# Molecular Evolution of Gene Expression and Protein Function in the Genera *Flaveria* (Asteraceae) and *Alternanthera* (Amaranthaceae): Phospho*enol*pyruvate Carboxylase and Glycine Decarboxylase

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1. Sascha Engelmann, Corinna Zogel, Maria Koczor, Ute Schlue, Monika Streubel, Peter Westhoff (2007): **Evolution of the $C_4$ Phosphoenolpyruvate Carboxylase Promoter of the $C_4$ Species *Flaveria trinervia*: the Role of the Proximal Promoter Region**. Submitted to BMC Plant Biology for publication.

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## I.1. $C_4$ Photosynthesis and Photorespiration

The evolution of oxygenic photosynthesis marks a central event in the development of life on Earth. The atmosphere of the early Earth was largely anaerobic, thereby preventing the emergence of advanced eukaryotic life forms. The advent of oxygenic photosynthesis, which evolved in the ancestors of recent cyanobacteria at least 2,5 billion years ago, permitted the development of advanced life by creating the ozone layer that shields the earth from UV radiation and by providing a ubiquitous terminal oxidant for respiration (Blankenship, 1992; Blankenship and Hartman, 1998; Summons et al., 1999).

Today, three types of oxygenic photosynthesis occur among higher plants. The most common and most primitive one of these is the $C_3$ pathway, while $C_4$ photosynthesis and the crassulacean acid metabolism (CAM) represent more evolutionarily recent photosynthetic variants (Ehleringer and Monson, 1993). More than 90% of all land plants, including agronomically important crop species such as wheat, barley, soybean and rice, assimilate $CO_2$ via $C_3$ photosynthesis (Kutschera and Niklas, 2007). In $C_3$ species, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) serves as the primary enzyme for $CO_2$-fixation. Rubisco catalyzes the transfer of $CO_2$ to ribulose-1,5-bisphosphate (RuBP) to produce two molecules of 3-phosphoglycerate (PGA), and processing of PGA in the Calvin cycle finally results in the formation of triosephosphates and the regeneration of RuBP. Rubisco is a bifunctional enzyme because it is also able to use $O_2$ as a substrate instead of $CO_2$, and in this case the oxidative degradation of RuBP results in the generation of one molecule each of PGA and 2-phosphoglycolate. Phosphoglycolate is metabolically useless and even toxic if it accumulates in the cell (Ogren, 1984), and therefore it is subsequently converted to PGA by the action of ten different enzymes distributed between three different organelles (chloroplasts, peroxisomes and mitochondria).

RuBP oxygenation by Rubisco and the following recycling reactions are termed photorespiration or $C_2$ oxidative photosynthetic carbon cycle (Tolbert, 1997). In the course of the recycling process, 75% of the carbon originally lost by the oxygenase activity of Rubisco are recovered for use in the Calvin cycle, while 25% are released as $CO_2$. The loss of $CO_2$ by photorespiration and the additional energy costs of the recycling reactions lead to a decrease of photosynthetic efficiency in $C_3$ plants. High temperatures increase the oxygenation activity of Rubisco (Wingler et al., 2000), and in hot and dry climates photorespiration can reduce the overall efficiency of $C_3$ photosynthesis by more than 30% (Jordan and Ogren, 1984; Brown and Byrd, 1993; Brown et al., 2005).

Despite of being a rather wasteful process, different positive aspects of photorespiration are also discussed. It is suggested that the photorespiratory mechanism in $C_3$ plants is important for energy dissipation to prevent photoinhibition, especially under stress conditions that lead to reduced rates of photosynthetic $CO_2$ assimilation (Kozaki and Takeba, 1996). Furthermore, photorespiration generates metabolites which can be used in other metabolic pathways, for example, provision of glycine for the synthesis of glutathione, a component of the antioxidative system in plants (Noctor and Foyer, 1998; Wingler et al., 2000).

The existence of the $C_4$ photosynthetic pathway was discovered in the 1960s by Hugo P. Kortschak, Marshall D. Hatch and Charles R. Slack (Kortschak et al., 1965; Hatch and Slack, 1966). $C_4$ photosynthesis represents an addition to the conventional $C_3$ cycle that occurs only in angiosperms. The leaves of $C_4$ species typically contain two different photosynthetic cell types, namely mesophyll- and bundle-sheath cells. The vascular bundles of a $C_4$ leaf are wrapped by one or two layers of bundle-sheath cells that are surrounded by a ring of mesophyll cells which are in contact with the intercellulars. This particular arrangement of leaf cells, which was described as “Kranz anatomy“ (Haberlandt, 1904), is a prerequisite for the correct functioning of the $C_4$ cycle.

Primary fixation of inorganic carbon in $C_4$ species takes place in the mesophyll cytoplasm by phosphoenolpyruvate carboxylase (PEPC), which catalyzes the carboxylation of phosphoenolpyruvate (PEP) to yield the $C_4$ dicarboxylic acid oxaloacetate (OAA). Three biochemically distinct types of $C_4$ photosynthesis exist (NAD-ME, NADP-ME and PCK), and the PEPC reaction is the only enzymatic step common to all versions of the $C_4$ pathway (Hatch, 1987; Sage, 2004). In $C_4$ plants of the NADP-ME type, OAA is converted to malate and subsequently transported symplastically to the bundle-sheath cell chloroplasts. Here, malate is decarboxylated via NADP-dependent malic enzyme so that pyruvate and $CO_2$ are produced, and pyruvate moves back to the mesophyll cell chloroplasts where it is used for the regeneration of PEP by pyruvate-orthophosphate dikinase. In the NAD-ME and PCK type $C_4$ species, aspartate – which is generated by transamination of OAA – serves as the $CO_2$-transporting metabolite from mesophyll to bundle-sheath cells. In NAD-ME species, aspartate enters the mitochondria of the bundle-sheath cells where it is metabolized to $CO_2$ and pyruvate. The latter one is converted to alanine in the bundle-sheath cell cytosol and is then transferred to the mesophyll cells where it is converted back to pyruvate and then to PEP. In PCK-type plants, transamination of aspartate in the bundle-sheath cell cytoplasm results in the formation of OAA. Decarboxylation of OAA by PEP-carboxykinase then leads to the

production of $CO_2$ and PEP, and PEP can return directly to the mesophyll compartment for carboxylation by PEPC (Hatch, 1987; Leegood and Walker, 1999; Sage, 2004).

The common principle shared by all three $C_4$ variants is the enrichment of $CO_2$ in the bundle-sheath compartment. Here, $CO_2$ is efficiently refixed by Rubisco and assimilated in the conventional Calvin cycle. The unique mode of carbon assimilation, consisting of primary carbon fixation in the mesophyll cells by PEPC and subsequent decarboxylation of $C_4$ acids in the bundle-sheath cells, can be regarded as a pump that concentrates $CO_2$ at the site of Rubisco, which is exclusively located in the bundle-sheath cells of $C_4$ plants. $CO_2$ concentrations in the chloroplasts of $C_3$ leaves are considerably lower (130 µl/l) than in the bundle-sheath cell chloroplasts of $C_4$ species (2000 µl/l) (Ehleringer et al., 1991). Therefore, the oxygenase activity of Rubisco is greatly reduced and photorespiration almost completely suppressed in $C_4$ plants.

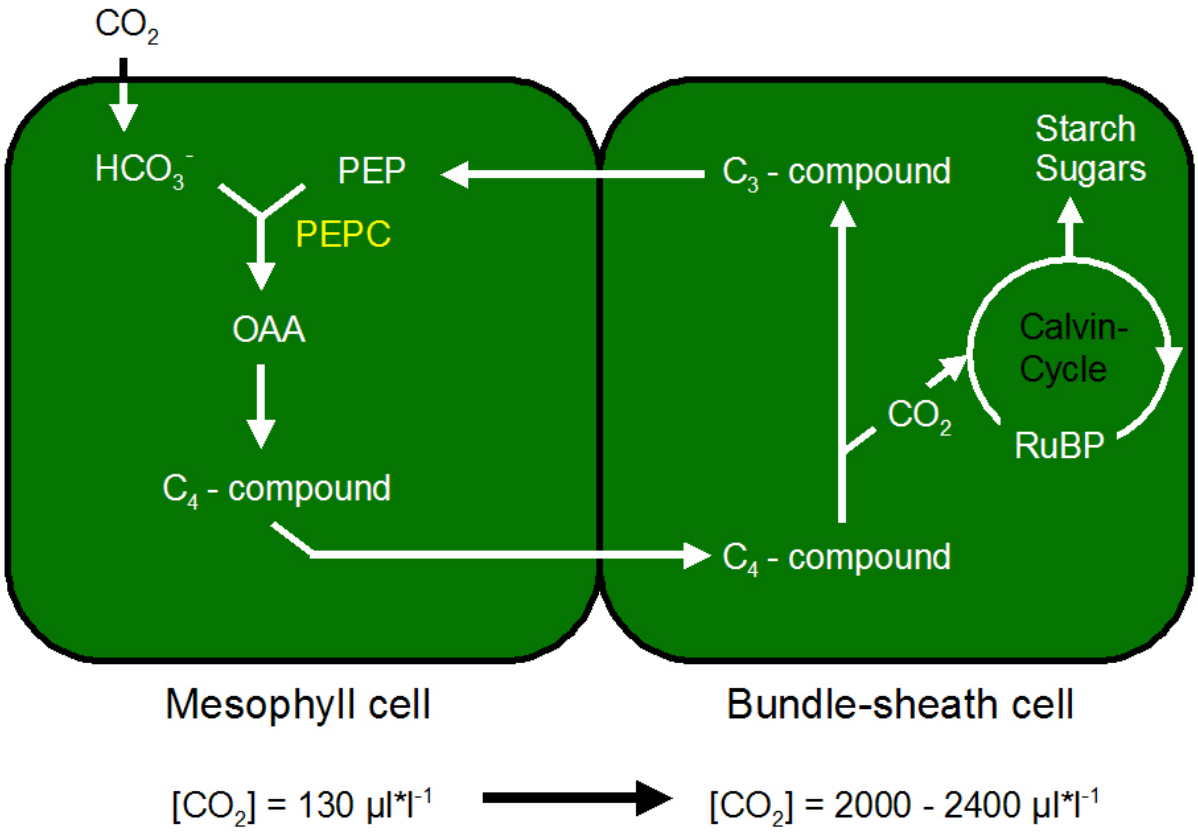


**Figure 1:** The principle reactions of the $C_4$ photosynthetic pathway. The $C_4$ cycle acts as a $CO_2$ pump which accumulates $CO_2$ in the bundle-sheath cells. PEP: phosphoenolpyruvate; PEPC: phosphoenolpyruvate carboxylase; OAA: oxaloacetate; RuBP: ribulose-1,5-bisphosphate.

Compared to $C_3$ photosynthesis, the operation of the $C_4$ cycle requires two additional ATP to reduce a $CO_2$ molecule, with the additional ATP associated with the regeneration of

PEP from pyruvate (Ehleringer and Monson, 1993). Because of this higher energy demand, $C_4$ plants photosynthesizing under non-photorespiratory conditions show a lower quantum yield of photosynthesis than $C_3$ plants. However, the light-use efficiency of photosynthesis in $C_3$ plants decreases with rising temperature due to increased levels of photorespiration, and therefore the quantum yields of $C_3$ and $C_4$ species are almost identical at leaf temperatures between 25°C and 30°C (Ehleringer and Björkman, 1977). In $C_4$ leaves, the high assimilatory capacity of PEPC ensures that $CO_2$ does not become a rate-limiting factor of photosynthesis under high-light conditions, and this enables $C_4$ species to achieve elevated photosynthetic capacities when compared to those of $C_3$ plants (Pearcy and Ehleringer, 1984). Other advantages of $C_4$ over $C_3$ photosynthesis can be detected when we compare the water- and nitrogen-use efficiencies of $C_3$ and $C_4$ plants. As a consequence of the $CO_2$-concentrating mechanism in $C_4$ species, the degree of stomatal opening exerts only little influence on photosynthetic rates. Therefore, the loss of water by transpiration is greatly reduced in the $C_4$ leaf. $C_4$ plants produce one gram of biomass for every 250-350 grams of water transpired, whereas in $C_3$ plants, this ratio is one gram of biomass for every 650-800 grams of water transpired (Taylor et al., 1983). The better nitrogen-use efficiency of $C_4$ compared to $C_3$ species is a result of the substantiantly lower amounts of Rubisco in $C_4$ leaves. In $C_3$ leaves, up to 50% of the soluble nitrogen is located in the Rubisco protein (Hatch, 1987). Although $C_4$ plants exhibit equivalent or higher maximum photosynthetic rates than $C_3$ plants, they contain three to six times less Rubisco (Ehleringer and Monson, 1993).

An important aspect of $C_4$ photosynthesis is the division of labour between mesophyll and bundle-sheath cells, and the partitioning of the photosynthetic reactions between these two cell types depends upon the strict compartmentalization of the $C_4$ assimilatory enzymes. In NADP-ME type $C_4$ species, PEPC, NADP-malate dehydrogenase and pyruvate-ortho-phosphate dikinase are specifically located in the mesophyll cells, whereas Rubisco and NADP-dependent malic enzyme are only found in the bundle-sheath compartment (Hatch, 1987). Enzymes of the photorespiratory $C_2$ carbon cycle (Baldy and Cavalié, 1984) and the sulphur (Schmutz and Brunold, 1984) and nitrogen (Rathnam and Edwards, 1976) assimilation pathways also accumulate differentially in mesophyll and bundle-sheath cells. Studies on the $C_4$ species maize revealed that mesophyll-specific expression is mainly regulated at the transcriptional level, while bundle-sheath-specific expression is likely controlled at both transcriptional and posttranscriptional levels (Sheen, 1999).

To fulfill the requirements of the $C_4$ cycle, a close physical interaction of the two photosynthetic cell types is indispensable. Diffusion of photosynthetic metabolites between

mesophyll and bundle-sheath cells is facilitated by a high frequency of plasmodesmata, and a direct exposure of bundle-sheath cells to intercellular space is avoided in order to minimize the rate of $CO_2$ leakage (Dengler and Nelson, 1999). The outer wall of the bundle-sheath cells in some grasses is often impregnated with suberin to enhance the resistance of the wall to $CO_2$ efflux, and in species without a suberin barrier there is a tendency for chloroplasts to occur on the inner (centripetal) side of the cell. Thus, the large vacuole of the cell helps to slow down the escape of $CO_2$ (Sage, 2004).

The vast majority of $C_4$ plants uses Kranz-anatomy to concentrate $CO_2$ at the site of Rubisco. However, recently the phenomenon of single-celled $C_4$ photosynthesis has been identified in a number of species, e.g. in the aquatic monocots *Hydrilla verticillata* and *Egeria densa* (Reiskind et al., 1997; Bowes et al., 2002), as well as in the terrestic species *Bienertia cycloptera* and *Borszczowia aralocaspica* (Freitag and Stichler, 2000; Voznesenskaya et al., 2001; Freitag and Stichler, 2002; Voznesenskaya et al., 2002). Nevertheless, these single-cell types of $C_4$ photosynthesis are regarded as rare exceptions that evolved only under very special environmental conditions (Westhoff and Gowik, 2004).

## I.2. The Evolution of $C_4$ Photosynthesis

The $C_4$ photosynthetic pathway independently evolved several times during the evolution of angiosperms. Today, this polyphyletic origin is reflected by the occurrence of different $C_4$ variants in at least 19 families of mono- and dicotyledonous plants (Sage, 2004). On the geological timescale, $C_4$ photosynthesis represents a relatively recent phenomenon. There is evidence that this new metabolic pathway at first arose in grasses at least 20 to 30 million years ago during the Oligocene/Miocene epochs (Kellogg, 1999), while Chenopods were probably the first $C_4$ dicots, appearing 15-20 million years ago (Sage, 2004). The oldest known fossil records of $C_4$ plants date back 12,5 million years ago (Cerling, 1999), and by the end of the Miocene (5 million years ago) a widespread global expansion of $C_4$-dominated grasslands took place (Cerling et al., 1997).

What was the selective pressure that was responsible for the evolution of $C_4$ species from $C_3$ ancestors? It is known that during the Mesozoic era (251-65 million years ago) the atmospheric $CO_2$ concentrations were four to eight times higher than today (Ehleringer and Monson, 1993). At this time, the oxygenase activity of Rubisco in $C_3$ organisms was negligible due to the elevated $CO_2$ and low $O_2$ levels in the atmosphere (Sage, 1999). After the Cretaceous, atmospheric $CO_2$ levels dropped, while $O_2$ levels increased (Ehleringer and Monson, 1993), and therefore photorespiration became prominent which resulted in a

decrease of net carbon assimilation by photosynthesis. To overcome the problem of carbon-loss by photorespiration, for $C_3$ plants it was not possible to develop a new, oxygenase-free form of Rubisco because the active site of this enzyme is constrained by similarities in the oxygenase and carboxylase reactions (Andrews and Lorimer, 1987). The photorespiratory problem could finally be solved by the evolution of the $C_4$ pathway, a mechanism that was able to enhance the $CO_2$-concentration at the active site of Rubisco. The addition of a $CO_2$-concentrating module to the ancient $C_3$ cycle must have been much easier in genetic terms than the development of a complete novel type of carbon assimilation, and this view is supported by the polyphyletic origin of $C_4$ photosynthesis and the fact that all $C_4$ enzymes are also present as non-photosynthetic isoforms in $C_3$ species and in non-photosynthetic tissues of $C_4$ plants. It is assumed that these $C_3$ isoforms in the ancestors of the $C_4$ species served as the starting point for the evolution of the $C_4$ genes (Monson, 1999).

During the evolution of the $C_4$ cycle, changes must have occurred in the $C_4$ progenitor genes to adapt to the requirements of the new photosynthetic pathway. $C_4$ genes are highly expressed, while the $C_3$ isoform genes are only moderately transcribed (Hermans and Westhoff, 1990; Cretin et al., 1991; Ernst and Westhoff, 1997), and therefore the effectiveness of gene expression had to be increased. To guarantee the correct compartmentalization of the $C_4$ enzymes, organ- and cell-specific expression patterns had to evolve (Hatch, 1987), and, in some cases, these enzymes had to develop different kinetic and regulatory properties when compared to their $C_3$ counterparts (Ku et al., 1996). Apart from these changes related to the biochemical aspects of the new metabolic pathway, alterations in leaf anatomy were necessary to establish a functioning $C_4$ cycle. Bundle-sheath cells also exist in $C_3$ species where they are involved in phloem loading and unloading (Kinsman and Pyke, 1998), but they are usually smaller than those of $C_4$ species and contain only few chloroplasts, which is associated with reduced photosynthetic activity (Metcalfe and Chalk, 1979). To evolve an effective $CO_2$ concentration mechanism, the distance between mesophyll and bundle-sheath cells had to decline to allow for rapid diffusion of metabolites (Raghavendra, 1980), and this was accomplished by reducing interveinal distances (Dengler and Nelson, 1999). Finally, the number of chloroplasts and mitochondria in the bundle-sheath cells had to be increased to create the necessary metabolic sinks for the new biochemical pathway (Sage, 2004).

A number of vascular plant species have been described as exhibiting photosynthetic characteristics intermediate between $C_3$ and $C_4$ plants (Monson et al., 1986). These $C_3$-$C_4$ intermediate plants are of special interest for investigating the evolution of $C_4$ photosynthesis

because it is assumed that they represent evolutionary intermediates which progress from $C_3$ to $C_4$ plants (Edwards and Ku, 1987; Monson and Moore, 1989). At least 24 species of both monocots and dicots have been described as being $C_3$-$C_4$ intermediates, including the genera *Neurachne*, *Eleocharis*, *Panicum*, *Mollugo*, *Alternanthera*, *Flaveria*, *Parthenium* and *Moricandia* (Rawsthorne, 1992). Most $C_3$-$C_4$ species tend to have a leaf anatomy intermediate to $C_3$ and $C_4$, with vascular strands surrounded by chlorenchymatous bundle-sheath cells reminiscent of the Kranz anatomy of leaves of $C_4$ plants. However, among them there is great variation in the degree of Kranz-cell development, reaching from species with $C_4$-like leaf structure (e.g. *Flaveria brownii*, *Neurachne minor*) to species with only poorly developed Kranz-anatomy (e.g. *Flaveria pubescens*, *Moricandia arvensis*) (Edwards and Ku, 1987).

A primary character for identifying $C_3$-$C_4$ intermediate species is the $CO_2$ compensation point ($\Gamma$), that is defined as the ambient $CO_2$ concentration at which the apparent rate of photosynthesis is just balanced by the apparent rate of photorespiration. The intermediate $\Gamma$ values of $C_3$-$C_4$ species indicate that they exhibit lower rates of apparent photorespiration than $C_3$ plants, yet higher rates than $C_4$ plants (Edwards and Ku, 1987). Some $C_3$-$C_4$ plants are to some extent able to fix $CO_2$ into the $C_4$ acids malate and aspartate (Monson et al., 1986), but most $C_3$-$C_4$ species do not possess a functional $C_4$ cycle. This raises the question how the reduced rates of photorespiration in these species can be achieved even in the absence of $C_4$ photosynthesis. Identical to the situation in $C_4$ species, the photorespiratory enzyme glycine decarboxylase (GDC) occurs exclusively in the bundle-sheath cells of all $C_3$-$C_4$ intermediate plants (Hylton et al., 1988). This indicates that the breakdown of photorespiratory phosphoglycolate cannot take place in the mesophyll cells of $C_3$-$C_4$ leaves, and therefore phosphogylcolate produced by Rubisco in the mesophyll cells has to move to the bundle-sheath compartment in order to become recycled to PGA for use in the Calvin-cycle.

In the bundle-sheath cells of $C_3$-$C_4$ intermediates, the mitochondria and peroxisomes are typically located in the centripetal region of the cells adjacent to the vascular tissue, whereas the chloroplasts can be found at the cell periphery towards the leaf mesophyll (Edwards and Ku, 1987; Rawsthorne et al., 1998). The relocation of GDC to the bundle-sheath compartment and the special arrangement of organelles result in the specific release of photorespiratory $CO_2$ in the centripetal part of the bundle-sheath cells, and so this $CO_2$ must pass through the overlying chloroplasts on its way to the leaf surface. In the chloroplasts, the $CO_2$ can efficiently be recaptured by Rubisco, and therefore photorespiratory loss of $CO_2$ in $C_3$-$C_4$ intermediate species is decreased compared to $C_3$ species (Rawsthorne et al., 1998).

Initial studies on the $C_3$-$C_4$ intermediate *Moricandia arvensis* showed that the loss of GDC activity from the mesophyll cells is due specifically to a lack of the P-subunit protein (GLDP), and the absence of additional GDC-subunits in the leaf mesophyll of other $C_3$-$C_4$ species suggests that they have developed further towards $C_4$ photosynthesis than *M. arvensis*. It is assumed that the confinement of GDC to the bundle-sheath cells might have constituted a biochemical starting point for the evolution of the $C_4$ syndrome (Morgan et al., 1993; Bauwe and Kolukisaoglu, 2003; Sage, 2004), and the subsequent increase of mesophyll PEPC activity might have been the next step in establishing a $C_4$-like $CO_2$-concentrating mechanism, followed by an enhancement and differential accumulation of other $C_4$ cycle enzymes (Sage, 2004). A schematic presentation of the main phases of $C_4$ evolution is shown in Figure 2.

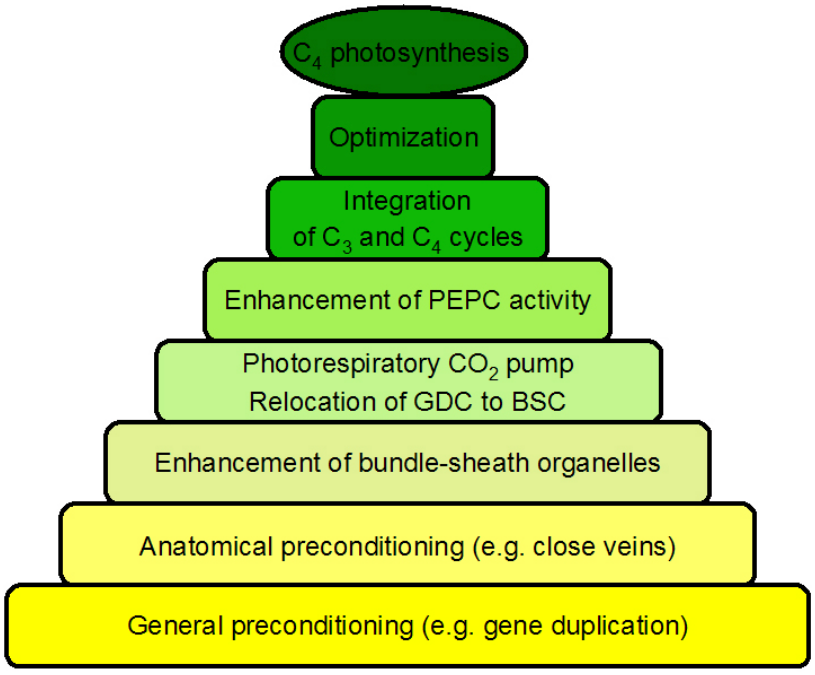


**Figure 2:** Simplistic model of the stepwise evolution of $C_4$ photosynthesis (adapted from Sage, 2004). GDC: glycine decarboxylase; PEPC: phospho-enolpyruvate carboxylase; BSC: bundle-sheath cell.

## I.3. The Phosphoenolpyruvate Carboxylase

Phosphoenolpyruvate carboxylase (PEPC) is a ubiquitous cytosolic enzyme that is present in all photosynthetic organisms such as plants, algae, cyanobacteria and photosynthetic bacteria, but also in most non-photosynthetic bacteria and protozoa. It is absent in animals, fungi and yeasts (Izui et al., 2004) and was first discovered in spinach leaves in 1953 (Bandurski and Greiner, 1953). Besides its important function in carbon fixation in $C_4$ and CAM species, PEPC plays primarily an anaplerotic role in non-photosynthetic tissues by repleneshing $C_4$-dicarboxylic acids utilized in the tricarboxylic acid cycle, thereby providing carbon skeletons necessary for nitrogen assimilation and amino acid biosynthesis (Melzer and O'Leary M,

1987). Non-photosynthetic isoforms of PEPC are also involved in the regulation of stomatal function (Tarczynski and Outlaw, 1990) and cytoplasmic pH maintenance (Latzko and Kelly, 1983), and some autotrophic bacteria assimilate $CO_2$ directly via PEPC (Lepiniec et al., 1994).

The active form of PEPC is a homotetramer consisting of four identical subunits with a molecular weight of about 95-110 kDA each (Izui et al., 2004). Using the bicarbonate form ($HCO_3^-$) of $CO_2$ as a substrate, it catalyzes the irreversible β-carboxylation of phosphoenol-pyruvate (PEP), resulting in the formation of oxaloacetate (OAA) and inorganic phosphate. PEPC is dependent on bivalent cations as cofactors and prefers $Mg^{2+}$ *in vivo*, but is able to utilize $Mn^{2+}$ and $Co^{2+}$ *in vitro* (O´Leary, 1982).

PEPC activity is influenced by various internal and external factors, including temperature, pH and metabolic effectors (Rajagopalan, 1994). Malate, aspartate and other citric acid cycle intermediates are general feedback inhibitors of PEPC, whereas sugar phosphates such as glucose-6-phosphate, fructose-1,6-bisphosphate and triosephosphates act as allosteric activators of the enzyme (Lepiniec et al., 1994).

Like many other photosynthetic enzymes, the $C_4$ isoform of PEPC is activated by light. $C_4$ PEPC extracted from illuminated maize leaves exhibits a two- to threefold higher activity and a significantly lower sensitivity towards the inhibitor L-malate than the corresponding dark form (Huber and Sugiyama, 1986; Doncaster and Leegood, 1987). Illumination causes a reversible phosphorylation of a conserved serine-residue in the N-terminal part of the protein (Jiao and Chollet, 1990; Chollet et al., 1996). This phosphorylation is mediated by a specific, light-dependent protein-serine/threonine kinase termed PEPC kinase (Vidal and Chollet, 1997), while dephosphorylation in the dark is catalyzed by a protein phosphatase of the type 2A (McNaughton et al., 1991; Rajagopalan, 1994). However, inactivation of PEPC kinase via RNAi in *Flaveria bidentis* ($C_4$) revealed that phosphorylation of PEPC is not essential to achieve high photosynthetic rates, and therefore the physiological function of reversible PEPC-phosphorylation remains ambiguous (Furumoto et al., 2007).

Light also affects the enzyme´s activity by causing pH changes in the leaves of $C_4$ plants. PEPC is very sensitive to even small pH variations and exhibits best activity at a pH of around 8,0 (O´Leary, 1982; Rajagopalan, 1994). During dark-light transitions, the cytosol of the mesophyll cells becomes alkalized, therefore shifting the cytoplasmic pH towards the pH optimum of PEPC. This results in an increase of enzymatic activity (Rajagopalan et al., 1993; Yin et al., 1993).

The different physiological roles of PEPC in higher plants are performed by several isoforms with distinct catalytic and regulatory properties. In all plant species examined so far, they are encoded by a small multigene family consisting of at least three genes (Chollet et al., 1996; Ernst and Westhoff, 1997; Dong et al., 1998; Besnard et al., 2003; Sanchez and Cejudo, 2003; Sullivan et al., 2004). The PEPC isoforms involved in $C_4$ metabolism are characterized by a lower affinity to the substrate PEP (i.e. higher $K_{0,5}$-PEP) than the non-photosynthetic PEPCs from both $C_3$ and $C_4$ plants (Ting and Osmond, 1973). The high $K_{0,5}$-PEP of a $C_4$ PEPC may be essential to assure the inactivation of the enzyme in the dark in order to avoid an unnecessary consumption of PEP in the absence of $C_4$ photosynthesis (Tovar-Mendez et al., 1998). Furthermore, $C_4$ PEPCs exhibit stronger activation by glucose-6-phosphate (Bauwe and Chollet, 1986; Bläsing et al., 2000) and a higher tolerance towards the feedback inhibitor L-malate (Engelmann et al., 2003).

## I.4. Molecular Evolution of $C_4$ PEPC in the Genus *Flaveria*

The genus *Flaveria* of the Asteraceae has been used extensively to study the evolution of $C_4$ photosynthesis. This small genus of 23 known species includes both strictly $C_3$ and $C_4$ plants, as well as a number of $C_3$-$C_4$ intermediate species (Powell, 1978; Edwards and Ku, 1987; McKown et al., 2005). There is evidence that at least some of the $C_3$-$C_4$ species are true evolutionary intermediates (Monson and Moore, 1989), suggesting that evolution towards $C_4$ photosynthesis is still continuing in this genus.

In the NADP-ME type $C_4$ species *F. trinervia* and the $C_3$ species *F. pringlei*, the different PEPC isoforms are encoded by a gene family consisting of three distinct gene classes, named *ppcA* to *ppcC*. The *ppcA* gene class may consist of two genes in both species, whereas *ppcB* and *ppcC* seem to be represented by single copy genes (Hermans and Westhoff, 1990; Poetsch et al., 1991; Hermans and Westhoff, 1992; Ernst and Westhoff, 1997). The photosynthetic $C_4$ isoform of PEPC in *F. trinervia* is encoded by the *ppcA* gene which is expressed at high levels in the leaves, whereas the *ppcA* gene of *F. pringlei* is only weakly transcribed with no apparent organ specificity. The *ppcB* and *ppcC* transcripts accumulate to moderate and comparable levels in leaves, stems and roots of *F. trinervia*, while in *F. pringlei* the *ppcB* gene is predominantly expressed in the roots and the *ppcC* gene is the most strongly expressed gene with preference for stems and roots. Except for the *ppcA* gene of *F. trinervia*, the physiological roles of the different PEPC genes in both species have not been determined yet (Ernst and Westhoff, 1997).

Molecular analyses revealed that the *ppcA*, *ppcB* and *ppcC* genes represent pairs of orthologous genes in the $C_4$ and the $C_3$ species, i.e. that the *ppcA1* gene of *F. trinervia* shows a higher sequence similarity to the corresponding *ppcA1* gene of *F. pringlei* than to its own *ppcB* and *ppcC* genes. Furthermore, the *ppcA* and *ppcB* genes are sister gene classes, indicating that they have originated from a common ancestor by gene duplication before the two plant lineages, leading to the present $C_3$ and $C_4$ *Flaveria* species, diverged (Hermans and Westhoff, 1990; Bläsing et al., 2002). Gene duplications are considered as being an important prerequisite for the evolution of the $C_4$ pathway because creating multiple copies of a gene allows for the modification and neofunctionalization of the copies without losing the function of the original gene (Lynch and Conery, 2000; Sage, 2004). Here, the duplication of a *ppcB*-type gene in the $C_3$ ancestor enabled one copy to develop into the photosynthetic PEPC gene present in the $C_4$ species *F. trinervia* (Fig. 3).

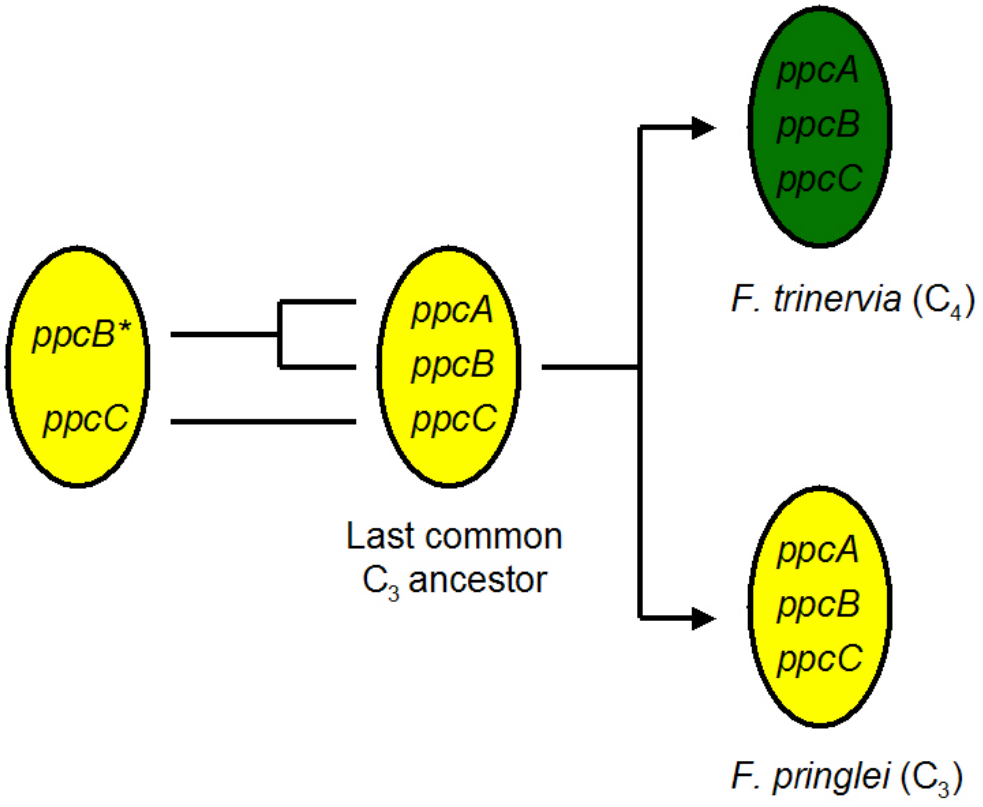


**Figure 3:** PEPC gene evolution in the genus *Flaveria*. Sequence analyses suggest that the *ppcA* gene class was formed by the duplication of a *ppcB*-type gene (*ppcB**) and was already present in the last common $C_3$ ancestor of *F. pringlei* and *F. trinervia* (Bläsing et al., 2002).

The orthologous *ppcA1* PEPC proteins of *F. trinervia* and *F. pringlei* show the expected differences in kinetic and regulatory properties: the $C_4$ *ppcA1* PEPC exhibits a $K_{0,5}$-PEP that is about ten-fold higher than the $K_{0,5}$-PEP of the corresponding enzyme from the $C_3$

species, and the $C_4$ PEPC is less sensitive towards the inhibitor L-malate and more strongly activated by glucose-6-phosphate than its $C_3$ counterpart (Svensson et al., 1997). Both proteins share 96% identical amino acid positions, and by reciprocal domain exchange experiments and site-directed-mutagenesis it was found out that a single amino acid in the carboxy terminus accounts for most of the observed differences in $K_{0,5}$-PEP (Bläsing et al., 2000). At this position, all $C_3$ PEPCs contain an alanine, while in $C_4$ PEPCs this alanine is replaced by a serine, suggesting that the $C_4$-invariant serine is of central importance for the evolution of a $C_4$ PEPC (Svensson et al., 2003). The increased activation by glucose-6-phosphate and the high malate tolerance are mediated by $C_4$-specific determinants which are located in the amino-terminal (amino acids 296-437) and carboxy-terminal regions (645-966) of the $C_4$ PEPC, respectively (Bläsing et al., 2000; Engelmann et al., 2002). Interestingly, the *ppcA* enzymes of the $C_3$-$C_4$ intermediate *F. pubescens* and the $C_4$-like plant *F. brownii* exhibit $K_{0,5}$-PEP values and malate inhibition constants that reflect their degree of $C_3$-$C_4$ intermediacy, indicating that the $C_3$ PEPC evolved step by step into a $C_4$ enzyme (Engelmann et al., 2003).

Apart from these changes in kinetic and regulatory enzyme properties, the evolution of a photosynthetic $C_4$ PEPC from a non-photosynthetic $C_3$ ancestor required the integration of new *cis*-regulatory elements into the PEPC gene in order to establish a high and mesophyll-specific expression in the leaves. Alterations in spatio-temporal expression patterns are believed to be the principal mechanisms for the development of novel morphological and biochemical traits (Doebley and Lukens, 1998; Carroll, 2000), and this view is supported by the fact that the increase of *ppcA* expression levels in $C_3$-$C_4$ intermediate *Flaveria* species preceeds the modifications of the coding regions. For example, the *ppcA* mRNA levels in the leaves of the less advanced $C_3$-$C_4$ intermediate plant *F. chloraefolia* are significantly higher when compared to the *ppcA* transcript levels in the leaves of $C_3$ *Flaveria* species (Engelmann et al., 2003). However, the corresponding *ppcA* PEPC of *F. chloraefolia* still exhibits $C_3$-like enzymatic characteristics (Bauwe and Chollet, 1986).

Studies on the *ppcA1* gene of *F. trinervia* revealed that the strong mesophyll-specific expression is largely regulated at the transcriptional level. 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 a high and mesophyll-specific expression (Stockhaus et al., 1997). 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 and mesophyll-specific expression of a β-glucuronidase (GUS) reporter gene

in transgenic *F. bidentis* ($C_4$) plants (Gowik et al., 2004). The orthologous *ppcA1* promoter of the $C_3$ species *F. pringlei* displays only weak activity in all interior leaf tissues in transgenic *F. bidentis*, but fusion of the $C_4$-DR to this $C_3$ PEPC promoter leads to the confinement of GUS expression to the mesophyll compartment. Analysis of the $C_4$-DR exhibited that the 41-bp module MEM1 (mesophyll expression module 1) is responsible for the $C_4$-characteristic spatial expression pattern of the *ppcA1* gene of *F. trinervia*. MEM1 homologous sequences have also been identified in *ppcA* promoters of other *Flaveria* species, including $C_4$, $C_4$-like and $C_3$ plants (Gowik et al., 2004). Comparison of MEM1 sequences from different species identified two $C_4$-specific elements: a conserved CACT tetranucleotide and a guanine at the 5´ outermost position of MEM1 (Fig. 4). The CACT motif is missing in the MEM1 homologues of $C_3$ *Flaveria* species, and instead of the $C_4$-characteristic guanine one finds an adenine there. To achieve high levels of reporter gene expression in the mesophyll cells, an interaction of the $C_4$-DR with the corresponding $C_4$-PR is required. This suggests that the quantity elements for the elevated expression of the $C_4$ PEPC gene are located within the PR of the 5´ flanking sequences (Gowik et al., 2004).

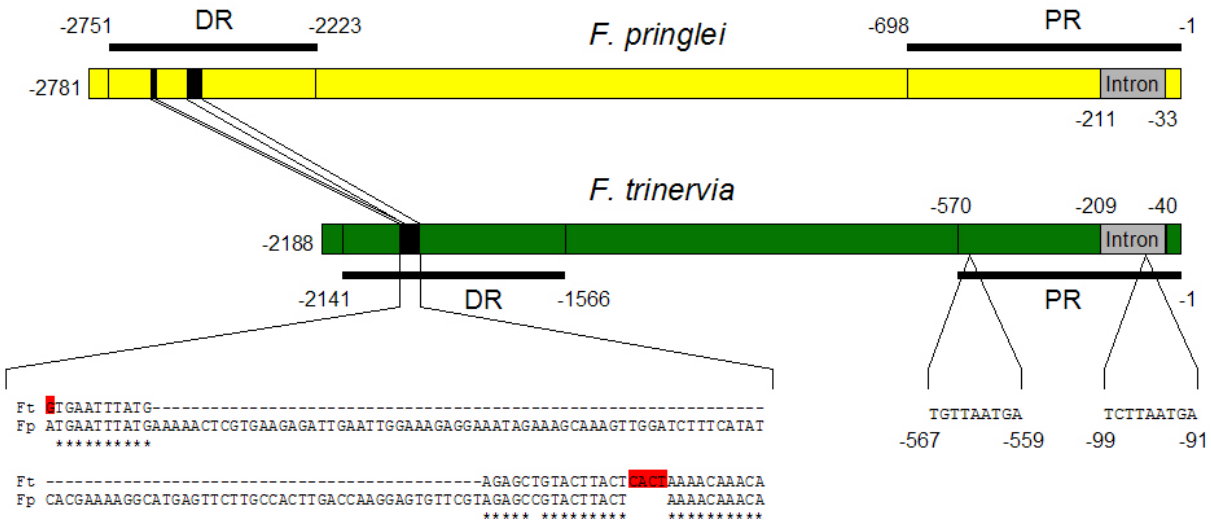


**Figure 4:** Structure of the *ppcA1* promoters of *F. trinervia* ($C_4$) and *F. pringlei* ($C_3$). The numbering of nucleotides refers to the translational start codon. The proximal (PR) and distal (DR) promoter regions are marked by black bars. The MEM1 module of the *F. trinervia ppcA1* promoter and its homologous sequences in the $C_3$ promoter are labelled by black boxes. In the $C_3$ promoter, the MEM1 homologue is divided into two parts by an insertion of 108 bp. The $C_4$-specific elements of MEM1 are highlighted in red colour. The two sequences shown in the $C_4$-PR represent the putative binding sites for FtHB1 to FtHB4.

Using a yeast one-hybrid system, Windhövel and colleagues (Windhövel et al., 2001) managed to identify four different proteins that interact with the PR of the *ppcA1* promoter of *F. trinervia*, but not with the corresponding region of the *ppcA1* promoter of *F. pringlei.* These proteins, named FtHB1 to FtHB4, belong to the class of zinc finger homeodomain proteins (ZF-HD). The $C_4$-PR possesses two putative binding sites for the FtHB proteins: one is located in an intron sequence within the 5´untranslated leader region (–99 to –91) while the other one – to which the FtHB proteins show a lower binding affinity – can be found upstream of the putative TATA-box (–567 to –559) (Windhövel et al., 2001). Homeobox proteins are known to act as transcriptional regulators of eukaryotic gene expression (Pabo and Sauer, 1992; Meshi and Iwabuchi, 1995; Chan et al., 1998), 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 establishing the $C_4$-characteristic expression pattern of the $C_4$ *ppcA1* gene.

## I.5. The Glycine Decarboxylase

Glycine decarboxylase (GDC) is a well-conserved, ubiquitous multimeric enzyme complex that occurs in all pro- and eukaryotes. In cooperation with serine hydroxymethyltransferase (SHMT) it catalyzes the reversible oxidative decarboxylation of two molecules of glycine to produce one molecule each of serine, $NH_3$ and $CO_2$, accompanied by the reduction of $NAD^+$ to NADH (Neuburger et al., 1986). In both directions of the concerted reaction of GDC and SHMT, tetrahydrofolate (THF) becomes methylenated to form $CH_2$-THF, an important donor of active one-carbon units for a variety of biosynthetic processes such as the biosynthesis of methionine, pyrimidines and purines (Bauwe and Kolukisaoglu, 2003). Apart from its participance in basic one-carbon metabolism, GDC fulfills an additional function in photosynthetic tissues of plants, where it is responsible for the rapid breakdown of glycine during the course of photorespiration.

The GDC complex is composed of four different subunits (P, T, L and H), with the 200 kDa P-protein homodimer being the actual decarboxylating unit. The T-protein is a 45 kDa aminomethyl transferase, and the homodimeric 100 kDa L-subunit represents a dihydrolipoamide dehydrogenase. The non-enzymatic 14 kDa H-protein acts as a mobile co-substrate that commutes between the other three GDC proteins (Douce and Neuburger, 1999). A stoichiometry of 4 P-protein : 27 H-protein : 9 T-protein : 2 L-protein has been determined (Oliver et al., 1990), but the detailed structure of the multienzyme complex is not yet known. In all eukaryotes, GDC is exclusively present in the mitochondria, and in green leaves of $C_3$

plants – where high rates of photorespiration can occur – GDC constitutes up to 50% of total matrix protein (Douce et al., 1994). This accumulation of GDC depends upon the induction of gene expression by light, as it has been observed for several genes encoding GDC subunits (Walker and Oliver, 1986; Kim et al., 1991; Srinivasan and Oliver, 1995; Vauclare et al., 1998). No functional GDC can be found in the mesophyll cells of $C_4$ and $C_3$-$C_4$ intermediate species, where GDC activity in the leaves is restricted to the bundle-sheath cells surrounding the vascular strands (Ohnishi and Kanai, 1983; Hylton et al., 1988; Morgan et al., 1993).

In *Arabidopsis thaliana*, small multigene families for all GDC components exist, except for the T-protein which is encoded by a single-copy gene (Bauwe and Kolukisaoglu, 2003). Recently, T-DNA insertion mutants for each of the two Arabidopsis P-protein genes have been isolated. The individual knockout of one of the two genes does not significantly alter the phenotype of the mutants when compared to wild-type plants, but the combined inactivation of both P-subunit genes is lethal even under non-photorespiratory conditions, i.e. in air enriched with 0,9% $CO_2$ (Engel et al., 2007). This clearly demonstrates that GDC activity in plants is indispensable not only for photorespiration, but also for basic one-carbon metabolism. On the other hand, a functional GDC is not obligatory in the cyanobacterial model strain *Synechocystis* sp. PCC 6803, although it has been shown that this organism possesses a complete photorespiratory metabolism (Hagemann et al., 2005; Eisenhut et al., 2006). The viability of *Synechocystis* mutants defective in GDC components is due to the presence of a bacterial-like glycerate pathway that can bypass several enzymatic reactions of the photorespiratory cycle, including the GDC-catalyzed reaction (Eisenhut et al., 2006; Hasse et al., 2007).

GDC is the only known bundle-sheath-specific enzyme in the leaves of $C_3$-$C_4$ intermediate plants, and it has been suggested that the inactivation of GDC in the mesophyll cells by avoiding the expression of the P-subunit (GLDP) gene might constitute a biochemical starting point for the evolution of the $C_4$ syndrome (Morgan et al., 1993; Bauwe and Kolukisaoglu, 2003). Therefore, the evolution of a $C_4$-specific GLDP gene from a $C_3$ ancestor requires the development of new *cis*-regulatory elements which are able to mediate a confinement of GLDP expression to the bundle-sheath compartment. The molecular analysis of $C_4$ GLDP promoter activity could serve as a tool to gain insight into the exact nature of bundle-sheath-specific gene expression, but to date it is not known which types of *cis*- and *trans*-regulatory factors constitute bundle-sheath cell expression modules and how regulatory networks for bundle-sheath-specific gene expression have evolved (Nomura et al., 2005).

## 2. Scientific Aims of this Work

This work covers different aspects of the molecular evolution of $C_4$ photosynthesis in the genera *Flaveria* (Asteraceae) and *Alternanthera* (Amaranthaceae). The main focus lies on the investigation of $C_4$-characteristic gene expression patterns in the $C_4$ leaf, using phospho-enolpyruvate carboxylase (PEPC) and the P-subunit of glycine decarboxylase (GLDP) to examine the establishment of mesophyll- and bundle-sheath-specific gene expression, respectively. In detail, the following experiments had to be performed:

1) The quantity elements necessary for an enhanced expression of the *ppcA1* gene of *F. trinervia* ($C_4$) in the leaf mesophyll seem to be located in the proximal region of the promoter. The zinc-finger homeodomain proteins FtHB1 to FTHB4 are good candidates for transcription factors that might be involved in the control of *ppcA1* expression levels (Windhövel et al., 2001). To test this assumption, a potential binding site for the FtHB proteins was removed from the proximal part of the *ppcA1* promoter, and the *in vivo* consequence of this deletion for promoter activity was analyzed in transgenic *F. bidentis* (Engelmann et al., 2007; **manuscript 1**).
2) While intensive work has been done to elucidate the molecular basis of mesophyll-specific gene expression in *Flaveria*, our knowledge of bundle-sheath-specific gene expression is still very limited. To identify potential *cis*-regulatory determinants that confer bundle-sheath-specificity, a functional analysis of the *GLDPA* promoter encoding the P-subunit of glycine decarboxylase from *F. trinervia* was performed in transgenic *F. bidentis* and *A. thaliana* (Engelmann et al., 2007; **manuscript 2**).
3) The photosynthetic $C_4$ isoforms of PEPC generally exhibit different kinetic and regulatory properties compared to their non-photosynthetic counterparts. The most significant differences are a lower affinity of $C_4$ PEPCs to the substrate PEP, a stronger activation by glucose-6-phosphate and a higher tolerance towards the inhibitor L-malate. Studies with the *ppcA1* PEPCs of the $C_3$-$C_4$ species *F. brownii* and *F. pubescens* revealed that they possess kinetic and regulatory characteristics which reflect their degree of $C_3$-$C_4$ intermediacy, indicating that the evolution of a $C_4$ PEPC occurs step-by-step (Engelmann et al., 2003). To compare the results obtained in *Flaveria* with another genus, the enzymatic properties of the *ppcA* PEPCs of a $C_4$, a $C_3$-$C_4$ and a $C_3$ *Alternanthera* species were investigated (Gowik et al., 2006, **manuscript 3**).

## 3. Theses

1) The proximal region of the *ppcA1* promoter of *F. trinervia* harbours quantity elements that allow a high level of gene expression in the leaf mesophyll when it is combined with the corresponding distal promoter region. However, an interaction of the zinc-finger homeodomain proteins FtHB1 to FtHB4 with the proximal region is not required in this context.
2) The *GLDPA* promoter of *F. trinervia* exhibits a bundle-sheath-preferential activity in the leaves of both transgenic *F. bidentis* ($C_4$) and *A. thaliana* ($C_3$). This indicates that the $C_4$-specific *cis*-regulatory determinants within the *GLDPA* promoter are also functional in a $C_3$ background, i.e. the necessary transcription factors for the correct interpretation of these sequences must already be present in *A. thaliana*. Promoter deletion studies in transgenic Arabidopsis resulted in the identification of distinct regions within the *GLDPA* promoter that harbour an expression enhancer or comprise *cis*-regulatory determinants which account for the bundle-sheath-preferential activity of the *GLDPA* promoter, respectively.
3) The *ppcA* PEPC from the $C_4$ species *Alternanthera pungens* shows a high $K_{0,5}$-PEP and a strong activation by glucose-6-phosphate, classifying this enzyme as a $C_4$ isoform of PEPC involved in photosynthesis. The *ppcA* PEPC from the $C_3$-$C_4$ intermediate species *A. tenella* still possesses $C_3$-characteristic enzymatic properties that are comparable to those of the *ppcA* PEPC from the $C_3$ plant *A. sessilis*. This contrasts with the situation in the genus *Flaveria*; here the *ppcA* PEPCs of the $C_3$-$C_4$ species *F. pubescens* and *F. brownii* exhibit intermediate enzymatic characteristics that reflect their degree of intermediacy.

## 4. Summary

$C_4$ photosynthesis depends upon the strict compartmentalization of the participating enzymes into either mesophyll- or bundle-sheath cells of the leaf. In this study, the molecular basis of mesophyll- and bundle-sheath-specific gene expression was investigated. The *ppcA1* and *GLDPA* genes from the $C_4$ species *Flaveria trinervia* were chosen for this analysis. The *ppcA1* gene of *F. trinervia* encodes the photosynthetic $C_4$ isoform of phosphoenolpyruvate carboxylase (PEPC) and is exclusively expressed in the leaf mesophyll. From previous work it is known that the 2188 bp comprising promoter of the *ppcA1* gene harbours all the necessary information for a high and mesophyll-specific expression of a GUS reporter gene in transgenic *F. bidentis* ($C_4$), and the $C_4$-specific *cis*-regulatory determinants could be restricted to a distal promoter region (DR) from –1566 to –2141 and a proximal region (PR) from –1 to –570. The DR contains the 41-bp module MEM1 that confers mesophyll-specificity to the promoter, while the quantity elements for a high transcriptional activity were thought to be located in the PR. In the present thesis, the existence of quantity determinants in the PR could be confirmed, and by deleting a potential transcription factor binding site from the PR it could be shown that the zinc-finger homeobox proteins FtHB1 to FtHB4 – which specifically interact with the $C_4$ PR *in vitro* – are not involved in the control of *ppcA1* expression levels.

The *GLDPA* gene of *F. trinervia* encodes the P-subunit of the photorespiratory enzyme glycine decarboxylase (GDC). A functional analysis of the 1571 bp comprising *GLDPA* promoter of *F. trinervia* was performed in transgenic *F. bidentis* ($C_4$) and *Arabidopsis thaliana* ($C_3$). A bundle-sheath-preferential activity of the *GLDPA* promoter was observed in both species, and promoter deletion experiments in transgenic Arabidopsis identified three regions within the promoter that play a role in the regulation of expression quantity or cell specificity.

$C_4$ cycle enzymes often exhibit different kinetic and regulatory properties compared to their non-photosynthetic isozymes. $C_4$ isoforms of PEPC are characterized by a low affinity to the substrate phosphoenolpyruvate (PEP), a strong activation by glucose-6-phosphate and a high tolerance towards L-malate. In this study, the enzymatic properties of the *ppcA* PEPCs from *Alternanthera pungens* ($C_4$), *A. tenella* ($C_3$-$C_4$) and *A. sessilis* ($C_3$) were examined. The *ppcA* PEPC from *A. pungens* could be characterized as a typical $C_4$ PEPC (high $K_{0,5}$-PEP, strong activation by glucose-6-phosphate), whereas the enzymatic characteristics of the *ppcA* enzymes from both *A. tenella* and *A. sessilis* could be regarded as $C_3$-specific.

## 5. Zusammenfassung

Die $C_4$ Photosynthese erfordert eine strikte Kompartimentierung der beteiligten Enzyme in Mesophyll- oder Bündelscheidenzellen. Im Rahmen dieser Arbeit sollten die molekularen Grundlagen mesophyll- und bündelscheidenspezifischer Genexpression untersucht werden. Zu diesem Zweck wurden die *ppcA1*- und *GLDPA*-Gene aus *Flaveria trinervia* analysiert. Das *ppcA1*-Gen aus *F. trinervia* kodiert die $C_4$-Isoform der Phosphoenolpyruvat-Carboxylase (PEPC) und wird ausschließlich im Mesophyll des Blattes exprimiert. Aus früheren Studien ist bekannt, daß der 2188 bp umfassende *ppcA1*-Promotor alle notwendigen Informationen für eine hohe, mesophyllspezifische Expression eines GUS-Reportergens in transgenen *F. bidentis*-Pflanzen ($C_4$) besitzt. Hierbei konnten die $C_4$-spezifischen *cis*-regulatorischen Sequenzen auf eine distale Promotorregion (DR) von –1566 bis –2141 und eine proximale Region (PR) zwischen –1 und –570 eingegrenzt werden. Die DR enthält das 41-bp Modul MEM1, welches dem Promotor Mesophyll-Spezifität verleiht, wogegen die Quantitätselemente für die hohe Expression in der PR zu liegen scheinen. In der vorliegenden Arbeit konnte die Existenz dieser Quantitätselemente in der PR nachgewiesen werden, und durch das Entfernen einer potentiellen Transkriptionsfaktor-Bindestelle in der PR konnte gezeigt werden daß die Zinkfinger-Homöoboxproteine FtHB1 bis FtHB4 – welche *in vitro* spezifisch mit der PR interagieren – nicht an der Kontrolle der *ppcA1*-Expressionsstärke beteiligt sind.

Das *GLDPA*-Gen aus *F. trinervia* kodiert die P-Unterheit der Glycin-Decarboxylase (GDC). Eine Funktionsanalyse des 1571 bp umfassenden *GLDPA*-Promotors wurde in transgenen *F. bidentis* und *Arabidopsis thaliana* durchgeführt. Eine bündelscheidenpräferentielle Aktivität des Promotors konnte in beiden Spezies beobachtet werden, und mit Hilfe von Promotor-Deletionsstudien in Arabidopsis konnten drei Regionen innerhalb des *GLDPA*-Promotors identifiziert werden, die eine Rolle bei der Regulation der Expressionsstärke oder Zellspezifität spielen.

$C_4$-Enzyme weisen oft andere kinetische und regulatorische Eigenschaften als ihre nicht-photosynthetischen Isozyme auf. Die $C_4$-Isoformen der PEPC zeichnen sich durch eine geringe Affinität zum Substrat Phosphoenolpyruvat (PEP), eine hohe Aktivierbarkeit durch Glukose-6-Phosphat und eine hohe Malattoleranz aus. In dieser Arbeit wurden die enzymatischen Kenngrößen der *ppcA*-PEPC aus *Alternanthera pungens* ($C_4$), A. tenella ($C_3$-$C_4$) und *A. sessilis* ($C_3$) bestimmt. Die *ppcA*-PEPC aus *A. pungens* konnte als typische $C_4$-PEPC klassifiziert werden (hoher $K_{0,5}$-PEP, starke Aktivierbarkeit durch Glukose-6-Phosphat), wogegen die *ppcA*-Enzyme aus *A. tenella* und *A. sessilis* $C_3$-typische Eigenschaften aufwiesen.

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## 7. Manuscripts

1. Sascha Engelmann, Corinna Zogel, Maria Koczor, Ute Schlue, Monika Streubel, Peter Westhoff (2007): **Evolution of the $C_4$ Phosphoenolpyruvate Carboxylase Promoter of the $C_4$ Species *Flaveria trinervia*: the Role of the Proximal Promoter Region**. Submitted to BMC Plant Biology for publication.

2. Sascha Engelmann, Christian Wiludda, Janet Burscheidt, Udo Gowik, Ute Schlue, Maria Koczor, Monika Streubel, Roberto Cossu, Hermann Bauwe, Peter Westhoff (2007): **The Gene for the P-subunit of Glycine Decarboxylase from the $C_4$ Species *Flaveria trinervia*: Analysis of Transcriptional Control in Transgenic *Flaveria bidentis* ($C_4$) and *Arabidopsis thaliana* ($C_3$)**. Submitted to Plant Physiology for publication.

3. Udo Gowik, Sascha Engelmann, Oliver Bläsing, Agepati S. Raghavendra, Peter Westhoff (2006): **Evolution of $C_4$ Phosphoenolpyruvate Carboxylase in the Genus *Alternanthera*: Gene Families and the Enzymatic Characteristics of the $C_4$ Isozyme and Its Orthologues in $C_3$ and $C_3$/$C_4$ *Alternantheras***. Planta 223 (2), 359-368

# Evolution of the $C_4$ Phosphoenolpyruvate Carboxylase Promoter of the $C_4$ Species *Flaveria trinervia*: the Role of the Proximal Promoter Region

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## Abstract

### Background

The key enzymes of photosynthetic carbon assimilation in $C_4$ plants have evolved independently several times from $C_3$ isoforms that were present in the $C_3$ ancestral species. The $C_4$ isoform of phosphoenolpyruvate carboxylase (PEPC), the primary $CO_2$-fixing enzyme of the $C_4$ cycle, is specifically expressed at high levels in mesophyll cells of the leaves of $C_4$ species. We are interested in understanding the molecular changes that are responsible for the evolution of this $C_4$-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* ($C_4$) 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 $C_4$-specific expression pattern. We demonstrate that the PR harbours *cis*-regulatory determinants which account for high levels of PEPC expression in the leaf. We further show that an intron in the 5′ 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 $C_4$ leaves.

## Background

About 90% of terrestrial plant species, including major crops such as rice, soybean, barley and wheat, assimilate $CO_2$ via the $C_3$ pathway of photosynthesis. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) acts as the primary $CO_2$-fixing enzyme of $C_3$ photosynthesis, but its ability to use $O_2$ as a substrate instead of $CO_2$ results in the energy-wasting process of photorespiration. The photosynthetic $C_4$ cycle represents an addition to the $C_3$ pathway which acts as a pump that accumulates $CO_2$ at the site of Rubisco so that the oxygenase activity of the enzyme is inhibited and photorespiration is largely suppressed. $C_4$ plants therefore achieve higher photosynthetic capacities and better water- and nitrogen-use efficiencies when compared with $C_3$ species [1].

$C_4$ 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 $C_4$ cycle depends upon the strict compartmentalization of the $CO_2$ assimilatory enzymes into either mesophyll or bundle-sheath cells [2]. Phosphoenolpyruvate carboxylase (PEPC), which serves as the actual $CO_2$ pump of the $C_4$ pathway, is specifically expressed in the mesophyll cells of $C_4$ leaves. This enzyme is not an unique feature of $C_4$ 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 $C_4$ photosynthesis suggests that the photosynthetic $C_4$ isoforms of PEPC have evolved independently several times from non-photosynthetic $C_3$ isozymes [4]. During the evolution of $C_4$ PEPC genes from ancestral $C_3$ genes, changes in expression strength and organ- and cell-specific expression patterns must have occurred. While $C_4$ PEPC genes are highly expressed in the mesophyll cells of the leaf, the $C_3$ isoform genes are only moderately transcribed in all plant organs [5-8].

To investigate the molecular evolution of a $C_4$ PEPC gene we are using the genus *Flaveria* (Asteraceae) as a model system. This genus includes $C_4$ and $C_3$ as well as $C_3$-$C_4$ intermediate species [9, 10] and thus provides an excellent system for studying the evolution of the $C_4$ photosynthetic pathway [11]. Previous studies on the *ppcA1* gene of *F. trinervia*, encoding the $C_4$ 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* ($C_4$) plants [13]. The orthologous, 2538 bp comprising *ppcA1* promoter of the $C_3$ species *F. pringlei* displays only weak activity in all interior leaf tissues in transgenic *F. bidentis*, but fusion of the $C_4$-DR to this $C_3$ PEPC promoter leads to a confinement of GUS expression to the mesophyll [13]. Analysis of the $C_4$-DR revealed that the 41-bp module MEM1 (mesophyll expression module 1) is responsible for the $C_4$-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 $C_4$-DR with the $C_4$-PR. This suggests that quantity elements for an elevated expression of the $C_4$ 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 $C_4$-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 $C_4$-characteristic expression pattern of the $C_4$ *ppcA1* gene.

In this study we have investigated the role of the proximal promoter region of the *ppcA1* gene of *F. trinvervia* with regard to its high and mesophyll-specific expression by transgenic analyses in the closely related $C_4$ 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 an intron located in the 5' untranslated segment of *ppcA1* is dispensable for the transcriptional control of this gene.

## 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 $C_4$ phosphoenolpyruvate carboxylase gene (*ppcA1*) of the $C_4$ 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 $C_4$-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 $C_4$ species is transformable by *Agrobacterium tumefaciens* mediated gene transfer [19] and was therefore chosen for these experiments.

Construct *ppcA*-$PR_{Ft}$-$DR(+)_{Ft}$ 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 $C_4$ *ppcA1* promoter contains quantity elements conferring high expression in the mesophyll cells we designed construct *ppcA*-$PR_{Fp}$-$DR(+)_{Ft}$. Here, the $C_4$-PR was exchanged for its counterpart from the orthologous *ppcA1* gene of the $C_3$ species *F. pringlei*. Deletion of the intron sequences in the 5′ untranslated segment of promoter construct *ppcA*-$PR_{Ft}$-$DR(+)_{Ft}$ resulted in the formation of construct *ppcA*-$PR_{Ft}\Delta$Intron-$DR(+)_{Ft}$. Thereby a putative binding site for the ZF-HD proteins FtHB1 to FtHB4 [14] was removed from the $C_4$-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 $C_4$-specific *ppcA1* expression pattern.

### The Proximal Region of the *ppcA1* Promoter of *F. trinervia* Harbours *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 $C_4$ leaves. To examine whether the PR actually harbours such quantity elements we analyzed the GUS expression patterns of constructs *ppcA*-$PR_{Ft}$-$DR(+)_{Ft}$ and *ppcA*-$PR_{Fp}$-$DR(+)_{Ft}$ (Fig. 1) in transgenic *F. bidentis***.**

In *F. bidentis* plants that had been transformed with promoter construct *ppcA*-$PR_{Ft}$-$DR(+)_{Ft}$, 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 $C_4$-PR by the corresponding region from the *ppcA1* promoter of *F. pringlei* (construct *ppcA*-$PR_{Fp}$-$DR(+)_{Ft}$) did not cause any alteration in the cellular GUS expression pattern when compared to *ppcA*-$PR_{Ft}$-$DR(+)_{Ft}$; 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 $C_4$-PR was substituted for the $C_3$-PR (Fig. 2D). This clearly demonstrated that the $C_4$-characteristic transcription-enhancing *cis*-regulatory elements must be located within the proximal region of the *ppcA1* promoter of *F. trinervia* and that they are missing in the equivalent part of the *ppcA1* promoter of the $C_3$ species *F. pringlei*.

**The Intron in the $C_4$-PR Is not Required for the Establishment of a $C_4$-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 $C_4$-characteristic expression pattern. We therefore deleted the intron sequences from the $C_4$-PR in construct *ppcA*-$PR_{Fp}$-$DR(+)_{Ft}$, resulting in the formation of construct *ppcA*-$PR_{Ft}\Delta$Intron-$DR(+)_{Ft}$ (Fig. 1). The histochemical analysis of transgenic *F. bidentis* plants demonstrated that the *ppcA*-$PR_{Ft}\Delta$Intron-$DR(+)_{Ft}$ 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*-$PR_{Ft}\Delta$Intron-$DR(+)_{Ft}$ (6,5 nmol MU/(mg*min)) and *ppcA*-$PR_{Ft}$-$DR(+)_{Ft}$ (5,9 nmol MU/(mg*min)). We infer from these results 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 $C_4$ leaves.

**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 $C_3$ to $C_4$ species [25]. This is consistent with the important function of PEPC during $C_4$ photosynthesis. The $C_4$-like species *F. brownii* and *F. vaginata* exhibited *ppcA* RNA levels that were comparable to those of the $C_4$ plants *F. bidentis* and *F. trinervia*, and even in *F. pubescens*, a $C_3$-$C_4$ intermediate with rather poorly developed $C_4$-characteristic traits, *ppcA* transcript accumulation in the leaves was significantly higher than in the $C_3$ species *F. cronquistii* and *F. pringlei* [25].

Searching for known plant *cis*-regulatory DNA elements in the PLACE database [26] 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 (GTTAGTT, [27]) and a CCAAT box [28], are present in all examined $C_3$-$C_4$, $C_4$-like and $C_4$ species, but are missing in the two $C_3$ 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 [29]. They are able to regulate the initiation of transcription by an interaction of CCAAT-binding transcription factors with the basal transcription initiation complex [30]. 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 [29]. 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 [31-33]. To test the physiological importance of the putative MYB and CCAAT binding sites (that are located within the PR of the *ppcA1* promoter of *F. trinervia*) it will be crucial to inactivate these sequences in construct *ppcA*-$PR_{Ft}$ΔIntron-$DR(+)_{Ft}$ by site-directed mutagenesis and to investigate whether this results in a decrease of reporter gene expression in the leaves of transgenic *F. bidentis* plants.

When searching for quantity elements in the PR of the *ppcA1* promoter of *F. trinervia*, one should always keep in mind that high levels of reporter gene expression in the leaf

mesophyll require the synergistic action of the distal and proximal promoter regions. The $C_4$-PR alone exhibits very low transcriptional activity in all interior leaf cell types of transgenic *F. bidentis* [34], indicating that the *cis*-regulatory elements for enhanced expression are only functional when the $C_4$-PR is combined with the cognate $C_4$-DR. One may speculate that a strong expression of the *ppcA1* gene in the mesophyll cells of *F. trinervia* depends on the interaction of *trans*-acting factors which bind to *cis*-regulatory elements within the PR with other transcription factors that are recruited to $C_4$-specific *cis*-regulatory determinants in the DR. In the future, further dissection of the $C_4$-PR of *F. trinervia* and expression analyses of additional DR-PR combinations from *ppcA* promoters of different *Flaveria* species in transgenic *F. bidentis* will be useful for uncovering the control of *ppcA* expression levels in $C_4$ leaves.

## Conclusions

In this study, we have demonstrated that the proximal region (–570 to –1) of the *ppcA1* promoter of *F. trinervia* ($C_4$) harbours *cis*-regulatory elements conferring high expression levels in leaf mesophyll cells of transgenic *F. bidentis* ($C_4$). It was further demonstrated that an intron in the 5′ untranslated leader region is dispensable for the establishment of the $C_4$-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 $C_4$ leaves.

## Methods

### Construction of Chimeric Promoters

DNA manipulations and cloning were performed according to Sambrook and Russell [35]. The construction of the promoter-GUS fusion *ppcA*-$PR_{Ft}$-$DR(+)_{Ft}$ has been described in detail [13]. Plasmids *ppcA*-S-Fp [36] and *ppcA*-$PR_{Ft}$-$DR(+)_{Ft}$ served as the basis for the production of *ppcA*-$PR_{Fp}$-$DR(+)_{Ft}$. The distal region (–2141 to –1566) of the *ppcA1* promoter of *F. trinervia* was excised from *ppcA*-$PR_{Ft}$-$DR(+)_{Ft}$ by digestion with *Xba*I. Insertion of this promoter fragment into *Xba*I-cut *ppcA*-S-Fp resulted in the generation of construct *ppcA*-$PR_{Fp}$-$DR(+)_{Ft}$.

For the production of construct *ppcA*-$PR_{Ft}$ΔIntron-$DR(+)_{Ft}$ a part of the *ppcA1* promoter from *F. trinervia* (–570 to –209) was amplified by PCR with primers S-Ft-F (5′-TGCTCTAGACCGGTGTTAATGATGG-3′) and S-Ft-R (5′-CTGAATATTGGGTATG-CTCAG-3′). Plasmid *ppcA*-$PR_{Ft}$-$DR(+)_{Ft}$ was used as the template for this PCR reaction. The amplified promoter fragment was cut with *Xba*I. The outermost 3′ region of the *ppcA1* promoter (–39 to –1) was generated by annealing the two oligonucleotides S-Ft-3′-1 (5′-GGTTGGAGGGGAATTAAGTATTAAGCAAGGGTGTGAGTAC-3′) and S-Ft-3′-2 (5′-CCGGGTACTCACACACCCTTGCTTAATACTTAATTCCCCTCCAACC-3′). Thereby a *Xma*I-compatible 5′ overhang was created next to position –1. The *ppcA*-S-Ft promoter plasmid [36] was digested with *Xba*I and *Xma*I and the released *ppcA1* promoter fragment was removed by agarose gel electrophoresis. The *Xba*I/*Xma*I-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*-$PR_{Ft}$ΔIntron. The distal region of the *ppcA1* promoter of *F. trinervia* (–2141 to –1566) was removed from of *ppcA*-$PR_{Ft}$-$DR(+)_{Ft}$ by incubation with *Xba*I and inserted into *Xba*I-cut *ppcA*-$PR_{Ft}$ΔIntron. The resulting plasmid was designated *ppcA*-$PR_{Ft}$ΔIntron -$DR(+)_{Ft}$.

**Plant Transformation**

In all transformation experiments the *Agrobacterium tumefaciens* strain AGL1 was used [37]. 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. [38] and Kosugi et al. [39]. For histochemical analysis of GUS activity the leaves were cut manually with a razorblade and the sections were transferred to incubation buffer (100 mM $Na_2HPO_4$, pH 7.5, 10 mM EDTA, 50 mM $K_4[Fe(CN)_6]$, 50 mM $K_3[Fe(CN)_6]$, 0.1% (v/v) Triton X-100, 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronid 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 [40]. The sequence alignments were created with the program DIALIGN 2.2.1 [41]. 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. cronquistii 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*-$PR_{Ft}$ΔIntron-$DR(+)_{Ft}$, the sequence alignments and wrote the manuscript. CZ produced construct *ppcA*-$PR_{Fp}$-$DR(+)_{Ft}$. 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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## Figure Legends

**Figure 1:**

Schematic presentation of the promoter-GUS fusion constructs used for the transformation of *Flaveria bidentis* ($C_4$).

**Figure 2:**

**(A)** to **(C)**: Histochemical localization of GUS activity in leaf sections of transgenic *F. bidentis* plants transformed with constructs *ppcA*-$PR_{Ft}$-$DR(+)_{Ft}$ **(A)**, *ppcA*-$PR_{Fp}$-$DR(+)_{Ft}$ **(B)** or *ppcA*-$PR_{Ft}\Delta$Intron-$DR(+)_{Ft}$ **(C)**. Incubation times were 6 h **(A, C)** and 20 h **(B)**.

**(D)**: GUS activities in leaves of transgenic *F. bidentis* plants. The numbers of independent transgenic plants tested (N) are indicated at the top of each column. Median values (black lines) of GUS activities are expressed in nanomoles of the reaction product 4-methylumbelliferone (MU) generated per milligram of protein per minute.

**Figure 3:**

Nucleotide sequence alignment of the proximal regions of *ppcA* promoters from *F. trinervia* ($C_4$, *ppcA*-Ft), *F. bidentis* ($C_4$, *ppcA*-Fb), *F. vaginata* ($C_4$-like, *ppcA*-Fv), *F. brownii* ($C_4$-like, *ppcA*-Fbr), *F. pubescens* ($C_3$-$C_4$, *ppcA*-Fpub), *F. cronquistii* ($C_3$, *ppcA*-Fc) and *F. pringlei* ($C_3$, *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.

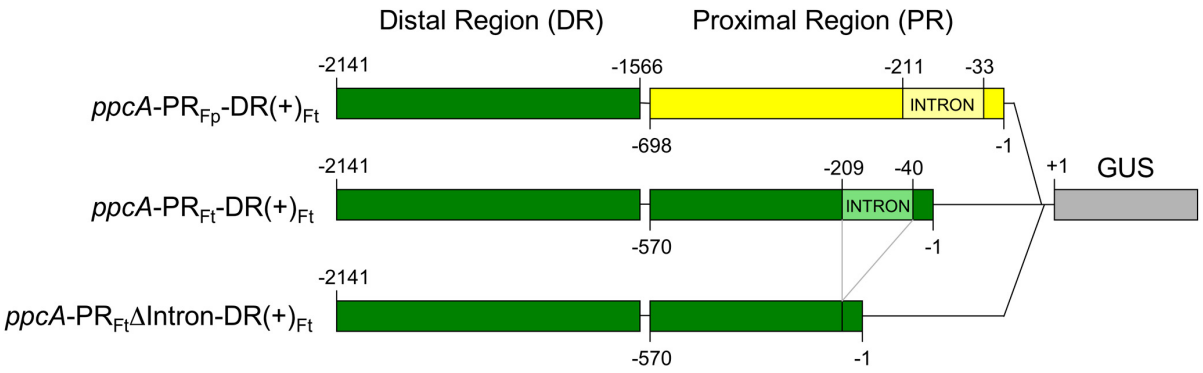
Distal Region (DR)
Proximal Region (PR)
-2141
-1566
-211
-33
ppcA-PRFp-DR(+)Ft
INTRON
-698
-1
-2141
-209
-40
+1
GUS
ppcA-PRFt-DR(+)Ft
INTRON
-570
-1
-2141
ppcA-PRFtΔIntron-DR(+)Ft
-570
-1

**Figure 1**

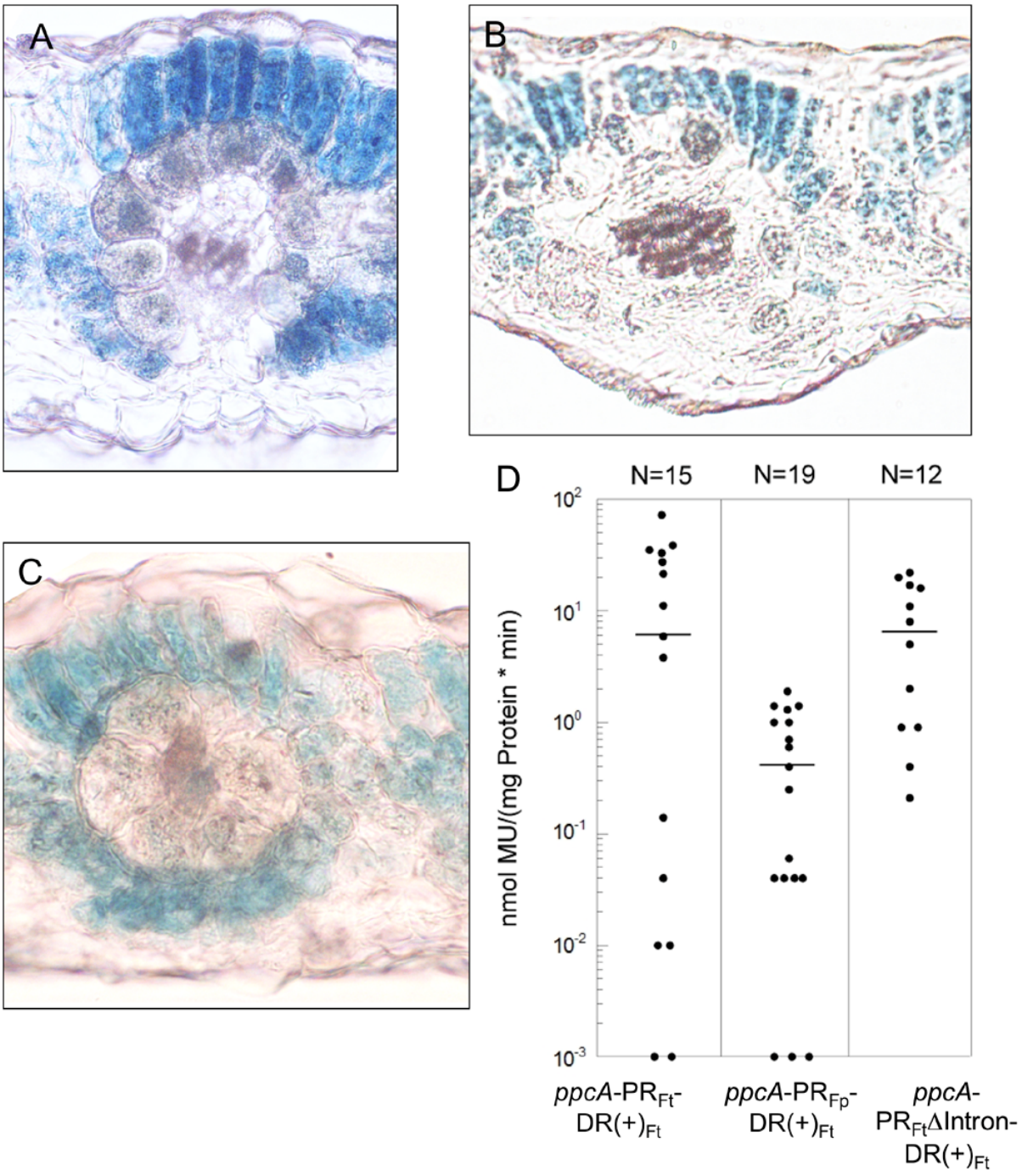
A
B
C
D
N=15
N=19
N=12
nmol MU/(mg Protein * min)
$10^{2}$
$10^{1}$
$10^{0}$
$10^{-1}$
$10^{-2}$
$10^{-3}$
*ppcA*-$PR_{Ft}$-$DR(+)_{Ft}$
*ppcA*-$PR_{Fp}$-$DR(+)_{Ft}$
*ppcA*-$PR_{Ft}\Delta$Intron-$DR(+)_{Ft}$

**Figure 2**

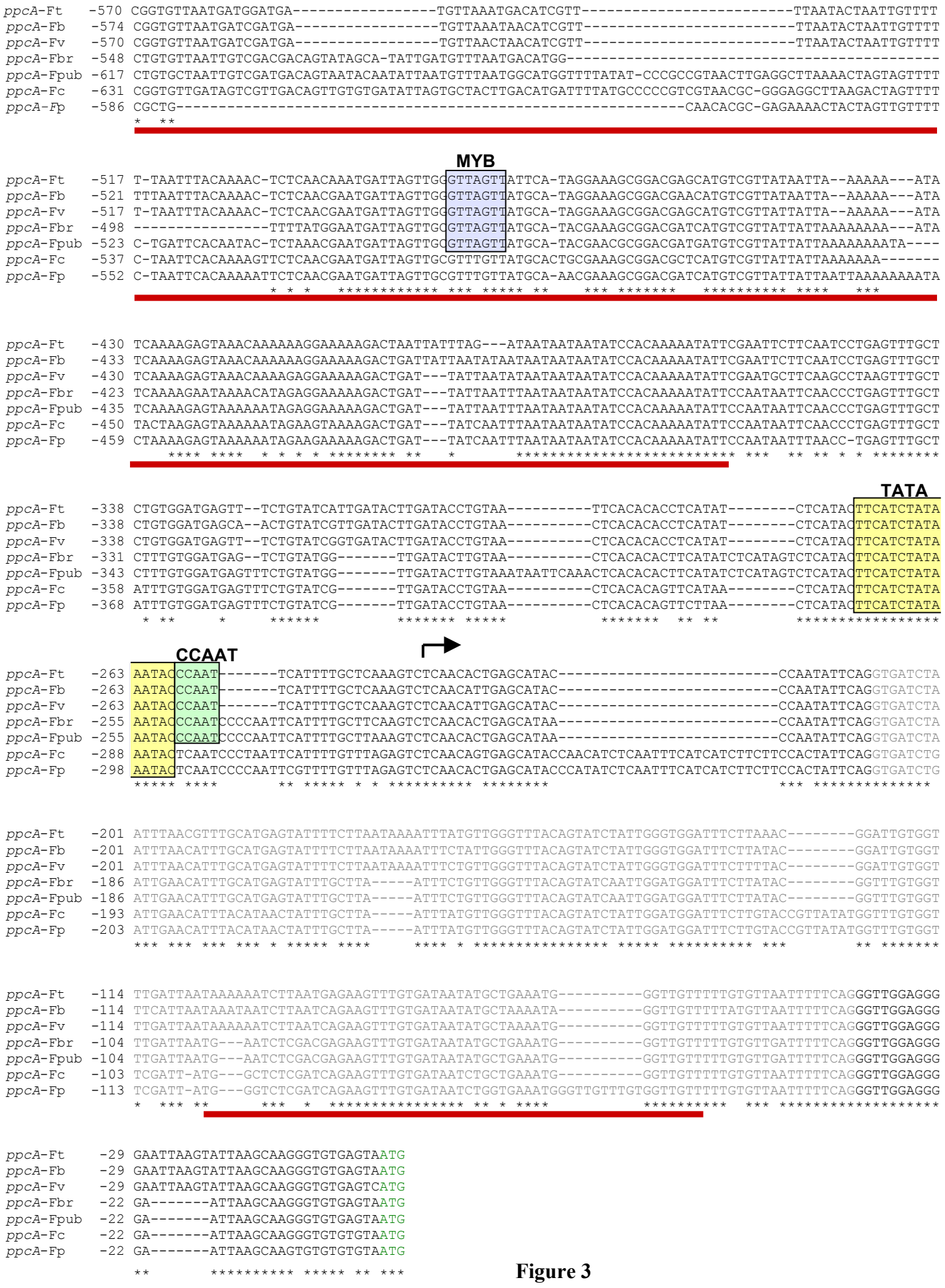


**Figure 3**

# The Gene for the P-subunit of Glycine Decarboxylase from the $C_4$ Species *Flaveria trinervia*: Analysis of Transcriptional Control in Transgenic *Flaveria bidentis* ($C_4$) and *Arabidopsis thaliana* ($C_3$)

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## Summary

Glycine decarboxylase (GDC) plays an important role in the photorespiratory metabolism of plants. In concert with serine hydroxymethyltransferase (SHMT) it catalyzes the oxidation of glycine, leading to the release of photorespiratory $CO_2$. GDC is composed of four subunits (P, H, L and T) with the P-subunit (GLDP) serving as the actual decarboxylating unit. In $C_3$ plants, GDC can be found in all photosynthetic cells whereas in leaves of $C_3$-$C_4$ intermediate and $C_4$ species its occurrence is restricted to bundle-sheath cells. The specific expression of GLDP in the bundle-sheath cells might have constituted a biochemical starting point for the evolution of $C_4$ photosynthesis. To understand the molecular mechanisms responsible for restricting *GLDP* expression to bundle-sheath cells, we performed a functional analysis of the *GLDPA* promoter from the $C_4$ species *Flaveria trinervia*. Expression of a promoter-reporter gene fusion in transgenic plants of the transformable $C_4$ species *Flaveria bidentis* ($C_4$) showed that 1571 bp of the *GLDPA* 5´ flanking region contain all the necessary information for the specific expression in bundle-sheath cells and vascular bundles. Interestingly, we found that the *GLDPA* promoter of *F. trinervia* exhibits a $C_4$-like spatial activity also in the $C_3$ plant *Arabidopsis thaliana*, indicating that a mechanism for bundle-sheath-specific expression is also present in this $C_3$ species. Using transgenic Arabidopsis, promoter deletion studies identified two regions in the *GLDPA* promoter, one conferring repression of gene expression in mesophyll cells and one functioning as a general transcriptional enhancer. Subsequent analyses in transgenic *F. bidentis* confirmed that these two segments fulfill the same function also in the $C_4$ context.

## Introduction

Net photosynthetic $CO_2$ assimilation rates in $C_3$ plants are reduced by photorespiration, a process that results from the oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). $C_4$ plants usually show no apparent photorespiration, and this is achieved by splitting the photosynthetic reactions between two morphologically and biochemically distinct cell types, the mesophyll and the bundle-sheath cells. Initial $CO_2$ fixation in $C_4$ plants occurs exclusively in the mesophyll cells and is performed by the enzyme phosphoenolpyruvate carboxylase (PEPC) to form a $C_4$ acid, oxaloacetate. Depending on the $C_4$ subtype, oxaloacetate is converted to either malate or aspartate, which subsequently move to the bundle-sheath and become decarboxylated, resulting in significant elevation of the $CO_2$ concentration in these cells. Final refixation of $CO_2$ is achieved by Rubisco, which, in $C_4$ plants, is only present in bundle-sheath cells. The enrichment of $CO_2$ in the vicinity of Rubisco effectively inhibits the enzyme´s oxygenase activity (Hatch, 1987).

In $C_4$ plants, the $CO_2$ assimilatory enzymes are compartmentalized into either mesophyll or bundle-sheath cells, and this is governed by differential gene expression. It has been shown that mesophyll-specific expression of $C_4$ cycle genes is mainly regulated at the transcriptional level (Schaffner and Sheen, 1992; Stockhaus et al., 1994; Rosche et al., 1998; Sheen, 1999), whereas bundle-sheath-specific expression is controlled at both transcriptional and post-transcriptional levels (Long and Berry, 1996; Marshall et al., 1997; Sheen, 1999; Patel et al., 2006).

There are indications that photorespiration also exists in $C_4$ plants, albeit at a much lower level than in $C_3$ plants (Osmond and Harris, 1971; Furbank and Badger, 1983; de Veau and Burris, 1989). This is likely due to the fact that photorespiration in $C_4$ species is strictly confined to the bundle-sheath cells in the leaves, while in $C_3$ plants photorespiration occurs in all photosynthetically active mesophyll cells (Ohnishi and Kanai, 1983).

The mitochondrial multienzyme complex glycine decarboxylase (GDC) plays a key role in the photorespiratory pathway. GDC is composed of four different subunits (P, H, T and L) and catalyzes, in cooperation with serine hydroxymethyltransferase (SHMT), the oxidative decarboxylation of glycine that originates from the breakdown of photorespiratory phosphoglycolate. In the course of these reactions, two molecules of glycine are converted to one molecule each of serine, $NH_3$ and $CO_2$ (Neuburger et al., 1986). Consistent with the compartmentation of the photorespiratory cycle, GDC is present in all photosynthetic cells of $C_3$ plant leaves, but strictly confined to the bundle-sheath cells in $C_4$ species (Ohnishi and

Kanai, 1983). None of the GDC subunits have been detected in the mesophyll cells of $C_4$ plants (Morgan et al., 1993).

Plant species possessing a $C_3$-$C_4$ intermediate type of photosynthesis are of special interest for studying the evolution of $C_4$-characteristic traits. Some $C_3$-$C_4$ plants are to some extent able to fix $CO_2$ into malate and aspartate (Monson et al., 1986), but most of these species do not have a $C_4$ metabolism. They can be characterized as "intermediate", for example, by their $CO_2$ compensation points, which are lower than those of $C_3$ plants, but higher than those of $C_4$ plants (Edwards and Ku, 1987; Rawsthorne, 1992). As in $C_4$ plants, functional GDC occurs only in the bundle-sheath cells of the leaves of $C_3$-$C_4$ intermediate plants, as it was first demonstrated for *Moricandia arvensis* (Rawsthorne et al., 1988a, 1988b). Later on, it was discovered that the loss of GDC activity in the mesophyll is due to a lack of the P-subunit (GLDP). Because of the absence of GDC activity in mesophyll cells of *M. arvensis* leaves, photorespiratory glycine moves to the bundle-sheath cells to be processed by GDC. The bundle-sheath cells of *M. arvensis* contain a large number of mitochondria which are arranged at the centripetal cell wall adjacent to the vascular tissue, whereas the chloroplasts are located at the cell periphery. This special distribution of organelles and the restriction of glycine oxidation to the bundle-sheath compartment result in an efficient recapture of released photorespiratory $CO_2$, thereby lowering the $CO_2$ compensation point when compared to a typical $C_3$ plant (Rawsthorne et al., 1998). $C_3$-$C_4$ intermediate species are thought to represent a stage in the evolutionary transition from $C_3$ to $C_4$ photosynthesis (Edwards and Ku, 1987). It was therefore tempting to speculate that the confinement of GDC to the bundle-sheath cells has been one of the biochemical starting points for the evolution of $C_4$ plants (Morgan et al., 1993; Bauwe and Kolukisaoglu, 2003; Sage, 2004). However, the possible effects of this relocation for $C_4$ evolution are discussed controversially (Edwards et al., 2001). The loss of the P-subunit seems to be the initial step to inactivate GDC in the mesophyll, and the absence of all GDC subunits in the leaf mesophyll of other $C_3$-$C_4$ intermediate species suggests that they have developed further towards $C_4$ photosynthesis than *M. arvensis* (Morgan et al., 1993).

A well established experimental system for investigating the evolution of $C_4$ characteristic traits is the genus *Flaveria* of the Asteraceae (Powell, 1978). This small genus comprising 23 known species includes both $C_3$ and $C_4$ species, but also a large number of $C_3$-$C_4$ intermediate species (Edwards and Ku, 1987). In this study we examined the promoter of the gene encoding the P-subunit of GDC from the $C_4$ species *Flaveria trinervia* to gain insight into the molecular basis of bundle-sheath-specific gene expression. Two genes encoding the

P-subunit of GDC have been identified in *F. trinervia*, *GLDPA* and the pseudogene *GLDPB* (Cossu, 1997). The *GLDPA* promoter was fused to a β-glucuronidase (GUS) reporter gene and promoter activity was analyzed in transgenic *Flaveria bidentis* ($C_4$) and *Arabidopsis thaliana* ($C_3$). Similar expression patterns were observed in these two species, which allowed the use of *A. thaliana* as a heterologous system for testing a series of promoter deletions to identify $C_4$-characteristic regulatory elements within the *GLDPA* promoter. These analyses resulted in the identification of regions within the *GLDPA* promoter that contribute mainly to the regulation of expression quantity or to the spatial expression pattern of the *GLDPA* gene, respectively.

# Results

### *In Situ* RNA Hybridization

Immunolabeling studies have shown that, in $C_4$ plants, the P-subunit of GDC (GLDP) accumulates exclusively in bundle-sheath cells of leaves (Hylton et al., 1988; Morgan et al., 1993; Yoshimura et al., 2004). In order to examine whether this $C_4$-characteristic localization of the P-protein is due to specific accumulation of *GLDPA* mRNA in this compartment we analyzed the expression pattern of the *GLDPA* gene in leaves of the $C_4$ species *F. trinervia* by *in situ* hybridization. As a probe we used a 2,4 kb fragment of the *GLDPA* cDNA from *F. trinervia*, and control hybridizations were performed with the corresponding sense probe.

In leaves of *F. trinervia*, transcripts of the *GLDPA* gene could only be detected in bundle-sheath and not in mesophyll cells (Fig. 1A). The *GLDPA* mRNA accumulated near the centripetal cell walls of the bundle-sheath cells, which was due to the concentration of cytoplasm in this region. The confinement of the P-protein to the bundle-sheath cells therefore is controlled by the specific accumulation of *GLDPA* mRNA in this compartment. The same result was obtained by *in situ* hybridization of the *GLDPA* probe to leaf cross-sections of the $C_4$-species *F. bidentis* (Fig. 1C).

### Expression of a GUS Reporter Gene under the Control of the *GLDPA* Promoter from *F. trinervia* in transgenic *F. bidentis*

The *in situ* RNA hybridization analysis showed that the occurrence of *GLDPA* transcripts is restricted to the bundle-sheath cells in *F. trinervia* and *F. bidentis* (Fig. 1). To test whether the available 1571 bp of the 5´ flanking region of the *GLDPA* gene (including the 5´ untranslated

region upstream of the AUG start codon) harbour all the necessary information for this bundle-sheath-specific expression pattern, we fused this region to a β-glucuronidase (GUS) reporter gene (construct *GLDPA-Ft*, Fig. 2A) and examined its expression behaviour in transgenic *F. bidentis* plants. The $C_4$-species *F. bidentis* is a close relative to *F. trinervia*, but unlike *F. trinervia* it is suitable for transformation by *Agrobacterium tumefaciens* mediated gene transfer (Chitty et al., 1994).

Histochemical analysis of the expression of the *GLDPA*-Ft promoter-GUS construct revealed an intense blue staining in bundle-sheath cells, but not in the mesophyll cells (Fig. 2B-D). GUS activity could also be observed in most vascular bundles, with the degree of GUS expression varying with the size of the veins. The small minor veins usually exhibited a strong blue staining, while higher-order vascular bundles showed only moderate GUS activity. Additional weak *GLDPA* promoter activity was also detected in the guard cells of the stomatal complexes (Fig. 2E).

**The Expression Pattern of the *GLDPA*-GUS Construct in *A. thaliana* is Similar to that in *F. bidentis***

Bundle-sheath cells are not a unique feature of $C_4$ plants. They are also present in many $C_3$ plants, but compared to the situation in $C_4$ species these cells exhibit fewer chloroplasts and mitochondria (Kinsman and Pyke, 1998; Leegood, 2002). To examine whether the *GLDPA* promoter of *F. trinervia* shows a cell-specific activity in a $C_3$ background we introduced the *GLDPA*-GUS construct into *A. thaliana*.

The histochemical analysis revealed GUS expression in the vascular tissue and in the surrounding bundle-sheath cells (Fig. 3C, D). Notably, very similar to the expression pattern in *F. bidentis*, no GUS activity could be detected in the mesophyll cells of transgenic Arabidopsis plants. The quantification of GUS levels showed that the median activity of the reporter protein was comparable in Arabidopsis and *F. bidentis* leaves (Fig. 2F, 3B).

To verify the results obtained from the histochemical GUS analysis, the *GLDPA* promoter was also fused to the green fluorescent protein (GFP) reporter gene *mgfp5-ER* (Siemering et al., 1996; Haseloff et al., 1997) and a Histone 2B/ yellow fluorescent protein (H2B-YFP) fusion gene (Boisnard-Lorig et al., 2001), which are targeted to the endoplasmic reticulum and the nucleus, respectively. These reporter proteins allow a non-destructive analysis by fluorescence or confocal laser microscopy, thereby avoiding any potential diffusion of the reporter protein which might occur during a histochemical staining procedure.

In both cases, the reporter proteins could be detected in bundle-sheath cells and vascular bundles, but not in mesophyll cells (Fig. 3E-L).

**Deletion Analysis of the *GLDPA* Promoter**

The "$C_4$-like" expression pattern of *GLDPA*-GUS in transgenic Arabidopsis provided the opportunity to functionally dissect the *GLDPA* promoter by using this $C_3$ model organism as an experimental system. To identify *cis*-regulatory determinants that are responsible for the activity of the *GLDPA* promoter in bundle-sheath cells and the vascular bundle, we produced a set of 5′ deletions and analyzed their expression specificity and level in transgenic Arabidopsis. The *GLDPA* promoter was subdivided into seven fragments that are referred to as region 1 to region 7 in the following (Fig. 4A).

The removal of a 182 bp segment (region 1) from the 5′ end of the *GLDPA* promoter, resulting in construct *GLDPA*-Ft-Δ1 (Fig. 4A), did not alter the spatial expression pattern of the GUS reporter gene when compared to the original full-length promoter, i.e., this promoter variant was still capable of directing GUS expression specifically in the vascular bundles and bundle-sheath cells of *A. thaliana* (Fig. 4B). This indicates the absence of *cis*-regulatory elements conferring cell specificity in this most distal part of the *GLDPA* promoter. However, quantitative GUS assays revealed an about 20-fold lower GUS activity in leaves of transgenic plants expressing *GLDPA*-Ft-Δ1 relative to leaves of *GLDPA*-Ft plants (Fig. 4D). The transcriptional activity of the promoter further decreased when deleting another 251 bp (region 2) from the 5′end of *GLDPA*-Ft-Δ1 (construct *GLDPA*-Ft-Δ2; Fig. 4A). GUS activity in leaves of Arabidopsis plants harbouring the *GLDPA*-Ft-Δ2 transgene was about 60 times lower than that of *GLDPA*-Ft (Fig. 4D), but the spatial expression pattern in the Arabidopsis leaf was still identical to that of the full-length promoter construct (Fig. 4C). Region 3 of the *GLDPA* promoter included the sequences between –1138 and –927. Deletion of this promoter fragment in construct *GLDPA*-Ft-Δ3 decreased the GUS activity below the sensitivity limit of the histochemical GUS assay. Hence, no GUS expression could be detected in leaf cross-sections of plants carrying this promoter construct. Similar low GUS activities (using the quantitative GUS assay) were also observed for the constructs *GLDPA*-Ft-Δ4 and *GLDPA*-Ft-Δ5 (Fig. 4A, D), while practically no GUS activity was observed for *GLDPA*-Ft-Δ6 (Fig. 4A, D).

This deletion analysis clearly demonstrated the pronounced importance of regions 1, 2 and 3 for the transcriptional activity of the *GLDPA* promoter in the leaves of transgenic

Arabidopsis plants. While truncation of regions 1 and 2 causes a dramatic decrease of transcriptional activity without affecting the spatial expression pattern, the additional deletion of region 3 results in a further reduction of promoter activity which impeded further analysis of cell type-specific expression within the leaf.

**Regions 1 and 2 of the *GLDPA* Promoter Together Function as a General Enhancer of Transcription in the Arabidopsis Leaf**

To investigate whether the *GLDPA* promoter fragment reaching from –1571 to –1139 (regions 1 and 2) was able to act as a transcriptional enhancer we combined this segment of the promoter with region 7 of the *GLDPA* promoter (–298 to –1). Region 7 harbours a putative TATA-box and the starting point of transcription, but – as reported above – this part of the promoter alone is not sufficient to drive GUS expression in the Arabidopsis leaf (Fig. 4D).

The transformation of construct *GLDPA*-Ft-1-2-7 (Fig. 5A) into Arabidopsis caused a substantial level of GUS expression in mesophyll and bundle-sheath cells as well as in the vascular strands of the leaves (Fig. 5B, C) and confirms that regions 1 and 2 contain transcriptional enhancers with no apparent cell type specificity within the leaf.

To test whether regions 1 and 2 of the *GLDPA* promoter function also in a heterologous promoter context we fused this segment in front of the proximal promoter region of the *ppcA1* gene of *F. trinervia* (Fig. 5A). The *ppcA1* gene encodes the $C_4$ isoform of phosphoenolpyruvate carboxylase (Hermans and Westhoff, 1992), and its complete 2188 bp promoter directs high and mesophyll-specific GUS expression in transgenic *F. bidentis* (Stockhaus et al., 1997). In contrast, the activity of the 570 bp long proximal *ppcA* promoter part (*ppcA*-$PR_{Ft}$) is extremely low and can hardly be visualized in histochemical GUS assays (Gowik et al., 2004). In plants in which the low activity permits a histological analysis, the *ppcA*-$PR_{Ft}$ promoter fragment directs a uniform expression in all cells of the leaves of *F. bidentis* including the vascular bundles (Akyildiz et al., 2007).

The fusion of regions 1 and 2 of the *GLDPA* promoter with the *ppcA*-$PR_{Ft}$ promoter fragment resulted in strong GUS expression in leaves of Arabidopsis (Fig. 5B). The GUS reporter gene was active in both mesophyll and bundle-sheath cells as well as in the vascular bundles (Fig. 5D), and the expression profile of this chimeric promoter is thus indistinguishable from that of *GLDPA*-Ft-1-2-7. We conclude from these experiments that regions 1 and 2 of the *GLDPA* promoter constitute a general transcriptional enhancer module

that, in combination with a basal promoter, stimulates the expression of a linked reporter gene in all types of interior leaf cells of Arabidopsis.

**Region 3 of the *GLDPA* Promoter Acts as a Mesophyll-Specific Repressor of Gene Expression**

The role of region 3 (–1138 to –927) in regulating *GLDPA* promoter activity was investigated by introducing the relevant promoter fragment into construct *GLDPA*-Ft-1-2-7, resulting in the production of the chimeric promoter *GLDPA*-Ft-1-2-3-7 (Fig. 6A). The addition of region 3 to *GLDPA*-Ft-1-2-7 caused a significant change in the spatial expression pattern of the GUS reporter gene. While *GLDPA*-Ft-1-2-7 plants expressed the GUS reporter gene in mesophyll and bundle-sheath cells as well as in the vascular tissue (Fig. 5C), GUS activity of *GLDPA*-Ft-1-2-3-7 plants was strictly confined to the bundle-sheath cells and the vascular compartment (Fig. 6B). These observations indicate that region 3 of the *GLDPA* promoter from *F. trinervia* functions as a mesophyll-specific repressor of gene expression in the Arabidopsis leaf.

**Analysis of *GLDPA* Promoter Regions 4, 5 and 6**

We have shown that the *GLDPA* promoter fragment comprising base pairs –1571 to –927 (regions 1 to 3) in combination with the most proximal promoter part (region 7) is sufficient to direct GUS expression in the bundle-sheath cells and vascular bundles of transgenic *A. thaliana* plants. Nevertheless, additional *cis*-regulatory determinants that could be involved in the spatial regulation of transcriptional activity might also be present in promoter regions 4, 5 and 6. In order to investigate the occurrence of *cis*-regulatory elements within these promoter regions it was necessary to raise the GUS expression levels of constructs *GLDPA*-Ft-Δ3 to *GLDPA*-Ft-Δ6 to a level that allowed a histochemical analysis. Since regions 1 and 2 of the *GLDPA* promoter contain a general transcriptional enhancer with no apparent leaf cell specificity we attached this *GLDPA* transcriptional enhancer module to the 5´ borders of the truncated promoters (Fig. 7A).

As expected, the transcriptional activity of these constructs was dramatically higher than that of their “enhancerless” counterparts and was therefore suitable for performing GUS-stainings *in situ* (Fig. 4D, 7B). In construct *GLDPA*-Ft-1-2-4-5-6-7, only region 3 was removed from the original full-length *GLDPA* promoter. While about 50 % of the transgenic lines displayed a uniform GUS expression in mesophyll and bundle-sheath cells and the vascular bundles (Fig. 7F), GUS expression in the other half of the plant lines was still

restricted to the bundle-sheath cells and the vascular tissue (Fig. 7C). The same distribution of transgenic plants displaying either a uniform or restricted expression of the reporter gene was also observed for the promoter constructs *GLDPA*-Ft-1-2-5-6-7 and *GLDPA*-Ft-1-2-6-7 in which regions 4 and 5 were further deleted (Fig. 7D, E, G, H). In contrast, as already reported above, the additional deletion of region 6 in construct *GLDPA*-Ft-1-2-7 resulted in a uniform expression pattern in the leaf (Fig. 5C). These findings suggest that additional *cis*-regulatory elements conferring repression of gene expression in the mesophyll are located in region 6 of the *GLDPA* promoter. However, when compared to the highly effective repressor elements located in region 3, these additional elements in region 6 do not provide robust repression.

**Analysis of Promoter Construct *GLDPA*-Ft-1-2-3-7 in Transgenic *F. bidentis***

A truncated promoter containing the transcription enhancing regions 1 and 2, the mesophyll repressor region 3 and the basal expression segment 7 generated the same spatial expression profile in the leaf of the $C_3$ plant Arabidopsis as the complete *GLDPA* promoter, i.e. the promoter regions 4, 5 and 6 (–926 to –299) were not essential for creating the $C_4$-characteristic spatial expression pattern of a reporter gene. We now wished to examine whether this chimeric *GLDPA*-Ft-1-2-3-7 promoter is capable of providing this C4 expression profile also in the $C_4$ background of *F. bidentis*. This chimeric promoter construct was therefore transformed into *F. bidentis*, and its expression was examined in the leaves of the transgenic plants (Fig. 8).

No differences between the spatial expression patterns of *GLDPA*-Ft-1-2-3-7 and the full-length promoter construct *GLDPA*-Ft were observed (compare Figs. 2 and 8). In both cases, GUS expression was found exclusively in the bundle-sheath cells and – with variable intensities – in the vascular strands. While GUS staining was strong in some minor veins (Fig. 8E), it was absent from other minor and all major vascular strands (Fig. 8B, D, E, F). These results indicate that regions 1 to 3 in combination with the basal TATA box-containing segment 7 of the *GLDPA* promoter are sufficient to direct reporter gene expression in bundle-sheath cells and the vascular bundles of both the homologous $C_4$ species *F. bidentis* and the heterologous $C_3$ plant *A. thaliana*.

## Discussion

The correct functioning of the $C_4$ photosynthetic cycle requires strict compartmentalization of $C_4$ enzymes in either mesophyll or bundle-sheath cells of the leaf. This cell type-specific accumulation of proteins is governed by differential gene expression (Sheen, 1999). To

broaden our knowledge on the molecular basis of bundle-sheath specific gene expression in $C_4$ plants we have performed a functional analysis of the 5´ flanking sequences of the *GLDPA* gene from *F. trinervia* ($C_4$). The *GLDPA* gene encodes the P-subunit (GLDP) of glycine decarboxylase (GDC), which is specifically located in the bundle-sheath cells of $C_4$ species (Morgan et al., 1993). To determine whether the bundle-sheath-specific accumulation of the GLDP protein in the $C_4$ leaf is paralleled by the accumulation of the corresponding mRNA we studied the occurrence of *GLDPA* transcripts within the leaf of *F. trinervia* and *F. bidentis* by *in situ* hybridizations. *GLDPA* RNA was exclusively found in the bundle-sheath cells of both C4 species, indicating that the presence of this protein in bundle-sheath cells, and its absence in mesophyll cells, is caused by differential *GLDPA* mRNA accumulation.

We then investigated whether the available 1571 bp of the 5´ flanking region of the *GLDPA* coding sequence harbour all the necessary information for this bundle-sheath-specific expression. Fusion of these sequences – including the 5' untranslated segment of the *GLDPA* gene – to the GUS reporter gene resulted in reporter gene activity in the bundle-sheath, but not in the mesophyll cells of transgenic *F. bidentis* plants. In addition, GUS activity could be detected in the vascular bundles. Here, GUS activity was clearly visible in minor veins, but was very low in major veins.

The expression of the reporter gene in the bundle-sheath cells and the absence of GUS activity in the mesophyll is consistent with the accumulation pattern of the *GLDPA* RNA. The additional activity of the GUS reporter gene in the vascular tissue, however, is in contrast to the lack of detectable *GLDPA* RNA in this tissue. There are two possible explanations which could account for this discrepancy in the patterns of RNA accumulation and reporter gene activity. First, there could be additional *cis*-regulatory sequences further upstream, within the introns or even downstream of the *GLDPA* gene which might control *GLDPA* transcription in the natural genome context, but are absent in the *GLDPA*-Ft promoter-GUS construct. These elements would be required for repressing reporter gene expression in the vascular tissue. Alternatively, the absence of *GLDPA* mRNA in the vascular tissue might be caused by low *GLDPA* RNA stability in this tissue (Parker and Song, 2004; Moore, 2005). While the 5´ untranslated region of the *GLDPA* mRNA is included in the *GLDPA* promoter construct, the 3´ untranslated segment is not. If this latter RNA segment or the coding region contain stability determinants leading to degradation of the *GLDPA* mRNA in the vascular tissue, the GUS mRNA lacking these segments would accumulate.

Interestingly, expression of the *GLDPA* promoter GUS construct in transgenic Arabidopsis showed a spatial activity of the *GLDPA* promoter that was very similar to that

observed in transgenic *F. bidentis* (Fig. 3). As found for the $C_4$ species, the *GLDPA* promoter of *F. trinervia* was inactive in the leaf mesophyll of the $C_3$ plant *A. thaliana*, but active in the bundle-sheath cells and the vascular bundle. The level of reporter gene expression was similar in both species. We therefore conclude that the $C_4$-characteristic *cis*-regulatory transcriptional determinants are recognized in the same spatial manner also in the $C_3$ context. This indicates that the transcription factors necessary for the correct interpretation of these *cis*-regulatory sequences are already present in this $C_3$ species and are operating in the same spatial pattern. Moreover, the similar spatial expression profiles in a $C_4$ and $C_3$ leaf allow to conclude that the gene regulatory network operating in mesophyll and bundle-sheath cells of dicot $C_3$ and $C_4$ species share common elements.

The exact physiological and biochemical functions of bundle-sheath cells in $C_3$ species are poorly understood. They are involved in phloem loading and unloading (van Bel, 1993), and for tobacco it was shown that the bundle-sheath cells of stems and petioles exhibit high activities of enzymes characteristic of $C_4$ photosynthesis, thus allowing the decarboxylation of four-carbon organic acids derived from the xylem and phloem (Hibberd and Quick, 2002). Additionally, a class of Arabidopsis mutants termed *dov* (*d*ifferential development *o*f *v*ascular associated cells) demonstrates that differential chloroplast development occurs between bundle-sheath and mesophyll cells in the Arabidopsis leaf (Kinsman and Pyke, 1998).

These observations from tobacco and Arabidopsis provide some evidence that bundle-sheath cells in $C_3$ plants are somehow predetermined to evolve $C_4$ characteristic features. The special physiology of bundle-sheath cells in *A. thaliana* and the fact that pre-existing transcription factors in this $C_3$ species are able to recognize heterologous $C_4$ characteristic *cis*-regulatory elements in the correct fashion provide further evidence for the view that the evolution of $C_4$ plants must have been relatively simple in genetic terms (Westhoff and Gowik, 2004). $C_4$-like spatial activities of $C_4$ promoters in transgenic $C_3$ plants have also been reported for the $C_4$ isoform of PEPC of maize (Matsuoka et al., 1994; Nomura et al., 2000), the pyruvate orthophosphate dikinase gene of maize (Matsuoka et al., 1993) and the phosphoenolpyruvate carboxykinase of *Zoysia japonica* (Nomura et al., 2005). On the other hand, the $C_4$-PEPC promoter of *F. trinervia* loses mesophyll specificity when it is introduced in Arabidopsis (Akyildiz et al., 2007). Similarly, the NADP-dependent malic enzyme promoter from maize loses its bundle-sheath specificity in rice (Nomura et al., 2005). This shows that the functionality of $C_4$-specific regulatory *cis*-elements in $C_3$ plants cannot be generalized.

The $C_4$-like expression pattern of the *GLDPA*-Ft promoter in Arabidopsis provided us with the possibility to dissect the functional organization of this promoter in Arabidopsis. A series of *GLDPA* promoter deletion and recombination constructs were analyzed and two major functional modules were identified and localized, a non-cell-type-specific transcriptional enhancer and a segment that represses gene expression in mesophyll cells.

The transcriptional enhancer is located within the outermost distal regions 1 and 2 of the *GLDPA* promoter comprising base pairs –1571 to –1139. The enhancer functioned in all interior leaf tissues of Arabidopsis, i.e. in mesophyll and bundle-sheath cells as well as in the vascular bundle. The transcription-enhancing activity of these regions was not restricted to the context of the *GLDPA* promoter but was also functional when combined with the proximal part of the *ppcA1* promoter of *F. trinervia*. The enhancer is thus not *GLDPA* gene-specific but functions as a general enhancer module.

The quantitative analysis of promoter activities (Fig. 4D) indicates that region 1 has a higher potential for transcriptional enhancement than region 2. A search for known *cis*-regulatory elements (Prestridge, 1991; Higo et al., 1999) revealed the presence of a motif with similarity to the simian virus 40 enhancer core (GTGGWWHG) at positions –1455 to –1448 in region 1. This motif is also present in a region of the *GLDPA* promoter of *F. pringlei* that is associated with an increase in expression quantity (Bauwe et al., 1995).

Region 3 (–1138 to –927) harbours *cis*-regulatory elements that confer cell specificity to the *GLDPA* promoter by repressing its activity in the mesophyll cells of the Arabidopsis leaf. A chimeric promoter consisting of the transcription-enhancing regions 1 and 2, region 3, and the proximal basal expression region 7 is also not active in the mesophyll cells of the $C_4$ species *F. bidentis*. This indicates that region 3 can repress expression in mesophyll cells also in the $C_4$ context, i.e. the mesophyll-repressing function of region 3 is conserved between the $C_3$ and the $C_4$ species. The lack of *GLDPA* expression in the mesophyll is thus caused by transcriptional regulation and not by post-transcriptional regulation as it was reported for the *FbRbc*S1 gene of *F. bidentis*. *FbRbc*S1 encodes the small subunit of Rubisco, and its bundle-sheath-specific expression is entirely established by selective *rbcS* transcript stabilization in the bundle-sheath cells (Patel et al., 2006).

Additional mesophyll-repressing *cis*-regulatory sequences are located in region 6 (–521 to –299). They can partially compensate for the lack of the mesophyll-repressing *cis*-regulatory sequences in region 3, when this segment is not present in the promoter construct. However, these *cis*-regulatory elements are not able to establish a robust repression of reporter gene activity in the mesophyll cells of Arabidopsis and appear to be of minor

importance. This is documented by the cell type-specific expression of construct *GLDPA*-Ft-1-2-3-7 that consists of the transcription-enhancing regions 1 and 2, region 3, and the proximal basal promoter region 7. *GLDPA*-Ft-1-2-3-7 directs a $C_4$-characteristic expression profile in *F. bidentis*. This demonstrates that the *cis*-regulatory motives present in region 6 are not necessary for the repression of *GLDPA* expression in mesophyll cells of this $C_4$ species. Moreover, we can infer from the expression profile of this truncated promoter that regions 4 and 5 are also not necessary to achieve a $C_4$-typical GUS expression pattern in both *Arabidopsis* and *F. bidentis*. Regarding the mechanism of mesophyll repression no predictions can be made at this moment, since searching for known *cis*-regulatory elements in region 3 did not identify any robust candidate motifs.

The *cis*-regulatory determinants for the mesophyll-specific repression of *GLDPA* expression in the leaf have not been determined yet, and no other gene system for bundle-sheath specific expression has been investigated in such a detail that its *cis*- and *trans*-regulatory elements are known. However, *cis*-regulatory elements for mesophyll-specific gene expression, have recently been identified at the nucleotide level (Gowik et al., 2004; Akyildiz et al., 2007) for the $C_4$ PEPC gene *ppcA1*. It was found that variation at two positions in a 41 bp element, a G to A transition and the presence or absence of the tetranucleotide CACT, determines whether the promoter is active in all interior tissues of the leaf of *F. bidentis* or only in the mesophyll cells (Akyildiz et al., 2007). Hence, for both genes, *GLDPA* and *ppcA1*, cell type-specific gene expression is achieved by repressing the activity of the gene in the respective other cell type. Further dissection of repressor regions 3 and 6 within the *GLDPA* promoter of *F. trinervia* should allow to precisely map the involved *cis*-regulatory elements. Such identification of a bundle-sheath-specific expression module consisting of a *cis*-regulatory sequence and the corresponding transcription factor would mark an important step in our understanding of the evolution of $C_4$ photosynthesis. Moreover, this information would also be of great value for attempts to convert a $C_3$ species into a $C_4$ species, as it has been proposed for rice (Mitchell and Sheehy, 2006).

## Experimental Procedures

### Construction of Chimeric Promoters

DNA manipulations and cloning were carried out according to Sambrook and Russell (2001). Construct *GLDPA*-Ft (Cossu, 1997) served as the basis for the series of *GLDPA* promoter deletions. In *GLDPA*-Ft, the 5´ upstream region of the *GLDPA* gene of *F. trinervia* from –

1571 to –1 (+1 describes the first base of the translational start codon) was fused to the GUS cDNA in the binary plant transformation vector pBI121 (Clontech Laboratories, Palo Alto, CA). Different 5´-deleted fragments of the *GLDPA*-promoter were generated by PCR amplification (Tables 1 and 2). The primers added an *Xba*I restriction site at the 5´ border of the DNA fragments and an *Xma*I site at the 3´ end. Therefore the deleted promoters could be inserted into *Xba*I/*Xma*I-cut pBI121, resulting in the formation of the constructs *GLDPA*-Ft-Δ1, *GLDPA*-Ft-Δ3, *GLDPA*-Ft-Δ4, *GLDPA*-Ft-Δ5 and *GLDPA*-Ft-Δ6. For the production of *GLDPA*-Ft-Δ2, plasmid *GLDPA*-Ft was digested with *Hind*III and *Eco72*I. The remaining plasmid was purified by gel electrophoresis and blunt ends were generated by treatment with the Klenow fragment of *Escherichia coli* DNA polymerase I. The vector was religated to form *GLDPA*-Ft-Δ2. The DNA-fragment comprising regions 1 and 2 of the *GLDPA*-Ft-promoter was generated by PCR-amplification with primers *GLDPA*-5´-Xba and Eco72-R. *Xba*I sites were introduced at both ends of the PCR product which allowed the insertion of the DNA fragment into *Xba*I-cut *GLDPA*-Ft-Δ3, *GLDPA*-Ft-Δ4, *GLDPA*-Ft-Δ5, *GLDPA*-Ft-Δ6 and *ppcA*-S-Ft (Stockhaus et al., 1994). The resulting plasmid constructs were named *GLDPA*-Ft-1-2-4-5-6-7, *GLDPA*-Ft-1-2-5-6-7, *GLDPA*-Ft-1-2-6-7, *GLDPA*-Ft-1-2-7 and *GLDPA*-Ft-1-2-*ppcA*-$PR_{Ft}$. The cloning of construct *GLDPA*-Ft-1-2-3-7 involved PCR-amplification of the promoter region between –1571 and – 927 using primers *GLDPA*-5´-Xba and gdcs3-R. The PCR-product was digested with *Xba*I and ligated with *Xba*I-cut *GLDPA*-Ft-7. The correct orientation of the inserted DNA fragment in *GLDPA*-Ft-1-2-3-7 was shown by sequencing.

To produce construct *GLDPA*-Ft::*H2B:YFP*, the *H2B:YFP* gene fusion was excised from plasmid pBI121-*35S::H2B:YFP* (Boisnard-Lorig et al., 2001) with *BamH*I and *Sac*I. The *GLDPA* promoter of *F. trinervia* was amplified from plasmid *GLDPA*-Ft (Cossu, 1997) with primers *GLDPA5´-Hind*III and *GLDPA3´-BamH*I. A pBI121 backbone was generated by removing the 35S::*H2B:YFP* insert from plasmid pBI121-*35S::H2B:YFP* via incubation with *Hind*III and *Sac*I, and a triple-ligation between pBI121 (*Hind*III/*Sac*I), the *GLDPA* promoter (*Hind*III/*BamH*I) and the *H2B:YFP* fragment (*BamH*I/*Sac*I) finally resulted in the formation of construct *GLDPA*-Ft::*H2B:YFP*.

The cloning of construct *GLDPA*-Ft::*mGFP5-ER* was achieved by PCR amplification of the *mgfp5-ER* gene (Haseloff et al., 1997) from genomic DNA of the *Arabidopsis thaliana* enhancer trap line E2443 (generated by Scott Poethig, http://www.arabidopsis.org/abrc/poethig.jsp) with primers 5´-mGFP5ER-*BamH*I and 3´-

mGFP5ER-*Sac*I. The PCR product was then cloned into *BamH*I/*Sac*I-cut *GLDPA*-Ft::*H2B:YFP* to yield *GLDPA*-Ft::*mGFP5-ER*.

**Plant Transformation**

In all transformation experiments the *Agrobacterium tumefaciens* strain AGL1 was used (Lazo et al., 1991). The promoter-GUS constructs were introduced into AGL1 by electroporation. Arabidopsis plants were transformed via the floral dip method according to Clough and Bent (1998). The transformation of *Flaveria bidentis* was performed as described by Chitty et al (1994). The integration of the transgenes into the genome of regenerated *F. bidentis* or T1 Arabidopsis plants was proved by PCR analyses.

**Measurement of GUS Activity and Histochemical Analysis of Reportergene Activity**

*F. bidentis* T0 plants used for GUS analysis were 40 to 50 cm tall and before flower initiation; the Arabidopsis T1 plants were examined around three weeks after germination. Fluorometrical quantification of GUS activity was performed according to Jefferson et al (1987) and Kosugi et al (1990). For histochemical analysis of GUS activity leaves were cut manually with a razorblade and the sections were transferred to incubation buffer (100 mM $Na_2HPO_4$, pH 7.5, 10 mM EDTA, 50 mM $K_4[Fe(CN)_6]$, 50 mM $K_3[Fe(CN)_6]$, 0.1% (v/v) Triton X-100, 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronid acid). After brief vacuum infiltration the sections were incubated at 37°C for 1 to 20 hrs. After incubation, chlorophyll was removed from the tissue by treatment with 70% ethanol. Fluorescence microscopy was performed using a Zeiss Axiophot (Carl Zeiss AG, Jena, Germany) equipped with an Olympus DP50 camera (Olympus Optical Co., LTD., USA) and a Zeiss GFP imaging filter system (BP 450-490, FT 510, BP 515-565). Bright field and fluorescence images were overlaid with Adobe Photoshop 7.0 (Adobe Systems Inc., San José, CA, USA). For confocal laser microscopy a Zeiss LSM 510 was used.

***In Situ* RNA Hybridization**

Non-radioactive *in situ* hybridization experiments were performed according to the protocol described by Simon (2002). Embedded leaves of *F. bidentis* and *F. trinervia* were cut into cross-sections of 20 µm thickness using a standard microtome. For the generation of the *GLDPA* probe a 2,4 kb cDNA-fragment of the *GLDPA* gene of *F. trinervia* was amplified by PCR (primers gdcsF-4 and gdcsR-4, see Table 1). The PCR-product was digested with *Bam*HI and *Xho*I and cloned into *Bam*HI/*Xho*I-cut pBluescript II KS (Stratagene). The use of

two different RNA-polymerases (T3, T7) then allowed the production of both antisense and sense probes for *in situ* hybridization.

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# Tables

**Table 1:** Oligonucleotide primers used for the construction of promoter fragments and for the synthesis of probes for *in situ*- and northern hybridizations

| Primer name | Sequence (5´- 3´) |
|---|---|
| *GLDPA*-5´-Xba | TGCTCTAGAAGCTTTACTCCTCTC |
| *GLDPA*-Xma | AAATCCCGGGTAGTGTAAGATGGG |
| *GLDPA*-Xba-2 | TGCTCTAGATGAAACAGGATGAGC |
| *GLDPA*-Xba-3 | TGCTCTAGATGAAACAGGATGAGC |
| *GLDPA*-Xba-4 | TGCTCTAGACAAGTTTGATACTAG |
| *GLDPA*-Xba-5 | TGCTCTAGACAAGTTTGATACTAG |
| *GLDPA*-Xba-6 | TGCTCTAGACATTTGATCTATAACG |
| Eco72-R | TGCTCTAGAGTGGAGATGATAGTTG |
| gdcs3-R | TGCTCTAGACAAAAGTTCAAAACTTG |
| gdcs-1F | CGCATTGTCGACAAGAACACT |
| gdcs-8R | GTGGTTGAAGAGTGCAGATG |
| gdcsF-4 | GAAAGGATCCTCGAATGCTTCATTGC |
| gdcsR-4 | TTCACTCGAGGAATTTAGCAGCACG |
| *GLDPA5´-Hind*III | AAGCTTTACTCCTCTCAACT |
| *GLDPA3´-BamH*I | TTAGGATCCAGTGTAAGATGGGGTCTA |
| 5´-mGFP5ER-*BamH*I | TTAGGATCCAAGGAGATATAACAATGA |
| 3´-mGFP5ER-*Sac*I | AATGAGCTCTTAAAGCTCATCATGTTT |

**Table 2:** Oligonucleotide primer combinations used for the construction of *GLDPA* promoter fragments

| Construct | Primer 1 | Primer 2 |
|---|---|---|
| *GLDPA*-Ft-Δ1 | *GLDPA*-Xba-2 | *GLDPA*-Xma |
| *GLDPA*-Ft-Δ3 | *GLDPA*-Xba-3 | *GLDPA*-Xma |
| *GLDPA*-Ft-Δ4 | *GLDPA*-Xba-4 | *GLDPA*-Xma |
| *GLDPA*-Ft--Δ5 | *GLDPA*-Xba-5 | *GLDPA*-Xma |
| *GLDPA*-Ft--Δ6 | *GLDPA*-Xba-6 | *GLDPA*-Xma |

## Figure Legends:

**Figure 1:**

*In situ* localization of *GLDPA* RNA accumulation in leaves of *F. trinervia* (A) and *F. bidentis* (C). Hybridizations with a *GLDPA* sense probe to leaf cross-sections of *F. trinervia* (B) and *F. bidentis* (D) served as negative controls. BS: bundle-sheath cell.

**Figure 2:**

Analysis of *GLDPA*-Ft promoter activity in transgenic *F. bidentis*.

(A) Structure of the chimeric *GLDPA*-Ft*::GUS* gene

(B) – (E) Histochemical localization of GUS activity (blue staining) in leaf sections of transgenic *F. bidentis* transformed with *GLDPA*-Ft. The photograph (E) was taken from the leaf surface displaying GUS activity in the stomatal guard cells of *F. bidentis* leaves. Incubation times were 1 h.

(F) GUS activities in leaves of transgenic *F. bidentis* plants transformed with *GLDPA*-Ft. GUS activities are expressed in nanomoles of the reaction product 4-methylumbelliferone (MU) per milligram of protein per minute. The numbers of independent transgenic plants investigated (N) and the median values of GUS activity (M) are indicated at the top of each column.

**Figure 3:**

Analysis of *GLDPA*-Ft promoter activity in transgenic *A. thaliana*.

(A) Schematic structure of the chimeric *GLDPA*-Ft*::GUS*, *GLDPA*-Ft::*mGFP5-ER* and *GLDPA*-Ft*::H2B:YFP* genes.

(B) GUS activities in leaves of transgenic *A. thaliana* plants transformed with *GLDPA*-Ft*::GUS*. GUS activities are expressed in nanomoles of the reaction product 4-methylumbelliferone (MU) per milligram of protein per minute. The numbers of independent transgenic plants investigated (N) and the median values of GUS activity (M) are indicated at the top of each column.

(C) + (D) Histochemical localization of GUS activity (blue staining) in leaf sections of transgenic *A. thaliana* transformed with *GLDPA*-Ft*::GUS*. Picture (D) was taken from the leaf surface displaying GUS activity in the vascular strands and the adjacent layer of bundle-sheath cells in Arabidopsis. Incubation times were 1 h.

(E) – (L) Fluorescence microscopy images taken from leaf cross-sections of Arabidopsis

plants transformed with the constructs *GLDPA*-Ft::*mGFP5-ER* (E-H) or *GLDPA*-Ft*::H2B:YFP* (I-L). For (E), (G), (I) and (K), images taken with a GFP imaging filter system were merged with corresponding bright-field images. Photographs in panels (F), (H), (J) and (L) were captured using the GFP imaging filter system alone. Selected bundle-sheath cells that express the reporter gene are highlighted by yellow arrows. BS: bundle-sheath cell; V: vascular tissue; X: xylem; P: phloem.

**Figure 4:**

Deletion analysis of the *GLDPA* promoter of *F. trinervia* in the $C_3$ plant *A. thaliana*.

(A) Overview of *GLDPA* promoter deletion constructs analyzed in transgenic *A. thaliana*. Due to the cloning strategy the *GLDPA* promoter of *F. trinervia* was subdivided into seven regions

(B) + (C) Histochemical localization of GUS activity in leaf sections of transgenic *A. thaliana* transformed with deletion constructs *GLDPA*-Ft-Δ1 (B) or *GLDPA*-Ft-Δ2 (C). Incubation times were 12 h (A) and 20 h (B).

(D) GUS activities in leaves of transgenic *A. thaliana* plants transformed with different *GLDPA* deletion constructs. GUS activities are expressed in nanomoles of the reaction product 4-methylumbelliferone (MU) per milligram of protein per minute. The numbers of independent transgenic plants investigated (N) and the median values of GUS activity (M) are indicated at the top of each column.

**Figure 5:**

Functional Analysis of *GLDPA* promoter regions 1 and 2 in transgenic *A. thaliana*.

(A) Chimeric promoter constructs used for plant transformation

(B) GUS activities in leaves of transgenic *A. thaliana* plants transformed with different promoter constructs. GUS activities are expressed in nanomoles of the reaction product 4-methylumbelliferone (MU) per milligram of protein per minute. The numbers of independent transgenic plants investigated (N) and the median values of GUS activity (M) are indicated at the top of each column.

(C) + (D) Histochemical localization of GUS activity in leaf sections of transgenic *A. thaliana* plants transformed with *GLDPA*-Ft-1-2-7 (C) and *GLDPA*-Ft-1-2-*ppcA*-$PR_{Ft}$ (D). Incubation times were 12 h.

**Figure 6:**

Functional Analysis of *GLDPA* promoter region 3 in transgenic *A. thaliana*.

(A) Schematic structure of construct *GLDPA*-Ft-1-2-3-7

(B) Histochemical localization of GUS activity in the leaf sections of a transgenic *A. thaliana* plant transformed with *GLDPA*-Ft-1-2-3-7. Incubation time was 20 h.

(C) GUS activities in leaves of transgenic *A. thaliana* plants transformed with promoter construct *GLDPA*-Ft-1-2-3-7. GUS activities are expressed in nanomoles of the reaction product 4-methylumbelliferone (MU) per milligram of protein per minute. The numbers of independent transgenic plants investigated (N) and the median values of GUS activity (M) are indicated at the top of each column.

**Figure 7:**

Analysis of promoter constructs *GLDPA*-Ft-1-2-4-5-6-7, *GLDPA*-Ft-1-2-5-6-7 and *GLDPA*-Ft-1-2-6-7 in transgenic *A. thaliana*.

(A) Schematic structure of constructs *GLDPA*-Ft-1-2-4-5-6-7, *GLDPA*-Ft-1-2-5-6-7 and *GLDPA*-Ft-1-2-6-7

(B) GUS activities in leaves of transgenic *A. thaliana* plants transformed with different promoter construct. GUS activities are expressed in nanomoles of the reaction product 4-methylumbelliferone (MU) per milligram of protein per minute. The numbers of independent transgenic plants investigated (N) and the median values of GUS activity (M) are indicated at the top of each column.

(C) – (H) Histochemical localization of GUS activity in leaf sections of transgenic *A. thaliana* plants transformed with *GLDPA*-Ft-1-2-4-5-6-7 (C, F), *GLDPA*-Ft-1-2-5-6-7 (D, G) or *GLDPA*-Ft-1-2-6-7 (E, H). Incubation times were 4 h (C, D, F, G) and 5 h (E, H).

**Figure 8:**

Analysis of the promoter construct *GLDPA*-Ft1-2-3-7 in transgenic *F. bidentis*.

(A) Schematic structure of construct *GLDPA*-Ft1-2-3-7

(B) , (D), (E), (F) Histochemical localization of GUS activity in leaf sections of transgenic *F. bidentis* plants transformed with *GLDPA*-Ft-1-2-3-7. Incubation times were 28 h.

(C) GUS activities in leaves of transgenic *F. bidentis* plants transformed with *GLDPA*-Ft-1-2-3-7. GUS activities are expressed in nanomoles of the reaction product 4-methylumbelliferone (MU) per milligram of protein per minute. The numbers of independent transgenic plants investigated (N) and the median values of GUS activity (M)

are indicated at the top of each column.

## Figures

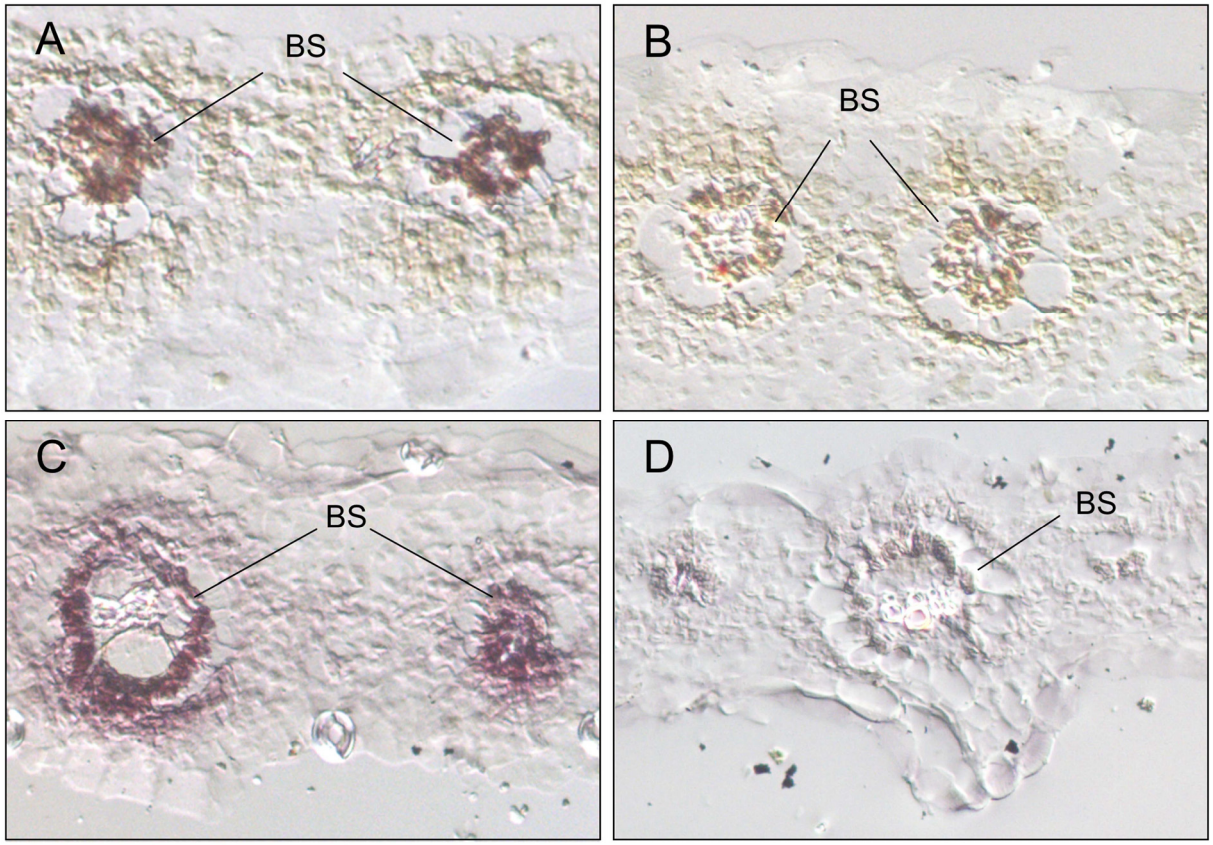
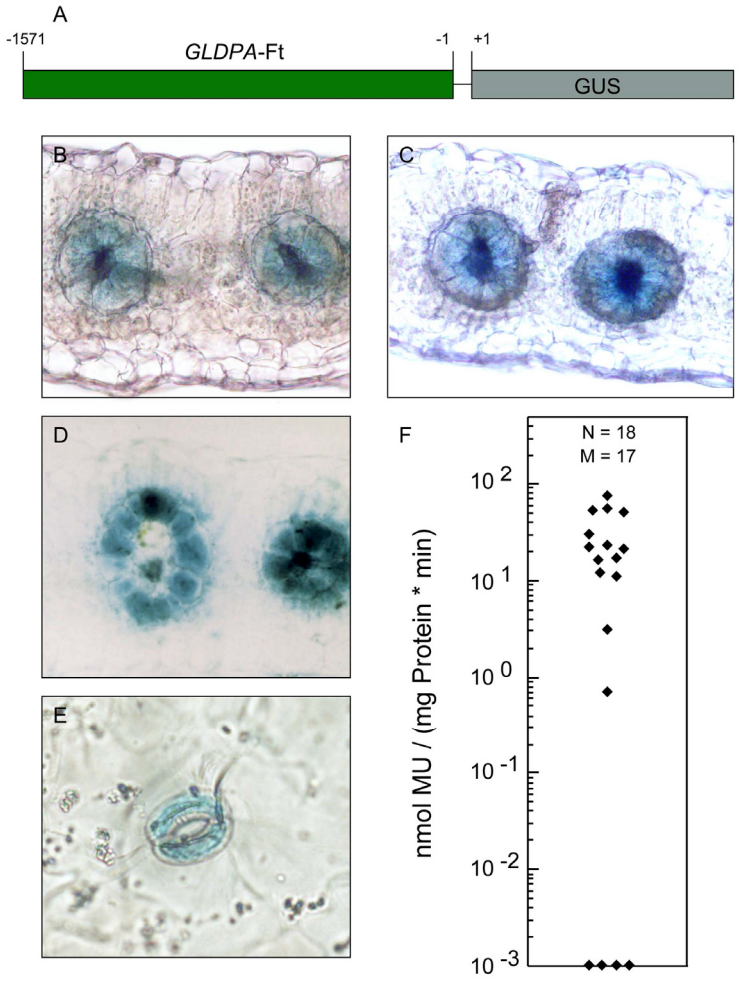



**Figure 1**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GLDPA-Ft
-1
+1
GUS
B
C
D
E
F
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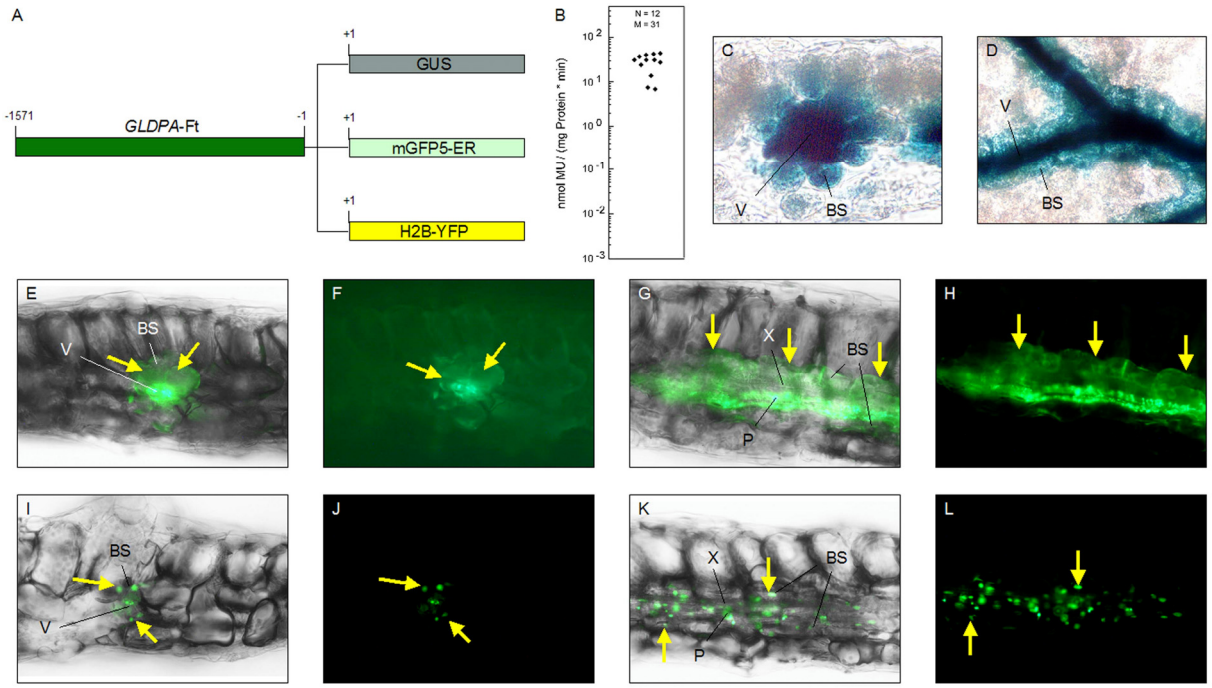
nmol MU / (mg Protein * min)
10 2
10 1
10 0
10 -1
10 -2
10 -3

**Figure 2**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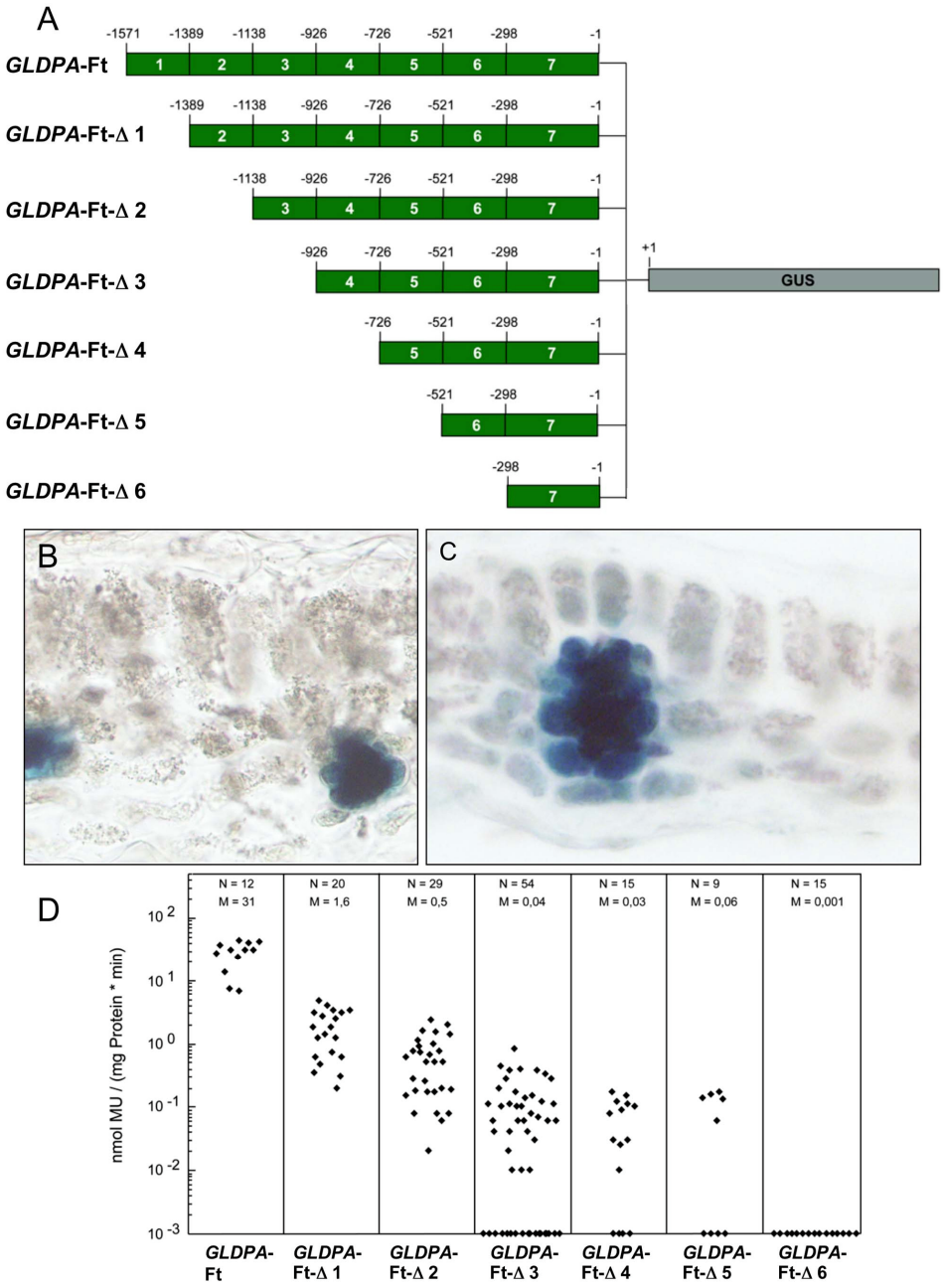
-1
+1
GUS
+1
mGFP5-ER
+1
H2B-YFP
B
N = 12
M = 31
nmol MU / (mg Protein * min)
C
V
BS
D
V
BS
E
V
BS
F
G
X
BS
P
H
I
BS
V
J
K
X
BS
P
L

**Figure 3**


A
-1571 -1389 -1138 -926 -726 -521 -298 -1
GLDPA-Ft
1 2 3 4 5 6 7
-1389 -1138 -926 -726 -521 -298 -1
GLDPA-Ft-Δ 1
2 3 4 5 6 7
-1138 -926 -726 -521 -298 -1
GLDPA-Ft-Δ 2
3 4 5 6 7
-926 -726 -521 -298 -1
GLDPA-Ft-Δ 3
4 5 6 7
+1
GUS
-726 -521 -298 -1
GLDPA-Ft-Δ 4
5 6 7
-521 -298 -1
GLDPA-Ft-Δ 5
6 7
-298 -1
GLDPA-Ft-Δ 6
7
B
C
D
nmol MU / (mg Protein * min)
N = 12 M = 31
N = 20 M = 1,6
N = 29 M = 0,5
N = 54 M = 0,04
N = 15 M = 0,03
N = 9 M = 0,06
N = 15 M = 0,001
GLDPA-Ft
GLDPA-Ft-Δ 1
GLDPA-Ft-Δ 2
GLDPA-Ft-Δ 3
GLDPA-Ft-Δ 4
GLDPA-Ft-Δ 5
GLDPA-Ft-Δ 6

# Figure 4

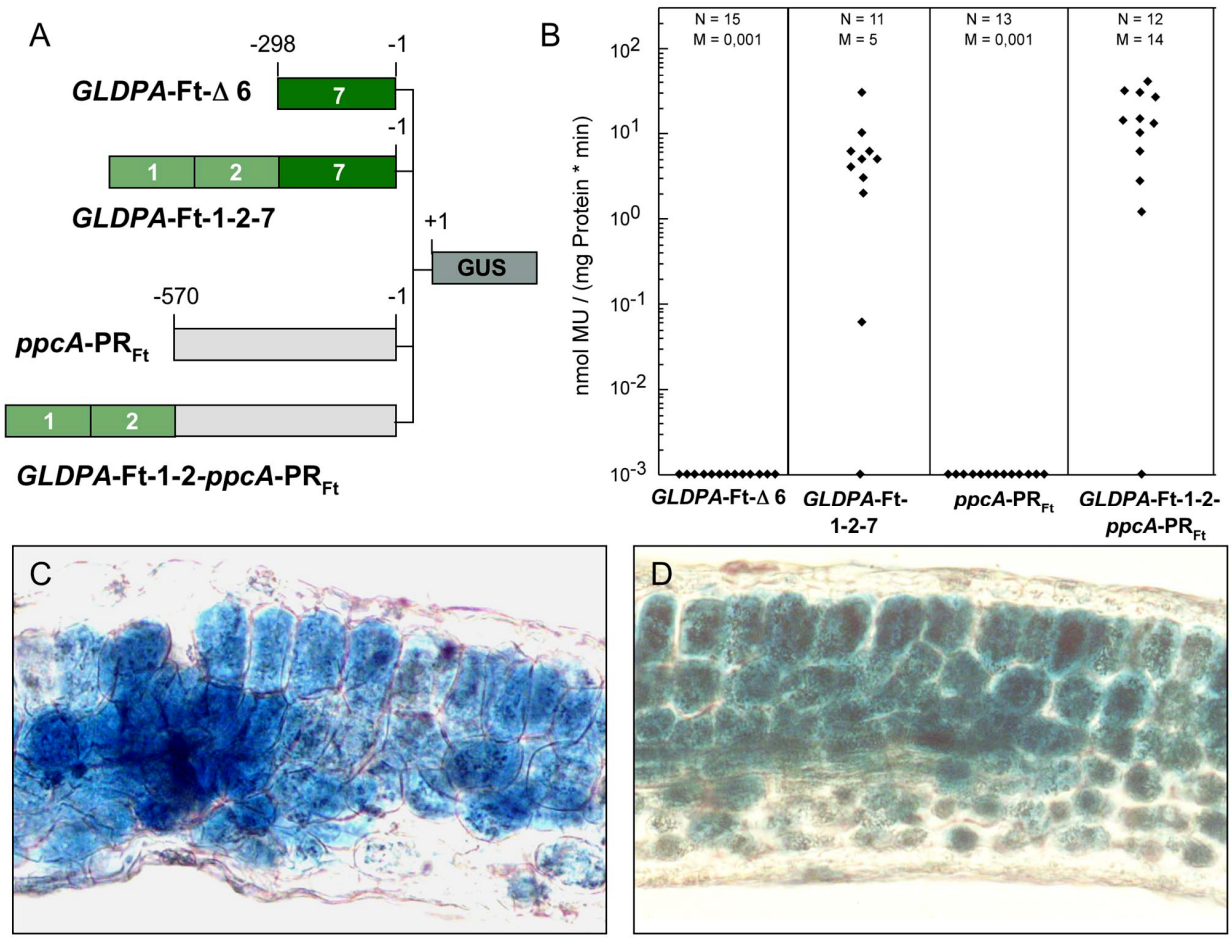
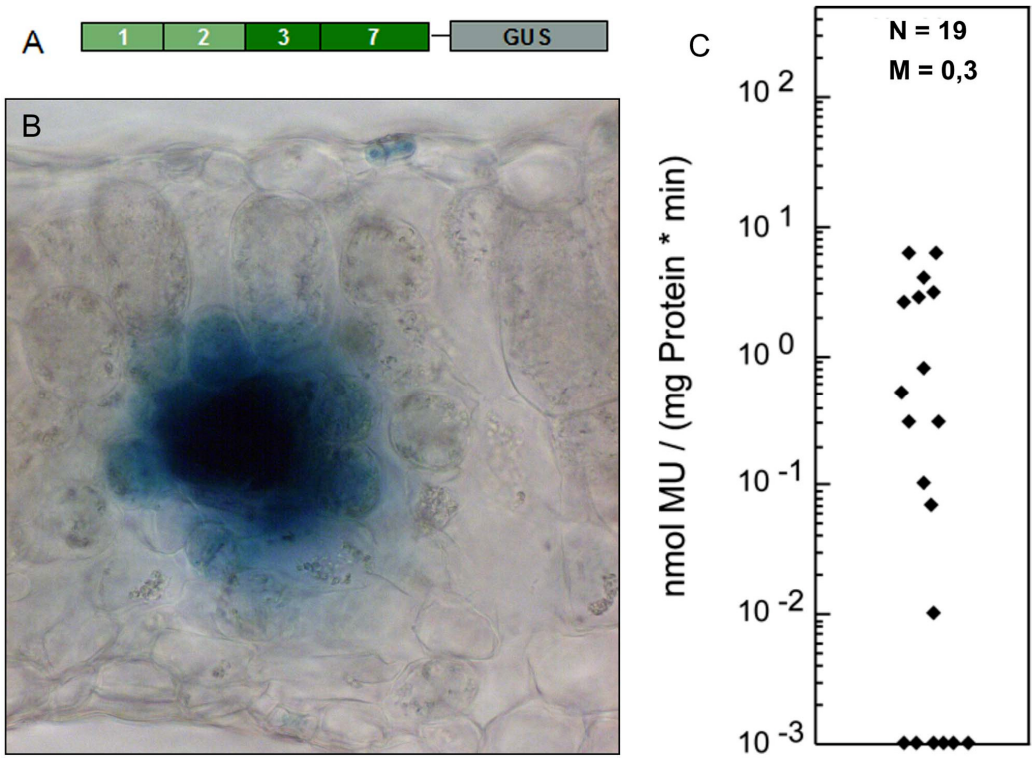

A
-298
-1
GLDPA-Ft-Δ 6
7
-1
1
2
7
GLDPA-Ft-1-2-7
+1
GUS
-570
-1
ppcA-PRFt
1
2
GLDPA-Ft-1-2-ppcA-PRFt
B
N = 15
M = 0,001
N = 11
M = 5
N = 13
M = 0,001
N = 12
M = 14
nmol MU / (mg Protein * min)
10^2
10^1
10^0
10^-1
10^-2
10^-3
GLDPA-Ft-Δ 6
GLDPA-Ft-1-2-7
ppcA-PRFt
GLDPA-Ft-1-2-ppcA-PRFt
C
D

**Figure 5**


A
1
2
3
7
GUS
B
C
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nmol MU / (mg Protein * min)
10 2
10 1
10 0
10 -1
10 -2
10 -3

**Figure 6**

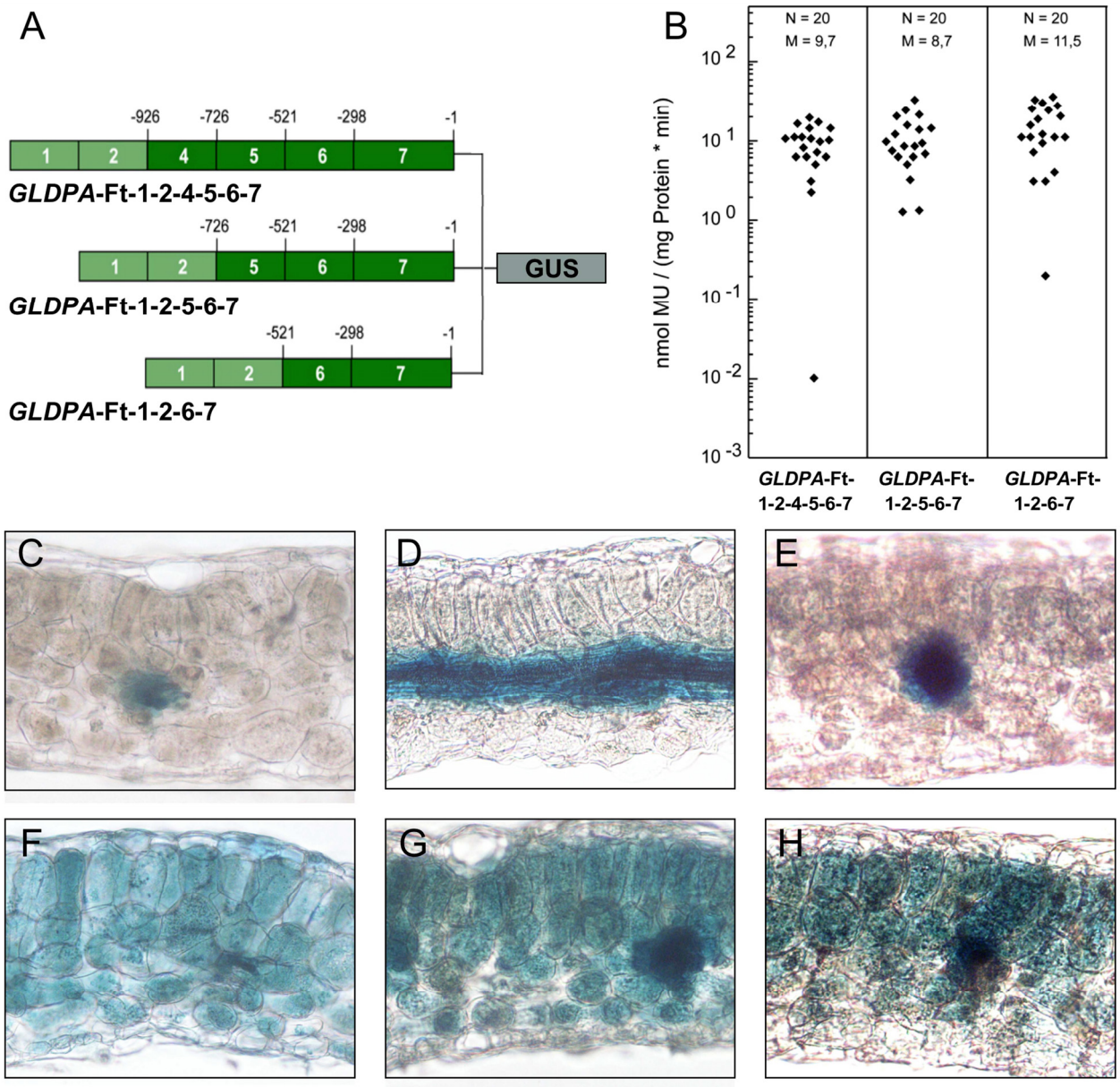
A
-926
-726
-521
-298
-1
1
2
4
5
6
7
GLDPA-Ft-1-2-4-5-6-7
-726
-521
-298
-1
1
2
5
6
7
GUS
GLDPA-Ft-1-2-5-6-7
-521
-298
-1
1
2
6
7
GLDPA-Ft-1-2-6-7
B
N = 20
M = 9,7
N = 20
M = 8,7
N = 20
M = 11,5
nmol MU / (mg Protein * min)
10 2
10 1
10 0
10 -1
10 -2
10 -3
GLDPA-Ft-1-2-4-5-6-7
GLDPA-Ft-1-2-5-6-7
GLDPA-Ft-1-2-6-7
C
D
E
F
G
H

**Figure 7**

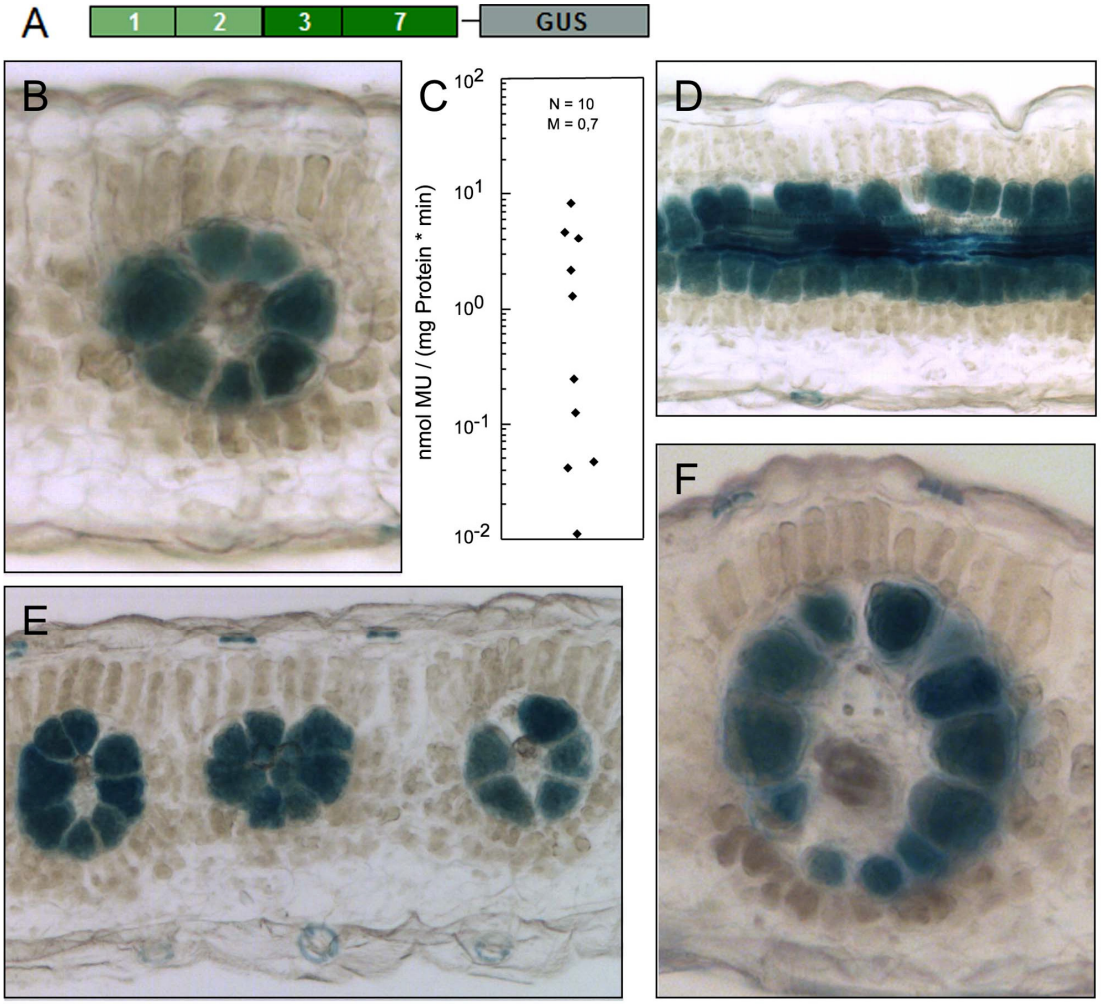
A
1
2
3
7
GUS
B
C
N = 10
M = 0,7
nmol MU / (mg Protein * min)
$10^2$
$10^1$
$10^0$
$10^{-1}$
$10^{-2}$
D
E
F

**Figure 8**

# Evolution of $C_4$ phosphoenolpyruvate carboxylase in the genus *Alternanthera*: gene families and the enzymatic characteristics of the $C_4$ isozyme and its orthologues in $C_3$ and $C_3/C_4$ *Alternantheras*

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## Abstract

Phosphoenolpyruvate carboxylase (PEPCase, EC 4.1.1.3) is a key enzyme of $C_4$ photosynthesis. It has evolved from ancestral non-photosynthetic ($C_3$) isoforms and thereby changed its kinetic and regulatory properties. We are interested in understanding the molecular changes, as the $C_4$ PEPCases were adapted to their new function in $C_4$ photosynthesis and have therefore analysed the PEPCase genes of various *Alternanthera* species. We isolated PEPCase cDNAs from the $C_4$ plant *Alternanthera pungens* H.B.K., the $C_3/C_4$ intermediate plant *A. tenella* Colla, and the $C_3$ plant *A. sessilis* (L.) R.Br. and investigated the kinetic properties of the corresponding recombinant PEPCase proteins and their phylogenetic relationships. The three PEPCases are most likely derived from orthologous gene classes named *ppcA*. The affinity constant for the substrate phosphoenolpyruvate ($K_{0.5}$ PEP) and the degree of activation by glucose-6-phosphate classified the enzyme from *A. pungens* ($C_4$) as a $C_4$ PEPCase isoform. In contrast, both the PEPCases from *A. sessilis* ($C_3$) and *A. tenella* ($C_3/C_4$) were found to be typical $C_3$ PEPCase isozymes. The $C_4$ characteristics of the PEPCase of *A. pungens* were accompanied by the presence of the $C_4$-invariant serine residue at position 775 reinforcing that a serine at this position is essential for being a $C_4$ PEPCase (Svensson et al. 2003). Genomic Southern blot experiments and sequence analysis of the 3′ untranslated regions of these genes indicated the

existence of PEPCase multigene family in all three plants which can be grouped into three classes named *ppcA*, *ppcB* and *ppcC*.

## Introduction

Phosphoenolpyruvate carboxylase (PEPcase, EC 4.1.1.3) is a key enzyme of $C_4$ photosynthesis where it catalyses the carboxylation of phosphoenolpyruvate resulting in the $C_4$ compound oxaloacetate. This is the initial reaction of the $C_4$ photosynthetic pathway and occurs in the mesophyll cells of the leaves of $C_4$ plants. The oxaloacetate is converted into malate or aspartate which is transferred into the bundle-sheath cells. There $CO_2$ is released from the $C_4$ acids raising the $CO_2$ concentration at the reaction site of RUBISCO and thereby preventing photorespiration (Hatch 1987).

Phosphoenolpyruvate carboxylase activity is controlled by various factors. Glucose-6-phosphate activates the enzyme, and malate and aspartate are feedback inhibitors (Bauwe and Chollet 1986; Andreo et al.1987). The enzyme is also regulated by reversible phosphorylation at a conserved serine residue near the amino terminus (Jiao and Chollet 1991; Duff and Chollet 1995).

Phosphoenolpyruvate carboxylase is not only a key enzyme of $C_4$ photosynthesis but other PEPCase isoforms are also involved in a variety of metabolic processes in both photosynthetic and non-photosynthetic tissues of all plants, e.g. replenishment of the tricarboxylic acid cycle intermediates, participation in pH maintenance and regulation of guard cell movement (Latzko and Kelly 1983). In all plants investigated so far at least three different PEPCase isoforms could be detected (Rajagopalan et al. 1994; Chollet et al. 1996; Svensson et al. 2003).

$C_4$ photosynthesis is of polyphyletic origin. This suggests that the photosynthetic ($C_4$) isoform of PEPCase must have evolved several times independently from non-photosynthetic ($C_3$) isozymes (Kellogg 1999). The $C_4$ PEPCase shows distinct kinetic and regulatory properties when compared to the $C_3$ isoforms. These differences involve a higher $K_{0.5}$ for the substrate phosphoenolpyruvate, a stronger activation by Glc6P and a lower sensitivity against the feedback inhibitor malate (Bauwe and Chollet1986). There is also evidence that $C_4$ and $C_3$ isoforms differ in their affinity towards the second substrate, i.e. bicarbonate (Bauwe 1986). Additionally, the expression of the $C_4$ PEPCase gene is altered as compared to the expression of genes encoding non-photosynthetic PEPCases (Sheen 1999). The $C_4$ PEPCase genes are

transcribed at high rates but exclusively in the mesophyll cells of the leaves whereas the non-photosynthetic isoforms show a low expression level and are more or less constitutively expressed in all plant organs (Ernst and Westhoff 1996; Koprivova et al. 2001).

To gain an insight into the changes underlying the evolution of $C_3$ to $C_4$ PEPCases we have analysed in detail, $C_3$, $C_3/C_4$ and $C_4$ PEPCases in the Asteracean genus *Flaveria*. These analyses revealed that only relatively small changes were required to convert a $C_3$ into a $C_4$ PEPCase. Particularly notable was the detection of a serine residue in the carboxyterminal part of the $C_4$ PEPCase that was also found in the $C_4$ PEPCases of all $C_4$ grasses and the $C_4$ dicot *Amaranthus hypochondriacus*. This serine was shown to be essential for the $C_4$-like $K_{0.5}$ of the substrate phosphoenolpyruvate (Bläsing et al. 2000). In all $C_3$ and CAM enzymes this serine is replaced by an alanine indicating that this $C_4$ to $C_3$ invariant amino acid position is of central importance for the evolution of a $C_4$ PEPCase (Svensson et al. 2003).

To compare the results obtained in *Flaveria* with other genera we have chosen the genus *Alternanthera*. *Alternanthera* is a member of the Amaranthaceae and, as *Flaveria*, contains $C_3$, $C_3/C_4$ and $C_4$ species (Chinthapalli et al. 2000). While the $C_4$ *Flaveria* species belong to the NADP-malic enzyme subgroup of $C_4$ plants, the $C_4$ *Alternantheras* use NAD-malic enzyme as decarboxylase (Rajendrudu et al. 1986). In these two dicot genera $C_4$ photosynthesis evolved clearly independently. The comparison of $C_4$ PEPCase evolution in *Flaveria* and *Alternanthera* should therefore allow to uncover molecular changes that are essential to all $C_4$ PEPCases regardless of their systematic position. In addition, genus-specific changes should be identified.

Full-length PEPCase cDNAs were isolated from the leaves of the $C_4$ plant *A. pungens* H.B.K., the $C_3/C_4$ intermediate plant *A. tenella* Colla and the $C_3$ species *A. sessilis* (L.) R.Br. and used for the production of the recombinant enzyme. In addition genomic sequences encoding the 3′ untranslated segments of PEPCase genes were isolated by vectorette PCR and analysed phylogenetically.

## Materials and methods

DNA modifying enzymes were purchased from MBI Fermentas (Vilnius, Lithuania) and Roche Diagnostics (Mannheim, Germany). L-Malate, Glc6P and NADH were purchased from Sigma, the trisodium salt of PEP from ICN (Eschwege, Germany) and NADH-malate dehydrogenase (porcine heart) from Serva (Heidelberg, Germany).

Seeds from *A. sessilis* (L.) R.Br. ($C_3$), *A. tenella* Colla ($C_3/C_4$) and *A. pungens* H.B.K. ($C_4$) were collected from a natural habitat in Hyderabad. Plants used for this study were grown in the greenhouse.

**RNA isolation and Northern analysis**

RNA was isolated from fully developed leaves of *A. sessilis*, *A. tenella* and *A. pungens* and analysed by northern blotting essentially as described (Westhoff et al. 1991; Rosche and Westhoff 1995). Filters were hybridized and washed at 60°C. The *ppcA* full-size cDNA of *A. sessilis* (see below) was used as a hybridization probe.

**Construction and screening of cDNA libraries**

Leaf cDNA libraries of *A. sessilis*, *A. tenella* and *A. pungens* were constructed using the ZAP-cDNA Synthesis Kit and the ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene) following the manufacturers instructions. Full-length PEPCase cDNAs were isolated from these libraries by standard procedures (Svensson et al. 1997). The full-length cDNA of *F. trinervia* (Poetsch et al. 1991) was first used to screen the *A. pungens* library resulting in a partial PEPCase cDNA (about 1,000 bp) in which the $C_4$-typical, carboxyterminal serine residue could be identified. This cDNA was then used to screen the *A. sessilis* library resulting in the identification of a full-size cDNA. This cDNA was then used to isolate full size cDNAs from the *A. tenella* and *A. pungens* libraries.

**Expression, purification and enzyme assay of recombinant PEPCases**

To insert the PEPCase coding sequences into the expression vector pTrc99A (Amersham Pharmacia Biotech) the region around the ATG was converted into a *Nco*I restriction site by performing a PCR reaction with a mutagenic primer (Ap: 5′-AGATCTAGACCATGGCAACAGTGAAGGTGG-3′; As and At: 5′-AGATCTAGACCATGGCAACAGTGAAGTTGG-3′) and a second primer which covers a *Sph*I restriction site 189 bp downstream of the ATG codon (5′-GTCCTTTCATATTCGGCAGC-3′). The PCR fragments were restricted with *Nco*I and *Sph*I and the original cDNA clones were cut with *Sph*I and *Kpn*I. The *Nco*I/*Sph*I and *Sph*I/*Kpn*I fragments were purified by gel electrophoresis and inserted into the *Nco*/*Kpn*I restricted pTrc99A vector by a triple ligation. The successful cloning procedure was confirmed by restriction and sequence analysis of the resulting expression plasmids.

The recombinant PEPCase enzymes were produced in the *Escherichia coli* strain PCR1 and purified basically as described for the PEPCase enzymes of *Flaveria* (Svensson et al. 1997). PEPCase activity was determined by coupling the reaction to NADH-malate dehydrogenase and measuring the consumption of NADH spectroscopically at 340 nm. One Unit (U) of enzyme was calculated from the activity oxidizing 1 μmol NADH per minute at 25°C. The standard assay mixture contained 50 mM Tricine/KOH, pH 8.0, 10 mM $MgCl_2$, 10 mM $KHCO_3$, 0.15 mM NADH and 6 U NADH-malate dehydrogenase in a total volume of 0.6 ml. The reaction was started by adding 40–50 mU of recombinant PEPCase. When indicated, the reaction mixture also contained 5 mM of the activator Glc6P. In order to determine the kinetic parameters ($K_{0.5}$-PEP, $k_{cat}$ and the Hill coefficient $h$) the Hill equation was fitted to the experimental data by non-linear regression analysis using the software package Kaleidagraph (Version 3.0.8, Synergy Software). Each kinetic measurement was repeated at least twice.

**Genomic Southern blot hybridization**

Total nucleic acids were isolated from leaf tissue (Westhoff et al. 1991) and DNA was recovered from the 2 M LiCl soluble nucleic acid fraction by isopropanol precipitation. The DNA was dissolved in double-distilled water and residual RNA was digested by RNase A treatment. After phenol/chloroform extraction, the DNA was precipitated with isopropanol, dissolved in double-distilled water and stored at −20°C until use. Total DNA of *A. sessilis, A. tenella* and *A. pungens* was digested with *Eco*RI, *Hind*III, *Pst*I and *Xba*I. Five microgram each of the restricted DNA was separated on 0.8% agarose gels and transferred to Hybond N+ membranes (Amersham Pharmacia Biotech). Hybridization and washing of the membranes were carried out as described (Ernst and Westhoff 1996). DNA from each plant was hybridized using the corresponding full-length cDNA as well as the 3′ untranslated region of the corresponding cDNA as probes. The probes were labelled by random priming (Feinberg and Vogelstein 1983).

**Genome walking**

The 3′ untranslated regions of the *ppc* genes of *A. sessilis* and *A. pungens* were isolated from total DNA using the Universal Genome Walker Kit (Clontech) following the manufacturers recommendations. For each plant species *Dra*I, *Eco*RV, *Pvu*II and *Stu*I DNA libraries were constructed. The gene-specific primers (GSP1 5′-TCTTGACCATGAAGGGTATTGC-3′; GSP2 5′-GCTGGTATGCAGAACACTGG-3′) were designed to recognize the very 3′ coding

region of *Alternanthera* PEPCases as represented by nucleotides 2,855–2,897 of the PEPCase cDNA sequences (the numbers of nucleotides refer to the translation initiation codons). The resulting PCR fragments were cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, USA). Eight *A. sessilis* and seven *A. pungens* clones differing by size could be isolated from the different libraries and were used for sequence analysis.

### Miscellaneous

Standard molecular biological techniques were carried out essentially as described in Sambrook and Russell (2001). Double stranded plasmid DNAs were sequenced with the T7 sequencing kit from Amersham Pharmacia Biotech. Full-length cDNAs and GW products were sequenced by Agowa GmbH (Berlin, Germany) and MWG Biotech (Ebersberg, Germany). Nucleic acid and protein sequences were analysed with the software packages MacMolly Tetra (Softgene GmbH, Berlin, Germany) and CLUSTAL W (Thompson et al. 1997). The phylogenetic tree was constructed using the program PAUP 4.0b10 (Swofford 2002) to perform a distance analysis.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under the accession numbers AY950665 (ApppcA cDNA), AY950666 (AtppcA cDNA), AY950667 (AsppcA cDNA), AY950668 (AsppcA 3′ flanking region), AY950669 (ApppcA1 3′ flanking region), AY950670 (ApppcA2 3′ flanking region), AY950671 (AsppcB 3′ flanking region), AY950672 (ApppcB 3′ flanking region), AY950673 (AsppcC 3′ flanking region) and AY950674 (ApppcC 3′ flanking region).

## Results

### Cloning and sequence analysis of *ppcA* cDNA clones from *A. pungens* ($C_4$), *A. tenella* ($C_3/C_4$) and *A. sessilis* ($C_3$)

Full-length cDNAs for the PEPCases of *A. sessilis* ($C_3$), *A. tenella* ($C_3/C_4$) and *A. pungens* ($C_4$) were isolated by screening leaf cDNA libraries with a full-length *ppcA* cDNA of *F. trinervia* which encodes the $C_4$ PEPCase of that genus. Putative full-size cDNAs were partially sequenced from both ends and their identity as PEPCase encoding sequences was verified by comparison with known PEPCase sequences. One full-size PEPCase cDNA clone each from *A. sessilis, A. tenella* and *A. pungens* was fully sequenced and the clones were named tentatively AsppcA, AtppcA and ApppcA, respectively.

All three cDNA clones contain long open reading frames of 2,898 bp encoding PEPCase proteins of 966 amino acids each (Fig. 1). The identity scores of the encoded proteins range from 93 to 97% (*A. sessilis*/*A. tenella*: 97%; *A. pungens*/*A. tenella*: 94%; *A. pungens*/*A. sessilis*: 93%). The similarity is lower in the 3′ untranslated regions. While the 3′ untranslated segments of the *A. pungens* and *A. tenella* cDNAs share 69% identical base pairs, the similarity of the 3′ untranslated sequence from the *A. sessilis* cDNA to the corresponding regions from the cDNAs of *A. pungens* and *A. tenella* is only 55%.

The PEPCase coding sequences show the typical conserved features of plant PEPCases (Fig. 1) (Kai et al. 2003). The PEPCase sequence from the $C_4$ plant *A. pungens* possesses a serine residue at position 775 which is equivalent to position 774 of the $C_4$ PEPCases of *Flaveria* suggesting that this PEPCase encodes the $C_4$ isoform of *A. pungens*. In contrast, the enzymes from *A. sessilis* ($C_3$) and *A. tenella* ($C_3$/$C_4$) hold the $C_3$-type specific alanine at that position.

**Kinetic and regulatory properties of the recombinant phosphoenolpyruvate carboxylase**

To prove the assumed properties of the PEPCase proteins encoded by the three cDNAs from *A. pungens*, *A. tenella* and *A. sessilis* recombinant proteins were produced in a PEPCase-negative strain of *E. coli* (Sabe et al. 1984) and the $K_{0.5}$ of the substrate phosphoenolpyruvate of the three enzymes were measured. This enzyme parameter was chosen because it reflects precisely the degree of $C_4$ characteristics expressed by the PEPC enzyme (Engelmann et al. 2003).

The PEPCase from *A. pungens* showed the highest $K_{0.5}$ value (Table 1). With 157 μM it is not that high as to be expected from *Flaveria* (*F. trinervia* PpcA $K_{0.5}$: 278 μM, Bläsing et al. 2000), but it is clearly $C_4$-like. In contrast the $K_{0.5}$ values of the PEPCases from *A. sessilis* and *A. tenella*, 36 and 42 μM, respectively, were found to be low and were typical of $C_3$-type enzymes (*F. pringlei* PpcA $K_{0.5}$: 29 μM, Bläsing et al. 2000). The $C_4$–$C_3$ characteristics of the three enzymes were also reflected by their behaviour to the allosteric activator Glc6P. While the enzyme from *A. pungens* is activated about eightfold, the activation of the enzymes from *A. sessilis* and *A. tenella* is only two- to threefold (Table 1). Finally, the non-activated enzyme from *A. pungens* showed the $C_4$-type sigmoidal saturation kinetics for the substrate phosphoenolpyruvate with a Hill coefficient of 1.6. The saturation curve became hyperbolic, when the enzyme was activated by Glc6P. In contrast the *A. sessilis* and *A. tenella* enzymes displayed only small deviations from Michaelis–Menten kinetics, both when investigated in their non-activated and activated states (Table 1). The *A. pungens* enzyme showed the highest

specific activity of the three enzymes. Its $V_{max}$ value was 1.2 times higher than that of the *A. tenella* and 1.7 times higher than that of the *A. sessilis* enzyme.

We conclude from these data that the PEPCase from the $C_4$ plant *A. pungens* is a typical $C_4$-type PEPCase, while the enzymes from the $C_3$ plant *A. sessilis* and the $C_3/C_4$ intermediate *A. tenella* should be classified as non- photosynthetic $C_3$ isoforms.

**PEPC expression levels in the leaves**

To compare the *ppcA* transcript levels in the leaves of the three plant species a northern analysis was performed. Figure 2 shows that large amounts of *ppcA*-PEPCase transcripts accumulate in the leaves of the $C_4$ plant *A. pungens*. In contrast the *ppcA* genes are only weakly expressed in the leaves of *A. tenella* ($C_3/C_4$) and *A. sessilis* ($C_3$) and their mRNA amounts are similar in the two species.

**Genomic Southern hybridization experiments**

To investigate the number of the *ppcA* genes as well as the total number of *ppc* genes in the genome of the three *Alternanthera* species Southern analyses were performed. For each plant species the corresponding full-length cDNAs as well as the 3′ untranslated regions (UTR) of the cDNAs were used as hybridization probes.

The *ppcA* specific 3′ UTR probe of *A. sessilis* resulted in a simple hybridization pattern when hybridized to the corresponding genomic DNA. This suggested that *ppcA* is most probably a single-copy gene (Fig. 3a). A more complex pattern with additional bands was obtained when the full-length cDNA was used as probe (Fig. 3b) indicating the existence of additional *ppc* genes in the *A. sessilis* genome which do not contain sequences similar to the 3′ untranslated region of the *A. sessilis ppcA* gene.

In the case of *A. tenella* and *A. pungens* even the hybridization with the *ppcA*-specific 3′UTR probe led to complex hybridization patterns (Fig. 3c, e). When the corresponding full-length cDNA probes were used additional signals became visible (Fig.3d, f). It has to be concluded that both *A. pungens* ($C_4$) and *A. tenella* ($C_3/C_4$) contain more than one *ppcA* isogene and that there are also other *ppc* gene classes in the genomes of these two plant species.

**Genome walking**

The Southern blot experiments indicated the presence of multi-membered *ppcA* gene families at least in *A. tenella* and *A. pungens*. To establish more precise relationships between the *ppc*

genes and to verify that the isolated cDNAs belong to one orthologous *ppc* gene class the "genome walking method" was used to isolate the 3′ untranslated segments of *ppc* genes from *A. sessilis* ($C_3$) and *A. pungens* ($C_4$). The genome walking method is a "vectorette-type PCR" combined with "suppression PCR" (Siebert et al. 1995) and allows the isolation of uncloned genomic DNA from one known region.

The very 3′ coding region was used for primer design because this region is well conserved in all plant PEPCases (Toh et al. 1994) and should represent the last exon of the *ppc* genes. With such primers it should be possible to amplify, most likely, the 3′ untranslated regions of many, if not all, existing PEPCase genes in the three *Alternanthera* species.

Eight PCR fragments from *A. sessilis* and seven PCR fragments from *A. pungens* were cloned and sequenced. In the case of *A. sessilis* two sequences were represented three times and one sequence was cloned twice from different genome walking libraries. In case of *A. pungens* identical pairs of sequences were cloned three times and one sequence was found once. In total three different 3′ untranslated regions could be isolated from *A. sessilis* and four from *A. pungens*.

Sequence alignments were constructed from the three non-identical *A. sessilis* sequences, the four non-identical *A. pungens* sequences and the 3′ untranslated regions of the cloned cDNAs of *A. sessilis*, *A. tenella* and *A. pungens* (Fig. 4). From all these sequences only the first 350 nucleotides downstream of the translational stop codon were used for comparison. The 3′ untranslated region of the *ppcA1* gene of *F. trinervia* was used as an outgroup. This alignment was used for phylogenetic tree construction using the distance method as implemented in PAUP 4.0b10 (Swofford 2002). The tree revealed three groups of sequences with a high degree of similarity (Fig.4). All groups contained *A. sessilis* as well as *A. pungens* sequences, specifically the 3′ untranslated regions of all cloned cDNAs belong to the same group. We conclude from these data that the tentative assignment of the isolated cDNAs to one single orthologous gene class (*ppcA*) is valid.

## Discussion

$C_4$ photosynthesis evolved several times independently during the evolution of higher plants and consequently also phosphoenolpyruvate carboxylase, the primary carboxylase of this photosynthetic pathway, evolved $C_4$ properties several times independently. The molecular changes, which were necessary to adapt the PEPCase genes to this new pathway, have been

studied in the genus *Flaveria*. To uncover molecular changes that are essential to all $C_4$ PEPCases regardless of their systematic origin and identify genus-specific changes the genus *Alternanthera* was chosen for comparison, since similar to *Flaveria*, this genus contains $C_3$, $C_3/C_4$ and $C_4$ species. PEPCase cDNAs could be isolated from the $C_4$ species *A. pungens*, the $C_3/C_4$ intermediate species *A. tenella* and the $C_3$ species *A. sessilis* and their enzymatic properties as well as their genomic representation were investigated.

**The *ppcA* gene of *A. pungens* encodes a $C_4$ isoform of PEPCase**

The *ppcA* PEPCase of *A. pungens* behaves as a typical $C_4$ enzyme documented by the high $K_{0.5}$ value for phosphoenolpyruvate and the strong effect of the activator Glc6P. As is typical for a $C_4$ enzyme, the Hill coefficient is greater than one in the non-activated state which indicates a cooperative binding of the substrate phosphoenolpyruvate. The cooperativity disappears when the enzyme is activated by Glc6P. The same behaviour was described for the $C_4$ PEPCase of the $C_4$ plant *F. trinervia* (Svensson et al. 1997). As to be expected for a $C_4$ PEPCase high amounts of *ppcA* transcripts were found to accumulate in the leaves of *A. pungens*.

The *ppcA* PEPC of *A. pungens* contains a serine residue at position 775. A serine residue at this position is characteristic for all $C_4$ PEPCases sequenced to date, i.e. the $C_4$-PEPCases of the grasses, the genus *Flaveria* and amaranth. In contrast, all $C_3$, i.e. the non-photosynthetic PEPCases which are involved in various pathways of basic plant metabolism as well as the photosynthetic PEPCases of CAM plants, harbour an alanine at this position. An alanine residue also occurs in all $C_3/C_4$ intermediate PEPCases of the genus *Flaveria* and, as found in this study, in the *ppcA* PEPCase of the $C_3/C_4$ intermediate plant *A. tenella*. For *Flaveria*, it was experimentally proven that the serine residue is a major determinant for the $C_4$ typical high $K_{0.5}$ value of the *ppcA* PEPCase of the $C_4$ species *F. trinervia* (Bläsing et al. 2000; Engelmann et al. 2002). Since this alanine-to-serine exchange occurred during the evolution of $C_4$ PEPCases in three different plant families in which $C_4$ photosynthesis evolved independently from each other, i.e. the Asteraceae (Engelmann et al. 2003), the Poaceae (Besnard et al. 2003) and the Amaranthaceae, one has to conclude that an alanine-to-serine exchange at this position is invariant for evolution for $C_4$ PEPCases from non-photosynthetic isoforms.

**The PEPCase sequences of *Alternanthera* species are encoded by gene families**

In all plant species investigated so far (*Sorghum*, Maize, *Flaveria*, *Arabidopsis*, sugarcane, soybean) small PEPCase gene families consisting of at least three PEPCase genes were found (Chollet et al. 1996; Dong et al. 1998; Besnard et al. 2003; Ernst et al. 1996; Sánchez and Cejudo 2003; Sullivan et al. 2004). This also applies for the three *Alternanthera* species analysed in this study. Genomic Southern blot analyses using the complete *ppcA* cDNAs as probes led to complex banding in each species indicating the existence of several *ppc* genes. This was confirmed by the random isolation of the 3′ regions of PEPCase genes from *A. pungens* ($C_4$) and *A. sessilis* ($C_3$) by vectorette PCR using a primer binding site that is conserved in the carboxyterminal part of all plant PEPCase genes.

The phylogenetic analysis of the obtained sequences indicate the presence of three classes of orthologous PEPCase genes in *A. sessilis* and *A. pungens* that were named *ppcA*, *ppcB* and *ppcC*. Two types of *ppcA* sequences were identified for *A. pungens* with one of them matching the isolated cDNA. This suggested the presence of at least two *ppcA*-type PEPCase genes in *A. pungens* which was supported by the genomic Southern blot hybridizations. More than one *ppcA*-type PEPCase is also predicted for the $C_3/C_4$ intermediate *A. tenella*. In contrast, all available evidence from the sequence and the genomic Southern blot analyses indicate only one single *ppcA*-type gene in the $C_3$ plant *A. sessilis*.

*Alternanthera* seem to be monophyletic and at least *A. sessilis* and *A. pungens* belong to the same phylogenetic line (Müller and Borsch 2005). So the last common $C_3$ ancestor of the present $C_3$, $C_3/C_4$ and $C_4$ *Alternanthera* species may have possessed only one single *ppcA*-type PEPCase gene which became duplicated in the course of $C_4$ evolution. However, this conclusion is too premature to be drawn. Because not all *ppcA* genes of *A. tenella* ($C_3/C_4$) and *A. pungens* ($C_4$) have been isolated and sequenced yet, it cannot be decided whether the gene duplication event occurred before the split of the lineages leading to *A. tenella* and *A. pungens* or whether the *ppcA* gene duplicated independently in the two lineages. Since auto- or alloploidy play important roles in plant evolution and speciation such genome duplications may have caused the formation of multiple *ppcA* genes in the $C_3/C_4$ and $C_4$ *Alternantheras* cf. (Wendel 2000; Soltis et al. 2003). On the other side, the presence of a single *ppcA* gene in *A. sessilis* may be a derived state, i.e. the last common $C_3$ ancestor of the present $C_3$, $C_3/C_4$ and $C_4$ *Alternanthera* species contained already two *ppcA*-type genes one of which became lost (Wolfe 2001). Since all members of the *ppc* gene families in the $C_3$, $C_3/C_4$ and $C_4$

*Alternanthera* species have not been isolated and characterized yet, it is not possible to favour one of these hypotheses.

**The *ppcA* PEPCases of *A. sessilis* and *A. tenella* are typical non-photosynthetic PEPCase enzymes**

The *ppcA* proteins of *A. sessilis* ($C_3$) and *A. tenella* ($C_3/C_4$) are more similar to each other than either of them is to the *ppcA* protein of *A. pungens*. This is reflected by the kinetic properties of the recombinant proteins. The values for $K_{0.5}$ PEP and activation by Glc6P are nearly the same for both enzymes and typical for non-photosynthetic PEPCases (Bläsing et al. 2002). The *ppcA* protein of *A. tenella* shows a slightly higher specific activity than the *A. sessilis* protein. It is not clear if there is any correlation between the specific activity and $C_4$ properties of PEPCases. Such a correlation could neither be found for the PEPCases of *Flaveria* or maize (Dong et al. 1998; Engelmann et al. 2003). With respect to its saturation kinetics for PEP the analysed *ppcA* PEPCase of the $C_3/C_4$ intermediate *A. tenella* is therefore not a $C_3/C_4$ intermediate PEPCase but rather $C_3$-like, i.e. of the non-photosynthetic PEPCase type. This raises the question whether there might be other *ppcA*-PEPCase genes with $C_3/C_4$ intermediate properties in this plant.

*PpcA* RNA amounts are similar in the leaves of *A. sessilis* and *A. tenella* and, as to be expected, are much lower than in the $C_4$ species *A. pungens*. This contrasts the results obtained with *Flaveria* where even the more $C_3$-like $C_3/C_4$ intermediates exhibit already enhanced amounts of *ppcA* transcripts (Engelmann et al. 2003). The rather $C_3$-like properties of the *ppcA* PEPCase of *A. tenella* are in line with the activities of the $C_4$ cycle enzymes, PEPCase, NAD-malic enzyme and pyruvate orthophosphate dikinase in this species which are only slightly elevated as compared to *A. sessilis* (Devi and Raghavendra 1993). At the present stage of investigation it cannot be decided if there is a $C_3/C_4$ intermediate PEPCase in *A. tenella*. However, the observed low *ppcA* transcript levels in the leaves of *A. tenella* and the physiological properties of *A. tenella* make it unlikely that there is such a gene.

***Alternanthera tenella* has been classified as a $C_3/C_4$ intermediate species based on its $CO_2$** compensation point which was found to be intermediate between that of the $C_3$ and $C_4$ species indicating a reduced apparent photorespiration compared to $C_3$ plants (Rajendrudu et al. 1986). This lowered apparent photorespiration in *A. tenella* is due to the compartmentation of the photorespiratory enzymes, i.e. glycine decarboxylase, in the bundle-sheath cells (Devi

et al. 1995). Together with *Moricandia arvensis* and *Parthenium argentatum*, *A. tenella* belongs to that group of $C_3/C_4$ intermediate plant species whose intermediate properties are predominantly caused by a photorespiratory $CO_2$ pump due to the restriction of glycine decarboxylase to the bundle-sheath compartment and not by the establishment of an at least rudimentary $C_4$ cycle (Sage 2004).

## Conclusion and outlook

The $C_4$ photosynthetic pathway arose several times independently during the evolution of angiosperms. In each case a $CO_2$ pumping device was developed with PEPCase being the primary carboxylase. The recruitment of a non-photosynthetic, i.e. $C_3$-type PEPCase gene for its functioning in the $C_4$ cycle involved changes in its primary structure to cope with the requirements of the altered metabolic context. The alanine-to-serine change in the carboxyterminal part of the enzyme appears to be indispensable and is conserved in all $C_3$ to $C_4$ transitions presently analysed, i.e. *Flaveria* and *Alternanthera*. Other alterations of the enzyme kinetic properties were realized by different modifications on the molecular level in both genera. For the *Flaveria* PEPCases it was shown that differences in a N-terminal region (aa 296–437) are responsible for the different response of the $C_3$ and the $C_4$ enzyme on the activator Glc6P (Engelmann et al. 2002). In the corresponding region of the *Alternanthera* enzymes (aa 297–438) no corresponding amino acid exchanges could be detected. Nevertheless in *Alternanthera* as in *Flaveria* the $C_4$ enzyme is more activated by Glc6P as the $C_3$ enzyme.

Nothing is known whether similar conservative alterations also occurred in the evolution of the *cis*-regulatory system for mesophyll-specific PEPCase expression. In *Flaveria* relatively small changes in *cis*-regulatory elements lead to different expression patterns of the photosynthetic *ppc* genes (Gowik et al. 2004). The genus *Alternanthera* may turn out to be a useful system to answer these questions.

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## Figure Legends

### Figure 1

Amino acid sequence alignment of the *ppcA* PEPCases of *A. pungens* (ApppcA), *A. tenella* (AtppcA) and *A. sessilis* (AsppcA). *Asterisks underline* identical amino acid residues in all three proteins. Amino acid residues of experimentally proven function (according to Kai et al. 2003) are labelled by *grey boxes*. Amino acid residues relevant for $C_4$ to $C_3$ differences in the $K_{0.5}$ PEP of the *Flaveria ppcA* enzymes are labelled by *black bars*

### Figure 2

RNA gel blot analysis of PEPCase RNA amounts in the leaves of *A. pungens*, *A. tenella* and *A. sessilis*. The hybridization pattern was obtained by using the *ppcA* cDNA of *A. sessilis* as a probe. One microgram of $polyA^+$ leaf RNA was analysed for each species. The exposure time was about 6 h

### Figure 3

**a–f** Genomic Southern blot analysis of the PEPCase genes of *A. pungens*, *A. tenella* and *A. sessilis*. Each genomic DNA was hybridized using the 3′ untranslated region of the corresponding *ppcA* cDNA (**a, c, e**) or the corresponding full-length *ppcA* cDNA (**b, d, f**) as hybridization probes. Five microgram of genomic DNA restricted with *Eco*RI (*E*), *Hind*III (*H*), *Pst*I (*P*) or *Xba*I (*X*) were loaded in each slot

### Figure 4

Phylogenetic analysis of the PEPCase gene family in *A. pungens*, *A. tenella* and *A. sessilis*. **a** Phylogenetic tree generated by the distance method of PAUP 4.0b10 (Swofford 2002). Sequence alignments were constructed from the 3′ untranslated sequences of the *ppcA* cDNAs of *A. pungens*, *A. tenella* and *A. sessilis* and from the genomic regions corresponding to the 3′ untranslated segments of *ppc* genes in *A pungens* and *A. sessilis*. In each case 350 base pairs downstream of the stop codon were used for the alignment. Bootstrap analysis was carried out with 1,000 replications and the obtained bootstrap values are indicated. The tree was rooted with the *ppcA1* sequence of *F. trinervia*. **b** Alignment of the *ppcA*, *ppcB* and *ppcC* 3′ untranslated sequences. The translational stop codons are typed in *bold letters*. The binding site of the GPS2 primer which was used to isolate these DNA fragments is *boxed*

## Tables

**Table 1:** Kinetic constants for the *Alternanthera* phosphoenolpyruvate carboxylases

| Enzyme | $K_{0.5}$(PEP) (µM) | | Hill coefficient h | | $V_{max}$ (mmol $kg^{-1}$ $s^{-1}$) | | $k_{cat}$ ($s^{-1}$) | | $k_{cat}/K_{0.5}$ ($s^{-1}$ $M^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | −Glc6P | +Glc6P | −Glc6P | +Glc6P | −Glc6P | +Glc6P | −Glc6P | +Glc6P | −Glc6P | +Glc6P |
| ap | 157±5 | 20±0.3 | 1.6±0.1 | 0.9±0.03 | 350±9 | 356±4 | 38±0.5 | 38±0.9 | $2.4±0.06×10^5$ | $19±0.37×10^5$ |
| at | 42±1 | 25±2 | 0.8±0.01 | 0.8±0.06 | 294±6 | 300±10 | 33±0.7 | 33±1.8 | $7.7±0.38×10^5$ | $14±1.20×10^5$ |
| as | 36±2 | 13±0.5 | 0.9±0.04 | 1.1±0.2 | 200±5 | 190±4 | 22±0.6 | 20±0.7 | $6.1±0.20×10^5$ | 16±0.60×10 |

Kinetic parameters were determined as described under “Materials and Methods”. Purified enzyme fractions exhibiting 40–50 milli unit (mU) activity were assayed with at least eight different concentrations of the substrate PEP. The kinetic parameters $K_{0.5}$, $V_{max}$ and the Hill coefficient were determined using nonlinear regression analysis. Each value represents the average of at least three separate measurements. Standard errors are given.

# Figures

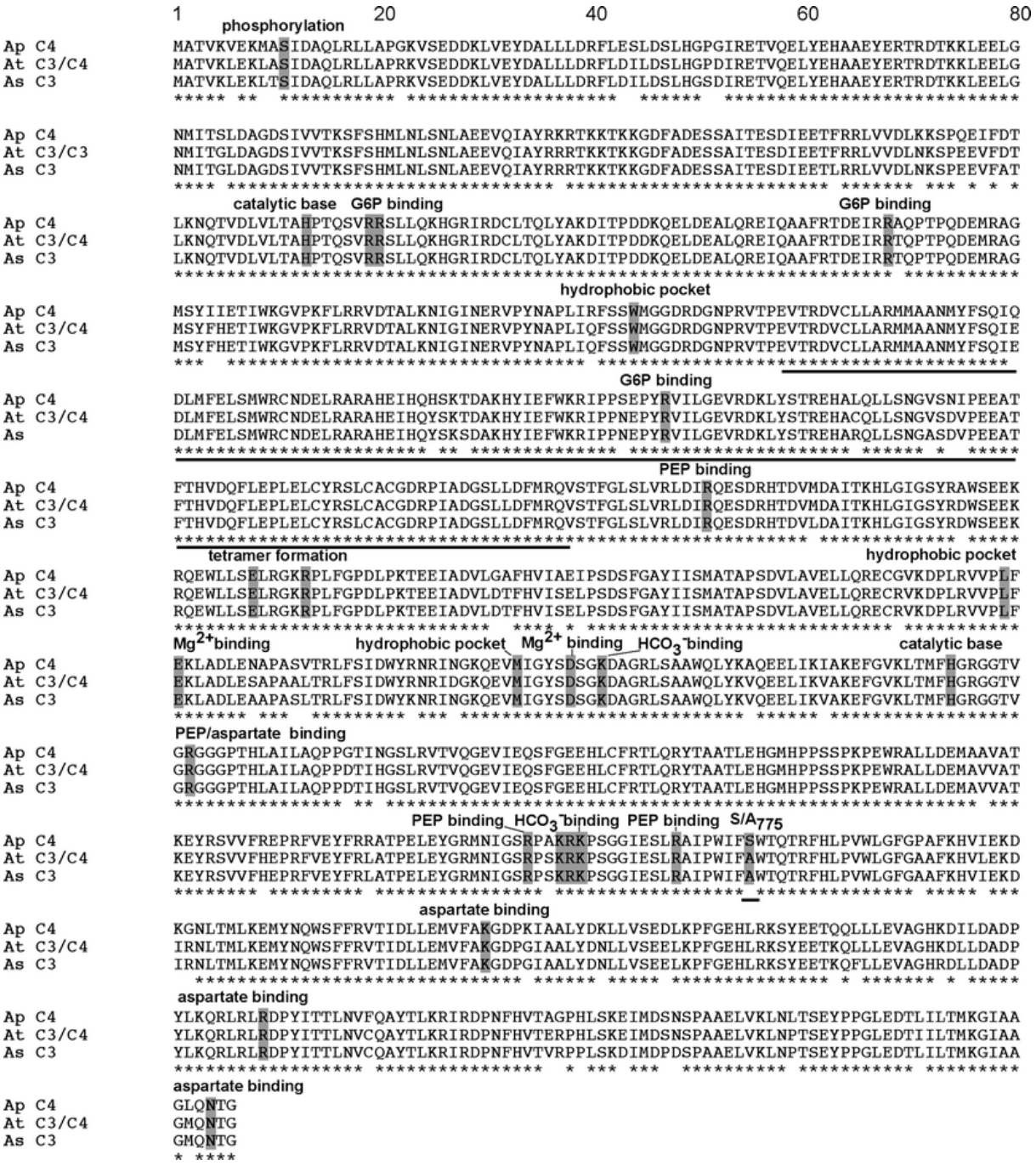


**Figure 1**

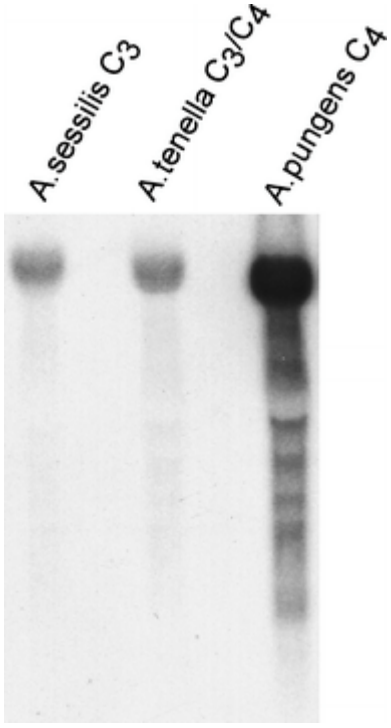


**Figure 2**

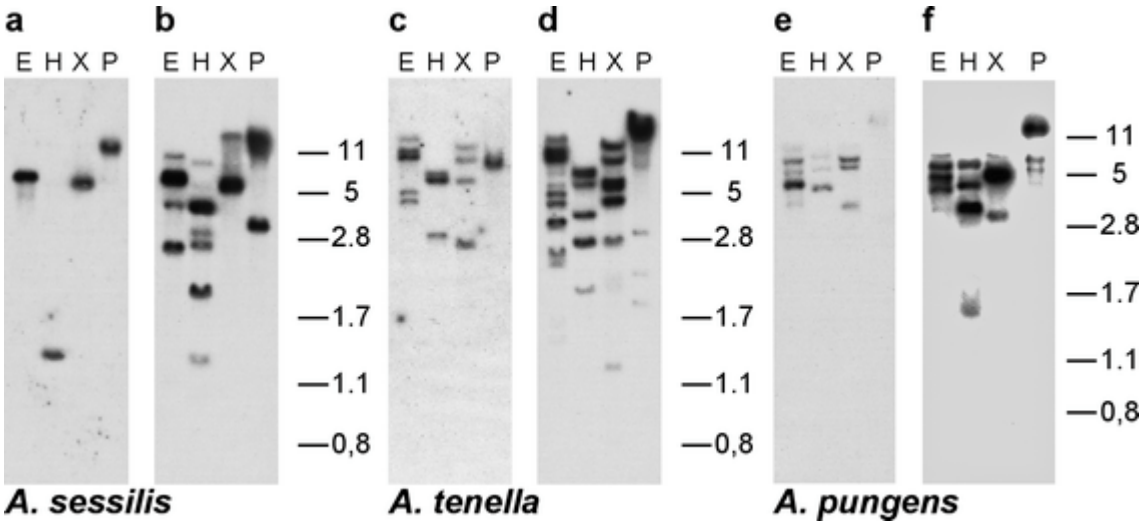
a
b
c
d
e
f
E H X P
E H X P
E H X P
E H X P
E H X P
E H X P
— 11
— 5
—2.8
—1.7
—1.1
—0,8
A. sessilis
A. tenella
A. pungens

**Figure 3**

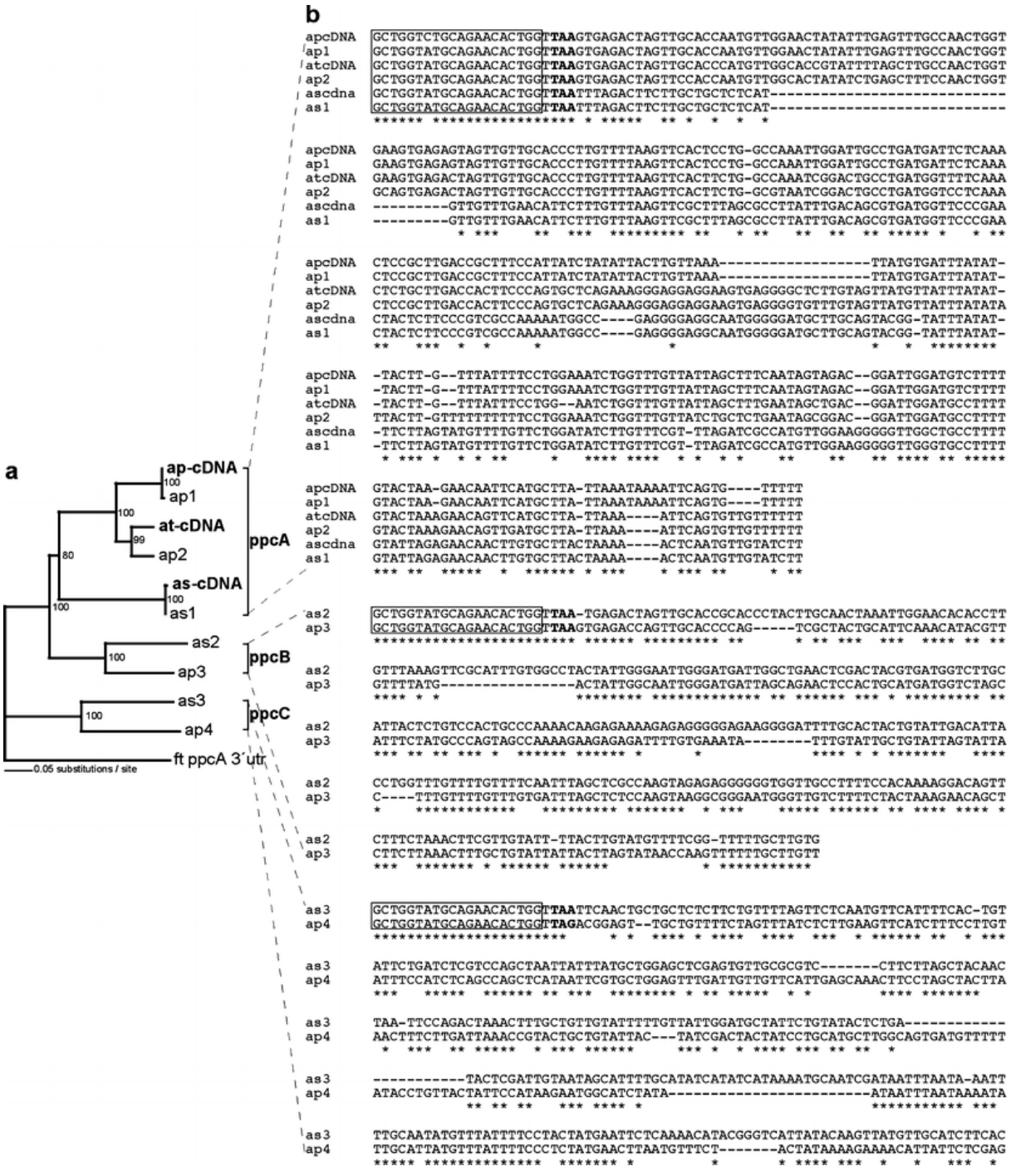
a
ap-cDNA
ap1
at-cDNA
ap2
as-cDNA
as1
as2
ap3
as3
ap4
ft ppcA 3´utr
ppcA
ppcB
ppcC
100
99
80
0.05 substitutions / site
b
apcDNA
ap1
atcDNA
ap2
ascdna
as1
as2
ap3
as3
ap4

**Figure 4**

Die vorliegende Dissertation habe ich eigenständig und ohne unerlaubte Hilfsmittel angefertigt. Die Dissertation wurde in dieser oder ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 9.11.2007

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