and Centothecoideae. C₄ photosynthesis occurs in all Chloridoideae (except *Eragrostis walteri* Pilg.), in a few Arundinoideae [i.e. *Aristida* L. (tribe Aristideae) or *Alloochaete* C.E. Hubb. (tribe Arundineae Dumort.)] and in most Panicoideae (Watson and Dallwitz 1992).

C₄ pathway involves several enzymes which catalyze the fixation and the transport of carbon dioxide. This results in a cycle reaction, called C₄ cycle or Hatch and Slack cycle, which concentrates carbon dioxide in the bundle sheath cells where photosynthesis is occurring. The first enzyme which is involved in the C₄ cycle, is the C₄°Phosphoenolpyruvate Carboxylase (PEPCase; EC 4.1.1.31). C₄°PEPCase gene is biotechnologically of potential interest (Ku et al. 1999) and it should be interesting to assess the diversity of this gene. PEPCase enzymes are encoded by an oligogenic family. A few C₄ and non-C₄ PEPCase isoforms have been characterised in maize, sorghum, wheat, rice and sugarcane. Each isoform is involved in a specific function such as initial fixation of atmospheric CO₂ (= C₄ PEPCase) and anaplerotic functions associated to nitrogen assimilation or amino-acid biosynthesis (for review see Lepiniec et al. 1994). Lepiniec et al. (1994) have postulated that C₄ PEPCase isoforms should derive from non-C₄ PEPCase isoforms. It is currently assumed that C₄ plants would independently appeared several times during evolution as attested by the phylogenetic reconstruction based on the different known gene sequences of C₄° and non-C₄° PEPCases (Lepiniec et al. 1994; Gehrig et al. 1998). An interesting point concerns the grasses, for which C₄ and non-C₄° isoforms have been shown to be well-differentiated. This suggests that grass C₄° PEPCases have diverged from non-C₄° PEPCases since a long time (Lepiniec et al. 1994). *Nevertheless, the grasses displayed very diversified photosynthetic pathways: C₃, C₄ PCK, C₄ NADP-ME or C₄ NAD-ME (Watson and Dallwitz 1992; Ku et al. 1996). Consequently, multiple appearances of grass C₄ photosynthetic pathway is supposed to have occurred (Sinha and Kellogg 1996) and C₄ isoform could have appeared several times. For a better understanding of the C₄ pathway evolution in grasses, sequencing of C₄ PEPCase RT-PCR products can be used to study the phylogenetic relationships between C₄ grass species.*

In the present study, we generated partial C₄°PEPCase cDNA sequences from various grass species. A phylogenetic approach was performed to estimate the evolutionary relationships between a few grass species and to discuss about the C₄ pathway evolution. This revealed that C₄°PEPCase isoform was very likely appeared only once during grass evolution.

Material and methods

Plant material

A subset of 12 species representative of all grass subfamilies displaying C₄ species was sampled (Table[°]1). The RNA extractions were performed from green leaves according to Besnard et al. (in preparation) protocol. C₄ PEPCase isoform has been reported to be highly expressed in green leaves in contrast to the other PEPCase isoforms (Lepiniec et al. 1994).

Table1: List and classification of the grass species analyzed in this study

Sub-family	Tribe	Species
Panicoideae Link.	Andropogoneae Dumort.	Saccharum officinarum L. <i>Saccharum spontaneum</i> L. <i>Hyparrhenia rufa</i> (Ness) Stapf <i>Eulalia aurea</i> (Borg) Kunth. <i>Themeda quadrivalvis</i> (L.) Kuntze <i>Heteropogon contortus</i> (L.) Beauv. ex Roem. & Schult. <i>Coix lacryma-jobi</i> L.
	Paniceae R. Br.	<i>Paspalum paniculatum</i> L. <i>Panicum maximum</i> Jacq.
Chloridoideae Burmeist.	Cynodonteae Dumort.	<i>Cynodon dactylon</i> (L.) Pers.
	Eragrostideae Stapf	<i>Eleusine indica</i> (L.) Gaertn.
Arundinoideae Burmeist.	Aristideae C.E. Hubb.	<i>Aristida mauritiana</i> Kunth.

RT-PCR and cDNA fragment sequencing

Reverse transcription was performed on total RNA using oligo-dT primers and the Reverse Transcriptase Kit (Gibco-BRL). A primer pair specific of grass C₄ PEPCase (Besnard et al. in preparation) was used to perform PCR amplifications. For each studied accession, a cDNA PEPCase segment of about 1,200 bp was generated. This was positioned 450 bp after the initiation codon according to the complete *Saccharum officinarum* and *Sorghum vulgare* sequences (EMBL accessions SOF293346 and X17379, respectively). PCR products were sequenced either directly or after cloning using the pGEM^T vector (PROMEGA).

Sequence analyses

For phylogenetic reconstruction, we used the CLUSTAL software (v.1.7). The known C₄ and non-C₄ PEPCase cDNA nucleotide sequences from sorghum, maize, wheat and rice plus the generated cDNA segments were aligned. We reconstructed a phylogeny based on all known grass PEPCase nucleotide sequences using distances (percent divergence) and the Neighbor

Joining algorithm. We used as outgroups non C₄ or C₄ PEPCase sequences from *Picea* (gymnosperm) and *Flaveria* (dicotyledonous) species to root the phylogram. In addition, reliability of the resulting tree was measured using bootstrap analysis of 1,000 replicates.

Results and discussion

Sequence data

For each studied accession, one sequence of a partial cDNA PEPCase was obtained. All the sequences displayed a quite high level of homology (from 78% to 96 %) with known grass C₄ PEPCases (EMBL accessions: X03613, X15238/9, X17379 and AF268091). Homology with grass non-C₄ PEPCase was comprised between 70 and 75 %. Using the same primers, PCR amplification did not occur in two C₃ grasses [*Osplimetus* (Panicoideae) and *Holcus* (Pooideae) genera; data not shown]. Lastly, due to their expression in green leaves and the high homology with grass C₄ PEPCase isoform, we assumed that the generated cDNA PEPCase segments corresponded to C₄ PEPCase isoform.

Phylogenetic reconstruction

We reconstructed a phylogeny based on all known grass PEPCase nucleotide sequences (Fig. 1). C₄ PEPCases formed a monophyletic clade (clade *D*) in the PEPCase phylogram while three grass non-C₄ PEPCase groups (clades *A*, *B* and *C*) were distinguished. Branch lengths are longer in the clade *D* suggesting a faster evolution of the C₄ gene in comparison with the other isoforms.

Phylogenetic relationships between species, deduced from C₄ PEPCase sequences, were quite different to those deduced from other nuclear data (Mathews and Sharrock 1996; Hsiao et al. 1999). First, *Aristida* (Arundinoideae) is placed between *Panicum* and *Paspalum*, two genera belonging to the tribe Paniceae (Panicoideae). However, bootstrap analysis showed that the *Panicum* position is not well-supported (only 39% for the bootstrap value). Second, the subfamily Chloridoideae displayed a basal position in our C₄ PEPCase clade, whereas other molecular data have supported that the subfamily Arundinoideae displayed such position (Mathews and Sharrock 1996; Hsiao et al. 1999). Consequently, these features have to be checked using more accessions and also with different analysis methods (Besnard et al. in preparation).

C₄ pathway evolution in grasses

The occurrence of only one C₄ PEPCase clade sustained that C₄ PEPCase isoform should have appeared only once in the grass evolution. Thus, we can assume that all grass C₄ photosynthetic pathways have derived from a common ancestor and convergent evolution hypothesis for this system inside grass family (Sinha and Kellogg 1996; Hsiao et al. 1999) would be debatable. Alternatively, due to adaptive implications of the C₄ photosynthetic pathway, we can assume that several independent disappearances of the C₄ system have likely occurred.

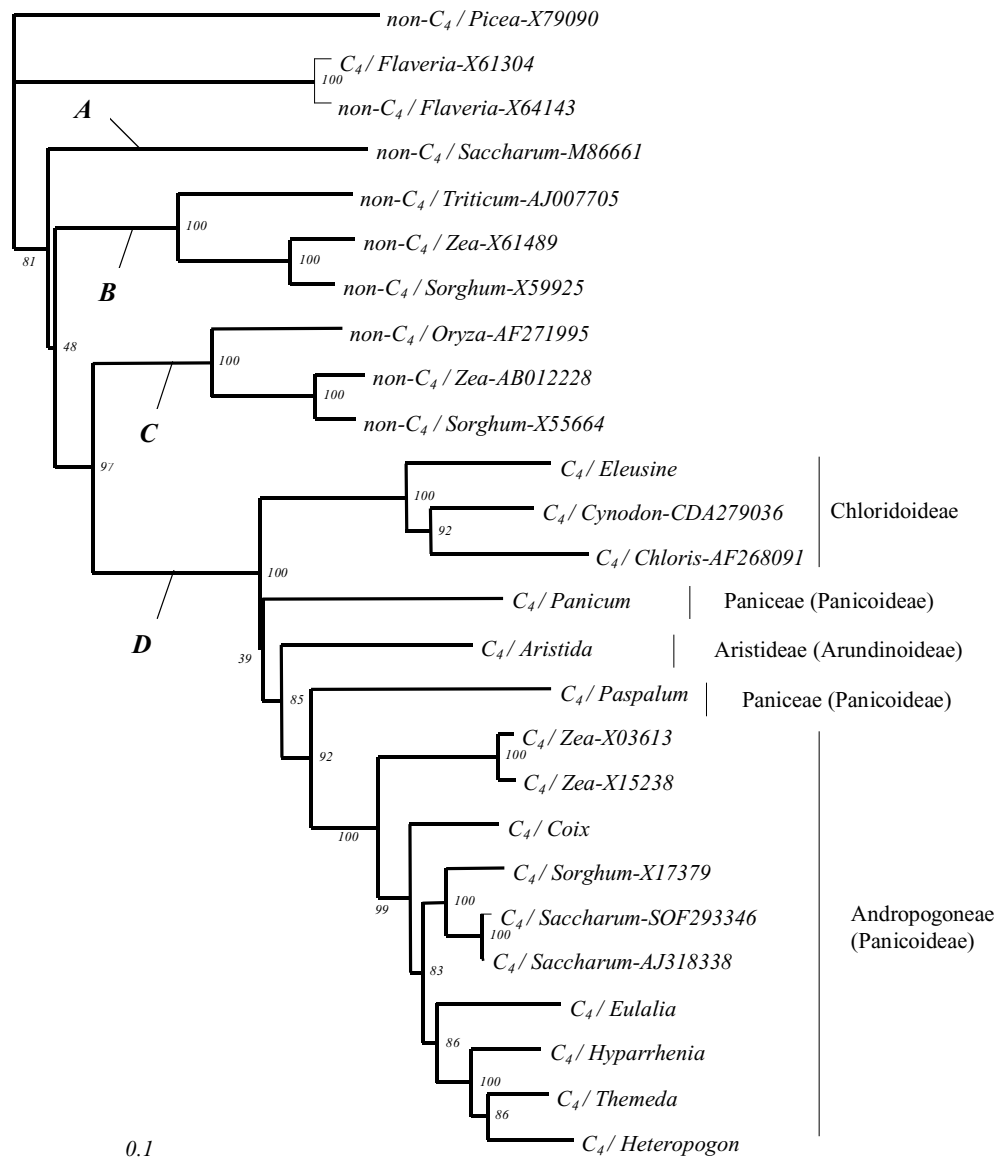


Fig. 1. Grass PEPCase phylogenetic tree based on percent divergences between nucleotide sequences and constructed using the Neighbor Joining algorithm. Bootstrap values (in percent) are noted on each corresponding node. The main grass PEPCase clades are indicated by the letters A, B, C and D.

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