Evolution of the C4 phosphoenolpyruvate carboxylase promoter of the C4 species Flaveria trinervia: the role of the proximal promoter region

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Abstract

Background: The key enzymes of photosynthetic carbon assimilation in C4 plants have evolved independently several times from C3 isoforms that were present in the C3 ancestral species. The C4 isoform of phosphoenolpyruvate carboxylase (PEPC), the primary CO2-fixing enzyme of the C4 cycle, is specifically expressed at high levels in mesophyll cells of the leaves of C4 species. We are interested in understanding the molecular changes that are responsible for the evolution of this C4-characteristic PEPC expression pattern, and we are using the genus Flaveria (Asteraceae) as a model system. It is known that cis-regulatory sequences for mesophyll-specific expression of the ppcA1 gene of F. trinervia (C4) are located within a distal promoter region (DR).

Results: In this study we focus on the proximal region (PR) of the ppcA1 promoter of F. trinervia and present an analysis of its function in establishing a C4-specific expression pattern. We demonstrate that the PR harbours cis-regulatory determinants which account for high levels of PEPC expression in the leaf. Our results further suggest that an intron in the S’ untranslated leader region of the PR is not essential for the control of ppcA1 gene expression.

Conclusion: The allocation of cis-regulatory elements for enhanced expression levels to the proximal region of the ppcA1 promoter provides further insight into the regulation of PEPC expression in C4 leaves.

Background

About 90% of terrestrial plant species, including major crops such as rice, soybean, barley and wheat, assimilate CO2 via the C3 pathway of photosynthesis. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) acts as the primary CO2-fixing enzyme of C3 photosynthesis, but its ability to use O2 as a substrate instead of CO2 results in the energy-wasting process of photorespiration. The photosynthetic C4 cycle represents an addition to the C3 pathway which acts as a pump that accumulates CO2 at the site of Rubisco so that the oxygenase activity of the enzyme is inhibited and photorespiration is largely suppressed. C4

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plants therefore achieve higher photosynthetic capacities and better water- and nitrogen-use efficiencies when compared with C₃ species [1].

C₄ photosynthesis is characterized by the coordinated division of labour between two morphologically distinct cell types, the mesophyll and the bundle-sheath cells. The correct functioning of the C₄ cycle depends upon the strict compartmentalization of the CO₂ assimilatory enzymes into either mesophyll or bundle-sheath cells [2]. Phosphoenolpyruvate carboxylase (PEPC), which serves as the actual CO₂ pump of the C₄ pathway, is specifically expressed in the mesophyll cells of C₄ leaves. This enzyme is not an unique feature of C₄ species; other PEPC isoforms with different catalytic and regulatory properties are found in both photosynthetic and non-photosynthetic tissues of all plants where they participate in a variety of metabolic processes, e.g. replenishment of citric acid cycle intermediates and regulation of guard cell movement [3].

The polyphyletic origin of C₄ photosynthesis suggests that the photosynthetic C₄ isoforms of PEPC have evolved independently several times from non-photosynthetic C₃ isoizymes [4]. During the evolution of C₄ PEPC genes from ancestral C₃ genes, changes in expression strength and organ- and cell-specific expression patterns must have occurred. While C₃ PEPC genes are highly expressed in the mesophyll cells of the leaf, the C₄ isoform genes are only moderately transcribed in all plant organs [5-8].

To investigate the molecular evolution of a C₄ PEPC gene we are using the genus *Flavia* (Asteraceae) as a model system. This genus includes C₃ and C₄ as well as C₃–C₄ intermediate species [9,10] and thus provides an excellent system for studying the evolution of the C₄ photosynthetic pathway [11]. Previous studies on the ppcA1 gene of *F. trinervia*, encoding the C₄ isoform of PEPC, revealed that the strong mesophyll-specific expression is largely regulated at the transcriptional level and that the available 2188 bp (with reference to the AUG start codon of the ppcA1 reading frame) of the 5’ flanking sequences contain all the essential cis-regulatory elements for high and mesophyll-specific expression [12]. Two parts of the ppcA1 promoter of *F. trinervia*, a proximal region (PR) up to -570 in combination with a distal region (DR) from -1566 to -2141, are sufficient to direct a high mesophyll-specific expression of a β-glucuronidase (GUS) reporter gene in transgenic *F. bidentis* (C₃) plants [13]. The orthologous, 2538 bp comprising ppcA1 promoter of the C₃ species *F. pringlei* displays only weak activity in all interior leaf tissues in transgenic *F. bidentis*, but fusion of the C₃-DR to this C₃ PEPC promoter leads to a confinement of GUS expression to the mesophyll [13]. Analysis of the C₃-DR revealed that the 41-bp module MEM1 (mesophyll expression module 1) is responsible for the C₄-characteristic spatial expression pattern of the ppcA1 gene of *F. trinervia*. Furthermore, it was shown that a high level of expression in the mesophyll requires an interaction of the C₄-DR with the C₄-PR. This suggests that quantity elements for an elevated expression of the C₄ PEPC gene are located within the PR of the 5’ flanking sequences [13].

Using the yeast one-hybrid system, Windhövel and colleagues [14,15] identified four different proteins which bind to the PR of the ppcA1 promoter of *F. trinervia*, but not to the corresponding part of the ppcA1 promoter of *F. pringlei*. These proteins (named FtHB1 to FtHB4) belong to the class of zinc finger homeodomain proteins (ZF-HD). Two regions of the C₄-PR specifically interact with the FtHB proteins in vitro: an intron sequence within the 5’ untranslated leader region and a DNA fragment that is located upstream of the putative TATA-box. To the latter one, the FtHB proteins showed a much lower binding affinity [14]. Homeobox proteins are known to act as transcriptional regulators of eukaryotic gene expression [16-18], and the fact that the FtHB homeobox proteins interact specifically with the PR of the ppcA1 promoter of *F. trinervia* makes them prime candidates for transcription factors that are involved in the establishment of the C₄-characteristic expression pattern of the C₄ ppcA1 gene.

In this study we have investigated the role of the proximal promoter region of the ppcA1 gene of *F. trinervia* with regard to its high and mesophyll-specific expression by transgenic analyses in the closely related C₄ species *F. bidentis*. We demonstrate that the proximal promoter region of the ppcA1 gene contains cis-regulatory elements that determine promoter strength. Furthermore, we show that the deletion of an intron located in the 5’ untranslated segment of ppcA1 does not alter promoter activity in transgenic *F. bidentis*.

**Results and discussion**

**Experimental strategy**

We are interested in elucidating the molecular events that are crucial for the evolution of the high and mesophyll-specific expression of the C₄ phosphoenolpyruvate carboxylase gene (ppcA1) of the C₄ plant *F. trinervia*. In this study we focus on the proximal promoter region (PR) of the ppcA1 gene with respect to its function in establishing the C₄-characteristic expression pattern. We performed a comparative analysis of three different promoter-GUS fusion constructs (Fig. 1) in transgenic *F. bidentis* plants. *F. bidentis* is a close relative to *F. trinervia*, but in contrast to *F. trinervia* this C₄ species is transformable by *Agrobacterium tumefaciens* mediated gene transfer [19] and was therefore chosen for these experiments.

Construct ppcA-PR₀₋₄₋DR₊₁₉₅ served as a reference because it was already known from previous experiments that a
combination of the distal (DR) and the proximal (PR) promoter regions was sufficient to direct a high and mesophyll specific expression of a GUS reporter gene in *F. bidentis* [13]. To find out if the PR of the C₄ *ppcA1* promoter contains quantity elements conferring high expression in the mesophyll cells we designed construct *ppcA*-PR₁⁻DR(+)₁. Here, the C₃-PR was exchanged for its counterpart from the orthologous *ppcA1* gene of the C₃ species *F. pringlei*. Deletion of the intron sequences in the 5’ untranslated segment of promoter construct *ppcA*-PR₁⁻ΔIntron-DR(+)₁ resulted in the formation of construct *ppcA*-PR₁⁻ΔIntron-DR(+)₁. Thereby a putative binding site for the ZF-HD proteins FtHB1 to FtHB4 [14] was removed from the C₄-PR. Hence, this chimeric promoter-GUS fusion could answer the question whether the intron-located putative binding site of the FtHB proteins is necessary for the establishment of the C₄-specific *ppcA1* expression pattern.

**The proximal region of the *ppcA1* promoter of *F. trinervia* harbour cis-regulatory elements for a high level of PEPC expression in the mesophyll**

Gowik et al. [13] assumed that the PR of the *ppcA1* promoter of *F. trinervia* comprises cis-regulatory determinants conferring high levels of expression in mesophyll cells of C₄ leaves. To examine whether the PR actually harbour such quantity elements we analyzed the GUS expression patterns of constructs *ppcA*-PR₁⁻DR(+)₁ and *ppcA*-PR₁⁻ΔIntron-DR(+)₁ (Fig. 1) in transgenic *F. bidentis*.

In *F. bidentis* plants that had been transformed with promoter construct *ppcA*-PR₁⁻DR(+)₁, GUS expression was exclusively detected in the mesophyll cells of the leaves (Fig. 2A). This observation shows that the DR and PR of the *ppcA1* promoter together are sufficient for a high and mesophyll-specific expression of the linked GUS reporter gene and therefore confirms the results obtained by Gowik et al. [13]. Replacement of the C₄-PR by the corresponding region from the *ppcA1* promoter of *F. pringlei* (construct *ppcA*-PR₁⁻DR(+)₁) did not cause any alteration in the cellular GUS expression pattern when compared to *ppcA*-PR₁⁻ΔIntron-DR(+)₁; GUS activity was still restricted to the mesophyll compartment (Fig. 2B). However, both chimeric promoters differed greatly in transcriptional strength. Quantitative GUS assays revealed that promoter activity was decreased by a factor of 15 when the C₄-PR was substituted for the C₃-PR (Fig. 2D). This clearly demonstrated that the C₄-characteristic transcription-enhancing cis-regulatory elements must be located within the proximal region of the *ppcA1* promoter of *F. trinervia*. The low expression level of construct *ppcA*-PR₁⁻ΔIntron-DR(+)₁ could be the result of an absence of transcription-enhancing cis-regulatory elements in the C₃-PR, but it might also be caused by problems in the interaction of the C₄-DR and the C₃-PR.

**The intron in the C₄-PR is not required for the establishment of a C₄-specific expression pattern of the *ppcA1* gene of *F. trinervia***

The 5’ untranslated region of the *ppcA1* gene of *F. trinervia* contains an intron between positions -209 and -40 (+1 refers to the starting point of translation). Introns are of prominent importance for the molecular evolution of eukaryotic genomes by facilitating the generation of new genes via exon-shuffling and by providing the possibility to create multiple proteins from a single gene via alternative splicing [20-22]. Furthermore, it has been shown that introns can affect many different stages of gene expression, including both transcriptional and post-transcriptional mechanisms [22-24].

Here, we wanted to investigate whether the first intron of the *ppcA1* gene of *F. trinervia* is essential for establishing the C₄-characteristic expression pattern. We therefore
deleted the intron sequences from the C₄-PR in construct ppcA-PR₁ΔIntron-DR(+)F₄, resulting in the formation of construct ppcA-PR₁ΔIntron-DR(+)F₄ (Fig. 1). The histochemical analysis of transgenic F. bidentis plants demonstrated that the ppcA-PR₁ΔIntron-DR(+)F₄ promoter was exclusively active in the mesophyll cells of the leaves (Fig. 2C). The quantitative examination of GUS activity (Fig. 2D) also revealed no significant differences between ppcA-PR₁ΔIntron-DR(+)F₄ (6.5 nmol MU/(mg*min)) and ppcA-PR₁ΔIntron-DR(+)F₄ (5.9 nmol MU/(mg*min)). These data suggest that the 5' located intron of ppcA1 does not contain any cis-regulatory elements that are essential for achieving high mesophyll-specific expression of a reporter gene. Accordingly, the specific binding of the FtHB proteins to this intron that was observed in vitro and in yeast one-hybrid experiments [14,15] has no in planta relevance concerning the regulation of ppcA1 expression in C₄ leaves. However, our results do not necessarily indicate that the intron is completely dispensable for the regulation of ppcA1 gene expression. It is known that C₄ gene transcription is modulated by various metabolites such as sugar hexoses [25-27], and we cannot exclude that the first intron of the ppcA1 gene of F. trinervia might be involved in the metabolic control of gene expression.

**Comparison of proximal ppcA promoter sequences from different Flaveria species**

As reported above, cis-regulatory elements for leaf-specific enhanced transcription of the ppcA1 gene of F. trinervia could be allocated to the PR of the 5' flanking sequences, but their exact nature and localization was still unclear. To identify potential cis-regulatory enhancing elements, a
sequence comparison between the PR of the ppcA1 gene of *F. trinervia* and equivalent promoter sequences from other *Flaveria* species was performed (Fig. 3). This approach was chosen because it was already known from northern analyses of ppcA transcript levels in different *Flaveria* species that ppcA RNA amounts in leaves increase gradually from C3 to C4 species [28]. This is consistent with the important function of PEP in C4 photosynthesis. The C4-like species *F. brownii* and *F. vaginata* exhibited ppcA RNA levels that were comparable to those of the C4 plants *F. bidentis* and *F. trinervia*, and even in *F. pubescens*, a C3–C4 intermediate with rather poorly developed C4-characteristic traits, ppcA transcript accumulation in the leaves was significantly higher than in the C3 species *F. cronquistii* and *F. pringlei* [28].

Searching for known plant cis-regulatory DNA elements in the PLACE database [29] resulted in the identification of two distinct sequence motifs which might be involved in the regulation of ppcA expression levels (Fig. 3). Both of them, a putative MYB transcription factor binding site (GTGTAGTT, [30]) and a CCAAT box [31], are present in all examined C3–C4, C4-like and C4 species, but are missing in the two C3 species (Fig. 3). Thus, these sequences are prime candidates for transcription-enhancing cis-regulatory elements. CCAAT boxes are common sequences that are found in the 5' untranslated regions of many eukaryotic genes [32]. They are able to regulate the initiation of transcription by an interaction of CCAAT-binding transcription factors with the basal transcription initiation complex [33]. There is no unifying expression pattern for plant genes containing putative CCAAT promoter elements, indicating that they may play a complex role in regulating plant gene transcription [32]. MYB proteins, on the other hand, comprise one of the largest families of transcription factors in plants, with almost 200 different MYB genes present in the Arabidopsis genome [34–36]. To test the physiological importance of the putative MYB and CCAAT-binding transcription factors with the basal transcription initiation complex, we have demonstrated that the proximal promoter of *F. trinervia* (C4) harbours cis-regulatory elements conferring high expression levels in leaf mesophyll cells of transgenic *F. bidentis* (C4). It was further demonstrated that the deletion of an intron in the 5' untranslated leader region does not affect the C4-specific ppcA1 expression pattern and strength, indicating that the previously isolated zinc finger-homeobox transcription factors that specifically interact with this intron in vitro are not involved in regulating ppcA1 expression levels. Sequence comparisons resulted in the identification of potential cis-regulatory elements in the proximal part of the ppcA1 promoter that might play a role in controlling ppcA1 expression quantity. Genetic manipulation of these sequences and subsequent analyses in transgenic *F. bidentis* will clarify whether they are able to direct high ppcA1 expression levels in C4 leaves.

**Conclusion**

In this study, we have demonstrated that the proximal region (-570 to -1) of the ppcA1 promoter of *F. trinervia* (C4) harbours cis-regulatory elements conferring high expression levels in leaf mesophyll cells of transgenic *F. bidentis* (C4). It was further demonstrated that the deletion of an intron in the 5' untranslated leader region does not affect the C4-specific ppcA1 expression pattern and strength, indicating that the previously isolated zinc finger-homeobox transcription factors that specifically interact with this intron in vitro are not involved in regulating ppcA1 expression levels. Sequence comparisons resulted in the identification of potential cis-regulatory elements in the proximal part of the ppcA1 promoter that might play a role in controlling ppcA1 expression quantity. Genetic manipulation of these sequences and subsequent analyses in transgenic *F. bidentis* will clarify whether they are able to direct high ppcA1 expression levels in C4 leaves.

**Methods**

**Construction of chimeric promoters**

DNA manipulations and cloning were performed according to Sambrook and Russell [38]. The construction of the promoter-GUS fusion ppcA-PR_{C4}·DR(+)_{Rt} has been described in detail [13]. Plasmids ppcA-S-Fp[39] and ppcA-PR_{C4}·DR(+)_{Rt} served as the basis for the production of ppcA-PR_{C3}·DR(+)_{Rt}. The distal region (-2141 to -1566) of the ppcA1 promoter of *F. trinervia* was excised from ppcA-PR_{C3}·DR(+)_{Rt} by digestion with XbaI. Insertion of this promoter fragment into XbaI-cut ppcA-S-Fp resulted in the generation of construct ppcA-PR_{C3}·DR(+)_{Rt}.

For the production of construct ppcA-PR_{C3}·ΔIntron·DR(+)_{Rt} a part of the ppcA1 promoter from *F. trinervia* (-570 to -209) was amplified by PCR with primers S-Ft-F (5'-TGCTCTAGACCGGTGTTAATGATTCG-3') and S-Ft-R (5'-CTGATTGTGGATATGCTCTAGACCT-3'). Plasmid ppcA-PR_{C3}·DR(+)_{Rt} was used as the template for this PCR reaction. The amplified promoter fragment was cut with XbaI. The outermost 3' region of the ppcA1 promoter (-39 to -1) was generated by annealing two oligonucleotides S-Ft-3'1 (5'-GGTGGAGGGGAAAATTAAATGATCGAAGGCTTTGACG-3') and S-Ft-3'2 (5'-GCCGCTACTCTACAC-CCTTGTTATATCCGCTCACC-3').
Figure 3

Nucleotide sequence alignment of the proximal regions of ppcA promoters from F. trinervia (C4, ppcA-Ft), F. bidentis (C4, ppcA-Fb), F. voginata (C4-like, ppcA-Fv), F. brownii (C4-like, ppcA-Fbr), F. pubescens (C3-C4, ppcA-Fpub), F. cronquistii (C3, ppcA-Fc) and F. pringlei (C3, ppcA-Fp). Identical positions in all ppcA sequences are marked by an asterisk. The intron sequences in the 5' untranslated leader regions are marked by grey nucleotides. The start site of the F. trinervia ppcA transcript is indicated by an arrow, the TATA-box by a yellow box, the putative MYB-binding site by a blue box, and the CCAAT-sequences by a green box. Fragments of the F. trinervia ppcA1 promoter that interact with the FtHB proteins in the yeast one-hybrid system [14, 15] are marked by red bars. The translational ATG start codon is indicated by green nucleotides.
a XmaI-compatible 5' overhang was created next to position -1. The ppcA-S-Ft promoter plasmid [39] was digested with XbaI and XmaI and the released ppcA1 promoter fragment was removed by agarose gel electrophoresis. The XbaI/XmaI-cut ppcA-S-Ft plasmid was ligated with the two ppcA1 promoter fragments (-570 to -209/-39 to -1) and the resulting plasmid was named ppcA-PRf1ΔIntron. The distal region of the ppcA1 promoter of *F. trinervia* (-2141 to -1566) was removed from of ppcA-PRf1-ΔIntron by incubation with XbaI and inserted into XbaI-cut ppcA-PRf1ΔIntron. The resulting plasmid was designated ppcA-PRf1ΔIntron-DR(+).  

**Plant transformation**

In all transformation experiments the *Agrobacterium tumefaciens* strain AGL1 was used [40]. The promoter-GUS constructs were introduced into AGL1 by electroporation. The transformation of *Flaveria bidentis* was performed as described by Chitty et al. [19]. The integration of the transgenes into the genome of regenerated *F. bidentis* plants was proved by PCR analyses.

**Measurement of GUS activity and histochemical analysis**

*F. bidentis* plants used for GUS analysis were 40 to 50 cm tall and before flower initiation. Fluorometrical quantification of GUS activity in the leaves was performed according to Jefferson et al. [41] and Kosugi et al. [42]. For histochemical analysis of GUS activity the leaves were cut manually with a razorblade and the sections were transferred to incubation buffer (100 mM NaHPO₄, pH 7.5, 10 mM EDTA, 50 mM K₄[Fe(CN)₆], 50 mM K₃[Fe(CN)₆], 0.1% (v/v) Triton X-100, 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid). After brief vacuum infiltration the sections were incubated at 37°C for 6 to 20 hrs. After incubation chlorophyll was removed from the tissue by treatment with 70% ethanol.

**Computer analyses**

DNA sequence analyses were performed with MacMolly Tetra [43]. The sequence alignments were created with the program DIALIGN 2.2.1 [44]. Sequence data mentioned in this article can be found in GenBank under accession numbers X64143 (F. trinervia ppcA1), X64144 (F. pringlei ppcA1), AY297090 (F. vaginata ppcA1), AY297089 (F. conquisitii ppcA1), AY297087 (F. bidentis ppcA1), EF522173 (F. brownii ppcA1) and EF522174 (F. pubescens ppcA1).

**Authors' contributions**

SE carried out the histochemical and quantitative GUS assays, the cloning of construct ppcA-PRf1ΔIntron-DR(+), the sequence alignments and wrote the manuscript. CZ produced construct ppcA-PRf1-ΔIntron-DR(+). MK, US and MS performed the transformation of *F. bidentis*. PW coordinated the design of this study and participated in drafting the manuscript. All authors read and approved the final manuscript.

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